Fractal dimension in mass spectra from herbal extracts: Hypothesis for a new method of phytocomplex characterization

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Fractal analysis; Phytochemicals; ESI; Mass spectrometry; Quality control.

ABSTRACT

In order to explore new possibilities to optimize the quality control of herbal products, a non-conventional method of analysis of complex systems, the fractal analysis, applied to ElectroSpray Ionisation (ESI) mass spectrometry, was evaluated. The ESI spectra obtained with herbal commercial products were converted into bitmap images and submitted to fractal analysis using the so-called “box counting” method. The fractal dimension \( D_B \) so obtained permitted to classify single plant extracts. Also the ESI spectrum obtained with a mixture of plant extracts contained in a commercial herbal product provided a fractal pattern; statistical analysis on several replicates obtained with different batches indicated that \( D_B \) tends to display a normal distribution around a mean value, which might be suggested as a typical reference for that product. \( D_B \) of ESI spectra of plant extracts which underwent thermal treatment indicates that this measure maybe useful also to evaluate the changes occurring with aging of the product. In conclusion, \( D_B \) might be proposed as a new promising technique of investigation to be used, coupled to mass spectrometry, as a summary measurement of complexity of the overall phytochemical composition and stability of a herbal product.

KEYWORDS

Fractal analysis; Phytochemicals; ESI; Mass spectrometry; Quality control.

INTRODUCTION

Metabolomic approach is a fast and effective analytical method able to give a fingerprinting of a phytochemical commercial product, in order to assist the standardization and the quality control process[1-2]. Several methods have been proposed, but often they are complex and time-consuming[3]. In previous papers[4-6] we found that the metabolomic fingerprinting of plant extracts is highly effective to characterize different plant extracts by using ElectroSpray Ionisation (ESI) mass spectrometry linked to a multivariate statistical method of data evaluation. In particular, when operating in negative ion mode, the procedure led to a clear characterization of different extracts, with different samples of the same species appearing well-clustered and separated from the other ones. Moreover, the same method was able to provide a reliable characterization of mix-
Fractal analysis can be performed on biological phenomena presenting complex patterns\textsuperscript{[7]}, such as cellular differentiation\textsuperscript{[8,9]}, tumor expansion\textsuperscript{[10,11]}, heart rate\textsuperscript{[12]} and even gene expression\textsuperscript{[13]}. Also pharmacology has been reported to be disseminated of fractal patterns\textsuperscript{[14-18]}, as well as electrophysiology\textsuperscript{[19]}. A fractal approach has been also described for chromatographic profiles of herbal products\textsuperscript{[20]} and colorimetric profile of fruit juice\textsuperscript{[21]}; the authors of the former article\textsuperscript{[20]} found that fractal fingerprints are more stable than the original chromatographic profile. When the phenomenon can be presented graphically, several procedures of fractal analysis have been suggested by using the simple image of the phenomenon, i.e. an histological section, an electrocardiographic trace, or a time-dependent series. The analysis of the phenomenon may depend on the characteristics of the image, that is of its details, in order to find any self-similarity pattern, one of the characteristics of fractal objects\textsuperscript{[22]}. Therefore, fractal analysis often is an approximate measurement of the fractal pattern(s) of the phenomenon, that is a measure of its complexity in terms of a change in detail with change in scale. Given that and ESI spectrum, either as negative or positive ion mode, is a complex system, we first tried to investigate if it can display any fractal pattern, and therefore to use the fractal pattern as a method to characterize the product under the aspect of stability, and possibly also to obtain any fingerprinting option for the characterization of the phytocomplex. The expected result would be a simplification of the entire process of phytocomplex characterization (see comparative flow-charts in Figure 1).

EXPERIMENTAL

Mass spectrometry

Sample preparation

The freeze-dried herbal extracts were suspended in water:ethanol (30:70) mixture. The suspension was left in ultrasonic bath for 22 minutes and centrifuged for 5 minutes at 13000 rpm. The supernatant was centrifuged and the pellet resuspended again in water:ethanol (30:70), sonicated and centrifuged. The combined solutions so obtained were employed for the determination of their metabolomic profiles by direct infusion ESI (positive and negative mode).

The commercial products were Grintuss syrup adults (medical device), and Natura Mix syrup (herbal dietary supplement), both supplied by Aboca. The Grintuss syrup contains: honey, water, plantain leaves hydroalcoholic and freeze dried extract, Grendelia robusta flowered tops freeze-dried extract, Helichrysum italicum flowered tops freeze-dried extract, essential oils of Eucalyptus, star anise and lemon. The Natura Mix syrups contains: honey, purified apple juice, water, Vaccinium myrtillus fruit juice, Malpighia punicifolia fruit juice, Sambucus nigra fruit juice, Rubus fruticosus fruit juice, freeze-dried royal jelly.

In some cases thermal treatment was applied to the batches, in order to simulate the aging of the product.
Direct Infusion ESI

The solutions of the samples were directly infused in the ESI source of an Agilent 1100 Series LC/MSD Trap at 11 µL/min with a nebulizer gas pressure of 15 psi for the negative ion mode and 13 psi for the positive ion mode. The dry gas flow and the entrance capillary temperature were 5 L/min and 325 ºC respectively for both ion modes. In the positive ion mode the capillary voltage was -4500 V, while in the negative ion mode it was 3000 V. Extracts were diluted in various amount with H₂O 0.1% HCOOH:MeOH (50:50, v:v) in order to optimize the instrumental performance.

Fractal evaluation of ESI spectra

The ESI spectra obtained with the herbal dietary supplement product (an example is shown in Figure 2) after data normalization, were converted into bitmap images and submitted to fractal analysis using the so-called “box counting” method. “Box counting” consists of superimposing the image with a series of grids of decreasing caliber (called “boxes”, of length r; the scale ε is equivalent to 1/r), and counting the boxes which contain any part of the original image (indicated as N) (Figure 3). Fractal dimension D can be defined as the relationship between N, the number of filled boxes, and the scale ε:

\[ N \propto \varepsilon^{-D} \]

The practical procedure determines how detail changes are found (i.e. the number of boxes filled by the image, N) with the change of the scale (ε), and then calculating the slope of the ln regression line for N and ε. Therefore the basic relation that permits to calculate the fractal dimension (here named as \( D_B \), the suffix “B” stands for “Boxes”) is:

\[ D_B = \ln N / \ln \varepsilon \]

The value of the fractal dimension is therefore computed taking the limit for \( \varepsilon \to 0 \) of the relation that links N to ε, that is the slope of the regression line:

\[ D_B = \lim_{\varepsilon \to 0} [\ln N / \ln \varepsilon] \]
The software HarFa

Harmonic and Fractal Image Analysis\textsuperscript{[23]} (5.5L light version) was used. HarFa calculates the slope of the linear portion of the ln function which gives the fractal dimension [specifically, the parameter defined by the software as $D_{BBW}$ was considered, which is based on the boxes filled by the black (B) images and by the black & white border (BW)]. The software calculates the linear portion of the line that is plotted on the data points. A routine calculates the length of analyzed data points segment ($L_{DP}$), in order to improve the accuracy of the fractal dimension estimation. Besides the classical routine, called Single Slope Analysis, the software permits also to obtain an Overall Slope Analysis, which consists of calculating all possible values of $L_{DP}$ (from 3 to count of data points). This tool gives a histogram of the fractal dimension count, where the most probable value of correct fractal dimension is that with the largest count\textsuperscript{[23]}.

RESULTS AND DISCUSSION

Figure 4 presents an exemplificative calculation of the fractal dimension $D_B$ obtained with the ESI spec-

![Figure 4: Exemplificative output from the analysis of an ESI spectrum (positive ion mode) using the routines available in HarFa software\textsuperscript{[23]} for the calculation of the fractal dimension. Upper figure: plot of the box count points; lower figure: histogram of the fractal dimension counts.](image-url)
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Multivariate analysis, such as cluster analysis (Figure 5), suggested the possibility of subdividing plant extracts on the basis of the fractal dimension of their spectra. These preliminary exploratory results indicated that a higher complexity of the pattern, that in turn led to an overall higher fractal dimension value, in comparison to the negative ion mode. TABLE 1 shows the fractal dimension $D_B$ obtained using the ESI spectra (positive and negative ion mode) of some herbal extracts, selected among those commonly used in phytotherapy and as dietary supplements. The results obtained suggest a direct relation between a higher fractal dimension value and the complexity of the spectrum, representing the occurrence of a more heterogeneous mixture of phytochemicals.

TABLE 1: Fractal dimension $D_B$ obtained with the ESI spectra (positive and negative ion mode) of some plant extracts.

<table>
<thead>
<tr>
<th>Plant Extract</th>
<th>Positive Ion mode</th>
<th>Negative Ion mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamamelis virginiana</td>
<td>1.4403</td>
<td>1.2656</td>
</tr>
<tr>
<td>Centella asiatica</td>
<td>1.2720</td>
<td>1.2325</td>
</tr>
<tr>
<td>Eschscholtzia californica</td>
<td>1.2397</td>
<td>1.2297</td>
</tr>
<tr>
<td>Melissa officinalis</td>
<td>1.3360</td>
<td>1.2848</td>
</tr>
<tr>
<td>Passiflora incarnata</td>
<td>1.3591</td>
<td>1.2813</td>
</tr>
<tr>
<td>Ruscus aculeatus</td>
<td>1.4241</td>
<td>1.3752</td>
</tr>
</tbody>
</table>

Fractal analysis might represent a tool for the phytochemical characterization of a single herbal extract. Also the ESI spectrum obtained with a mixture of plant extracts, such as those contained in a commercial herbal dietary supplement product (Grintuss syrup adults, medical device, Aboca), provided a fractal pattern; statistical analysis on several replicates obtained with different batches permitted to show that the fractal dimension tends to display a normal distribution around a mean/median value (Figure 6), which might be suggested as a typical feature for that product, to be possibly used also for reference purposes in quality control issues. In this context, the ESI method appears to show similar opportunities for a fractal approach like chromatographic or colorimetric methods previously presented in the literature\[20,21\], but with the advantage of a more complete and definite pattern of signals, proportional to the product composition.

Further, we tried to use the fractal approach to characterize the repeatability of the instrumental measure. Another commercial product containing herbal extracts (Natura Mix syrup, Aboca) was measured several times in the same day and in the following days. The results, both for positive ions and negative ions are shown in Figure 7, suggesting a limited rate of change among measurements in positive ion mode. However, the overall variability was higher with the negative ion mode,
and in addition, the net value of fractal dimension appeared very low, due to the scarceness of the overall ionic current signal. These results suggest that the fractal approach provides reproducible data, and is linked to the quality and complexity of the spectra.

To investigate the usefulness of the approach to evaluate the stability of a herbal product, ESI spectra obtained with plant extracts (Natura Mix syrup, Aboca) which received an artificial treatment with heat were also analyzed with fractal analysis. The procedure of thermal exposure of a pharmaceutical is a well recognized method for prediction of its chemical stability. The extracts underwent thermal treatment to simulate aging or extreme storage conditions. As Figure 8 shows, the fractal dimension appears to indicate variations following thermal treatment, suggesting that this measure could be useful also to evaluate the changes occurring with aging of the product, in order to detect the proper conditions of storage and stability control intervention.

Figure 7: Fractal dimension obtained with repeated measurements (n=10) of the same commercial herbal mixture extracts (Natura Mix syrup, Aboca). Upper graph: positive ion mode ESI; lower graph: negative ion mode ESI.

To present the variations occurring with the data in a more direct way, again drawing on the fractal world, we might use a fractal-like shape of immediate visual impact (for example, a fern) automatically generated with a fractal image-software (ChaosPro). The complexity of the shape of the fern is proportional to the value of the fractal dimension; for example, fractal dimension $D=1$ (left) or $D=1.30$ (right).

Figure 8: Thermal stability of a herbal product (Natura Mix syrup, Aboca) evaluated by fractal dimension analysis. The extract underwent thermal treatment to simulate aging or extreme storage conditions. “Not pasteurized”, is the extract before any pasteurization process; “basal”, after pasteurization; 15m at 40°C, 15 minutes of thermal treatment at 40°C; 30m at 40°C, 30 minutes of thermal treatment at 40°C; 7m at 50°C, 7 minutes of thermal treatment at 50°C.

Figure 9: Image representation of fractal dimension by means of ChaosPro software. The complexity of the shape of the fern is proportional to the value of the fractal dimension; example, fractal dimension $D=1$ (left) or $D=1.30$ (right).
Conclusions

Fractal dimension might be proposed as a new promising technique of investigation to be used, coupled to mass spectrometry, as a summary measurement of phytochemical complexity of the overall composition of a herbal product and of its stability.

References

[1] (a) L. Mattoli, M. Burico, V. Mercati, P. Traldi, E. Ragazzi; Planta Medica, 78, 1257 (2012); (b) Part of this paper has been presented as preliminary result at the ICNPR (International Congress on Natural Products Research), New York, USA, 28 July - 1 August (2012).