Impact of succussion on pharmaceutical preparations analyzed by means of patterns from evaporated droplets

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The aim of the present study was to investigate if patterns obtained from evaporating droplets of pharmaceutical preparations reveal the impact of succussion on such medicinal products. For this purpose, five pharmaceutical preparations (Echinacea $10^{-2}$, Baptisia $10^{-3}$, Baptisia $10^{-4}$, Luffa $10^{-4}$, and Spongia $10^{-6}$) were prepared according to the European Pharmacopoeia guidelines for the production of homeopathic remedies, in three variants each: with varying numbers of succussion strokes (i) 100, (ii) 10 (succussed samples), and (iii) zero (gently mixed, unsuccussed sample). System stability was studied by means of systematic positive control experiments. Patterns were evaluated by means of computerized image analysis regarding grey level distribution, texture, and fractality. For all investigated pharmaceutical preparations, significant differences were found between the succussed and gently mixed samples; whereas, all three samples (prepared with 100, 10 and zero succussion strokes) could be significantly differentiated for Luffa $10^{-4}$ and Spongia $10^{-6}$ for one image evaluation parameter each. Control experiments showed a reasonable stability of the experimental set-up.

It is known that shaking a solution may have impact on proteins it contains1,2; the introduction of air bubbles into the solution3, as also the action of sharing forces, may trigger oxidation processes and aggregation of these molecules4–6. Solely, an accidental dropping of a vial has been reported to modify some proteins in suspension2. In pharmaceutical preparations, in some cases shaking and the thereby induced aggregation of proteins may influence their properties; therefore, the development of measures mitigating the shaking influence, like for instance development of new coatings for pre-filled syringes, is important and is addressed in recent investigations6.

The impact of agitation upon liquid pharmaceutical products has been investigated by means of various analytical approaches, including methods analyzing the particle formation (micro-flow imaging, dynamic light scattering, light obscuration method), protein degradation (size exclusion chromatography, tryptic digestion/HPLC), formation of free radicals (hydroxyphenyl fluorescein assay), and flow dynamics occurring during agitation (high speed imaging). Furthermore, different spectroscopy methods (fluorescence spectroscopy, Fourier transform infrared spectroscopy) and calorimetric methods (differential scanning calorimetry) have been applied for accessing the characteristics of agitated samples1–6. Here we propose for the first time to apply the droplet evaporation method (DEM) to access the characteristics of agitated pharmaceutical preparations in a comparably quick and integral manner.

Recently, methods based on droplet evaporation find application in various fields of science and technology, as for instance in fabrication of novel materials, microelectronics, ink-jet printing, coating technologies, bioassay manufacturing, condensation of solutes7–9, and also for analytical purposes. Among DEM’s analytical applications the most studied one is medical diagnosis9,10. It is based on the idea that in the case of some diseases patterns formed in desiccated droplets of some specific corporal fluids (e.g. blood, serum, tears, sweat) would differ depending on whether the fluid was taken from a diseased or healthy donor, since the disease would specifically modify the composition of the fluid.

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In a previous study\textsuperscript{11}, we have proposed DEM as a tool for a phenomenological, multi-factorial characterization of pharmaceutical preparations in a low dilution range ($10^{-2}$-$10^{-6}$). The corresponding experimental procedure consists in the evaporation of droplets of the diluted pharmaceutical preparations under controlled conditions, the consecutive inspection of patterns formed in droplet residues under an optical microscope with dark-field, and computerized image evaluation. In the present study further investigations by means of the same experimental protocol were conducted to determine, if it is possible to ‘visualize’ through the formation of self-assembled patterns any differences between succussed and unsuccussed samples; and furthermore, if the number of succussion strokes ($N_s$) performed would show any impact on the patterns.

We have chosen to investigate the impact of shaking on pharmaceutical products according to the guidelines for homeopathic preparations, since the application of succussion is a mandatory procedure according to the European Pharmacopoeia\textsuperscript{12}. The corresponding processing of pharmaceutical preparations from a given liquid substance consists in subsequent dilution steps (in a defined dilution ratio), each followed by succussion (i.e. introduction of some kind of motion into the liquid, mostly vigorous).

The choice of the pharmaceutical preparations was based on both their pattern forming properties (dendrite formation was preferably chosen)\textsuperscript{13} and their presence in the product Sinusitis Hevert SL. We investigated five different pharmaceutical preparations of vegetal (Echinacea $10^{-2}$, Baptisia $10^{-3}$, Baptisia $10^{-4}$, Luffa $10^{-4}$) and animal (Spongia $10^{-4}$) origin, prepared in three different variants each: succussed by the application of 100 or 10 strokes (succussed samples), or without succussion (only gently mixed control sample). The agitation technique applied was adopted from the production protocol as used by the pharmaceutical company Hevert-Arzneimittel GmbH & Co.

A crucial point in analytical methods involving images as main experimental output is the image evaluation and the choice of proper evaluation tools and evaluation criteria or parameters. In many studies DEM images were analyzed exclusively by means of visual evaluation\textsuperscript{13}; despite the fact that the human eye is the most precise tool for form recognition, the visual evaluation of patterns may be subjective and it also strictly restricts the size of the image database to be evaluated. In previous studies we introduced the computerized measurement of several image evaluation parameters characterizing the images in terms of their grey level distribution, texture\textsuperscript{11}, and fractality\textsuperscript{14}. The parameter grey level distribution measures the image brightness\textsuperscript{15}, which in case of DEM images provides information on the structures size, thickness of branches, and their brightness. The size of the structure can be assessed in a more precise way by means of the parameter foreground pixel, which measures the structure’s area\textsuperscript{16}, however does not access the brightness. The parameter entropy is an attribute of the grey level co-occurrence matrix measuring how often different pixel brightness values occur in an image; in particular, entropy characterizes the heterogeneity of the brightness values distribution and describes so the image’s disorder\textsuperscript{17}. Finally, the parameter local connected fractal dimension measures the fractal dimension of structures in a pre-defined size range and accesses so the structures complexity\textsuperscript{18}. Moreover, in the present study we added the parameter lacunarity, a complementary measure to fractal dimension, characterizing the gaps in-between the structure elements\textsuperscript{19} and providing so information about the structure’s density.

**Results**

**Qualitative description of the patterns.** When analyzed by means of DEM, the five here investigated pharmaceutical preparations created visually recognizable and easily identifiable patterns (Fig. 1). In case of Echinacea $10^{-2}$, Baptisia $10^{-3}$, and Luffa $10^{-4}$ the patterns consisted of dendritic, fractal-like structures placed in the droplet center. Echinacea $10^{-2}$ created large, dense networks of very fine ramifications, Baptisia $10^{-3}$ created rather small, roundly shaped structures, and Luffa $10^{-4}$ structures made out of rather few and thick dendrites. Baptisia $10^{-4}$ created unspecific patterns consisting of lines, smears, and, in some cases, single dendrites distributed all over the droplet. Whereas, Spongia $10^{-6}$ created one to five filled, wavy forms per droplet, characterized by a concave and a convex side, placed near to each other and facing each other with the concave sides.

In general, in all pharmaceutical preparations, the impact of succussion on the patterns was visually perceptible in a varying, but rather small degree, and it seemed to decrease the structure’s ordering.

**Computerized pattern evaluation.** The results of the computerized pattern evaluation of the pharmaceutical preparations produced with different numbers of succussion strokes ($N_s = 100, 10, 0$), the corresponding systematic positive control experiments, and the F-tests of the analysis of variance for Echinacea $10^{-2}$, Baptisia $10^{-3}$, Baptisia $10^{-4}$, Luffa $10^{-4}$, and Spongia $10^{-6}$ are shown in Tables 1–5, respectively.

**Echinacea $10^{-2}$.** In case of Echinacea $10^{-2}$ (Table 1) application of succussion significantly increased the pattern evaluation parameters grey level distribution (GLD) and entropy (for $N_s = 10, 100$). Also, the fractality parameters local connected fractal dimension (LCFD) and lacunarity increased following the succussion, however, LCFD only for $N_s = 10$ and lacunarity only for $N_s = 100$.

All systematic control experiments performed did not show any significance between the randomization groups for the main effects.

**Baptisia $10^{-3}$.** As shown in Table 2, Baptisia $10^{-3}$ succussed samples ($N_s = 100, 10$) were characterized by significantly lower GLD, entropy, FP, and LCFD values compared to the unsuccussed samples, whereas lacunarity was significantly higher.

The systematic control experiments yielded a significant main effect for the parameters FP and entropy; the other three image analysis parameters did not show statistically significant differences between the randomization groups for the main effects. Thus, the main experiments’ outcome regarding FP and entropy can be distorted due to chamber gradients (see below) and was excluded from further evaluation.
Baptisia $10^{-4}$. In case of Baptisia $10^{-4}$ the parameter FP could differentiate significantly between all samples ($N_S = 0, 10, 100$); whereas the parameters GLD, entropy, LCFD, and lacunarity differentiated between the succussed ($N_S = 10, 100$) and unsuccussed ($N_S = 0$) samples (Table 3).

The systematic control experiments yielded a significant main effect for the parameter FP; the other four image analysis parameters did not show statistically significant differences between the randomization groups for the main effects. Thus, the main experiments’ outcome regarding FP can be distorted due to chamber gradients (see below) and was excluded from further evaluation.
way analysis of variance for the factors NS groups.

In the order NS 0 of analysis of variance of the main experiments the F value for the factor NS groups.

Table 1. Results of pattern evaluation of Echinacea 10–2 samples prepared with different numbers of succussion strokes (NS = 100, 10, or 0) and systematic positive control (SPC) experiments (on the left) and F-test of the two-way analysis of variance for the factors NS and day (on the right). Mean values with different letter codes (a, b, c) are significantly different (p < 0.05). LEGEND: N – number of patterns; NS – number of succussion strokes; GLD – grey level distribution; FP – foreground pixels; LCFD – local connected fractal dimension; LAC – lacunarity; * – p < 0.05; ** – p < 0.01; *** – p < 0.001; ns – not significant.

Table 2. Results of pattern evaluation of Baptisia 10–3 samples prepared with different numbers of succussion strokes (NS = 100, 10, or 0) and systematic positive control (SPC) experiments (on the left) and F-test of the two-way analysis of variance for the factors NS and day (on the right). Mean values with different letter codes (a, b, c) are significantly different (p < 0.05). LEGEND: N – number of patterns; NS – number of succussion strokes; GLD – grey level distribution; FP – foreground pixels; LCFD – local connected fractal dimension; LAC – lacunarity; * – p < 0.05; ** – p < 0.01; *** – p < 0.001; ns – not significant.

Luffa 10–4. For Luffa 10–4 GLD, FP, and LCFD decreased significantly in the succussed samples, whereas lacunarity increased (Table 4). Parameter lacunarity significantly differentiated all samples (NS = 100, 10, and 0); whereas parameter entropy showed no significance between the samples in the main experiments.

No systematic control experiment performed showed significant main effects between the randomization groups.

Spongia 10–6. In case of Spongia 10–6 (Table 5) parameter LCFD differentiated all samples and ranked them in the order NS 0 > 100 > 10; whereas lacunarity yielded significantly higher values only for the sample NS = 10. Parameter entropy differentiated the succussed samples (NS = 100, 10) from the unsuccussed ones. The parameters GLD and FP did not differentiate the samples.

No systematic control experiment performed showed significant main effects between the randomization groups.

Influence of succussion on DEM patterns. In order to summarize the experimental results, in Table 6 we considered as relevant only cases where the corresponding image analysis parameter was experimentally stable, which means that (i) the systematic positive control experiments were not significant, and (ii) in the F-test of analysis of variance of the main experiments the F value for the factor NS was higher than the F value for the interaction NS and day. This means that 16 out of 25 parameter/preparation combinations were retained.
Results of pattern evaluation of Baptisia 10⁻⁴ samples prepared with different numbers of succussion strokes (Nₛ = 100, 10, or 0) and systematic positive control (SPC) experiments (on the left) and F-test of the two-way analysis of variance for the factors Nₛ and day (on the right). Mean values with different letter codes (a, b, c) are significantly different (p < 0.05). LEGEND: N – number of patterns; Nₛ – number of succussion strokes; GLD – grey level distribution; FP – foreground pixels; LCFD – local connected fractal dimension; LAC – lacunarity; * – p < 0.05; ** – p < 0.01; *** – p < 0.001; ns – not significant.

Overall, we observed significant differences for at least one sample (Nₛ = 100 or 10) compared to Nₛ = 0 in all analyzed comparisons (100, 16/16). In most cases (68.75% of comparisons, 11/16), the difference was between the succussed (Nₛ = 100, 10) and unsuccussed (Nₛ = 0) samples, without differentiating between the succussed samples. In 12.50% (2/16) of cases all samples (Nₛ = 100, 10, and 0) could be significantly differentiated; in 12.50% (2/16) of cases the Nₛ = 10 sample differed from the two others (Nₛ = 0, 10); and in one case (6.25%, 1/16) the Nₛ = 100 sample differed from the two others (Nₛ = 0, 10).

Generalizing, it can be said that the GLD did not show a general direction of the influence of the succussion on the patterns; whereas in patterns from the succussed samples the pattern evaluation parameter entropy increased, and LCFD decreased. Lacunarity was the unique parameter showing significant differences for all pharmaceutical preparations and in general showed increased values in the succussed samples. FP differentiated the samples only in case of one remedy (Luffa 10⁻⁴).

**Climatized chamber gradients.** Results of the F-test of the two-way analysis of variance with independent factors row and column from the systematic positive control experiments performed with *Echinacea* 10⁻⁴, *Baptisia* 10⁻⁴, *Baptisia* 10⁻³, *Luffa* 10⁻⁴, and *Spongia* 10⁻⁴ are shown in Table 7. As it can be noticed, factor row showed significance for most image evaluation parameters of the patterns obtained from the five pharmaceutical preparations (14 results out of 25; 14/25), whereas factor column was significant only in one case (*Luffa* 10⁻⁴, parameter lacunarity). The interaction between factors row and column resulted also significant in 8/25 cases, however mostly with lower F values than those observed for factor row.
way analysis of variance for the factors NS substances, since the sponge is roasted) through the introduction of air bubbles and/or particle formation2.

Results of pattern evaluation of Spongia 10−6 samples prepared with different numbers of succussion strokes (NS = 100, 10, or 0) and systematic positive control (SPC) experiments (on the left) and F-test of the two-way analysis of variance for the factors NS and day (on the right). Mean values with different letter codes (a, b, c) are significantly different (p < 0.05). LEGEND: N – number of patterns; NS – number of succussion strokes; GLD – grey level distribution; FP – foreground pixels; LCDF – local connected fractal dimension; LAC – lacunarity; * – p < 0.05; ** – p < 0.01; *** – p < 0.001; ns – not significant.

The quasi-randomization design applied in the differentiating experiments could eliminate the significant influence of chamber gradients (Table 7) in total in 13/14 cases and in the 16 retained experiments in 11/11 cases (Tables 1–5).

Discussion

The results of the present study show that in all five analyzed pharmaceutical preparations the succussion strokes applied during production significantly influenced the DEM patterns. It can be summarized that succussion induced the formation of structures characterized by a greater disorder (parameter interaction) and smaller complexity (parameter local connected fractal dimension), at the same time increasing the gaps between the structure elements (parameter lacunarity). In case of two preparations (Luffa 10−4 and Spongia 10−6), significant differences could be found between all samples (NS = 0, 10, and 100). The here chosen parameters have already been applied in structure analysis of patterns formed in course of phase transition of liquid pharmaceutical preparations41; moreover, raw material surfaces present in pharmaceutical triturations were also analyzed by means of fractal dimension18.

DEM patterns in the here analyzed dilution range 10−2–10−6 are in a first place a function of solute dry residue. Differences found between the patterns of succussed vs. not succussed samples might be linked with succussion-induced aggregation of large-size molecules18, or, in case of Spongia 10−6 (consisting only of mineral substances, since the sponge is roasted) through the introduction of air bubbles and/or particle formation1.

Whereas the patterns of Echinacea 10−2, Baptisia 10−3, Luffa 10−4, and Spongia 10−6 were concentrated in the central part of the droplet residue and fitted entirely on the photographed in 100× image, in case of Baptisia 10−4 the structures were rather unspecific and distributed almost evenly through the entire droplet residue (Fig. 1). In order to keep the magnification equal in the whole experimentation series, the part to be photographed was chosen by the experimenter (based on a visual check of the pattern, the part with most evident structures was

### Table 5

| Spongia 10−6 | SPC | Factor | SPN | F | p |
|-------------|-----|--------|-----|---|---|
| N | N | Mean | N | N | Mean |
| GLD | 100 | 131 | 1.97 b | 10 | 120 | 0.72 a | 10 | 119 | 0.80 a | 15.68 | 0.0001*** |
| 0 | 128 | 2.31 a | 10 | 115 | 0.82 a | 2.50 | 0.0249** |
| Entropy | 100 | 131 | 1.77 a | 10 | 120 | 1.03 a | 10 | 119 | 1.02 a | 21.56 | 0.0001*** |
| 0 | 128 | 1.83 a | 10 | 115 | 0.98 a | 7.05 | 0.0001*** |
| FP | 100 | 131 | 3.42 × 10 a | 10 | 120 | 1.72 × 10 a | 10 | 119 | 1.71 × 10 a | 0.0000*** |
| 0 | 128 | 3.42 × 10 a | 10 | 115 | 1.35 × 10 b | 0.0008 | 1.0180* |
| LCDF | 100 | 131 | 1.54 b | 10 | 120 | 1.48 a | 10 | 119 | 1.47 a | 16.65 | 0.0001*** |
| 0 | 128 | 1.47 c | 10 | 115 | 1.47 a | 1.35 | 0.0001*** |
| LAC | 100 | 131 | 0.16 b | 10 | 120 | 0.18 a | 10 | 119 | 0.20 a | 11.11 | 0.0001*** |
| 0 | 128 | 0.19 a | 10 | 115 | 0.18 a | 1.44 | 0.02207 ns |

### Table 6

| Echinacea 10−2 | Baptisia 10−3 | Baptisia 10−4 | Luffa 10−4 | Spongia 10−6 |
|---------------|---------------|---------------|-------------|--------------|
| GLD | 100 | 131 | 1.97 b | 10 | 120 | 0.72 a | 10 | 119 | 0.80 a |
| 0 | 128 | 2.31 a | 10 | 115 | 0.82 a |
| Entropy | 100 | 131 | 1.77 a | 10 | 120 | 1.03 a | 10 | 119 | 1.02 a |
| 0 | 128 | 1.83 a | 10 | 115 | 0.98 a |
| FP | 100 | 131 | 3.42 × 10 a | 10 | 120 | 1.72 × 10 a | 10 | 119 | 1.71 × 10 a |
| 0 | 128 | 3.42 × 10 a | 10 | 115 | 1.35 × 10 b |
| LCDF | 100 | 131 | 1.54 b | 10 | 120 | 1.48 a | 10 | 119 | 1.47 a |
| 0 | 128 | 1.47 c | 10 | 115 | 1.47 a |
| LAC | 100 | 131 | 0.16 b | 10 | 120 | 0.18 a | 10 | 119 | 0.20 a |
| 0 | 128 | 0.19 a | 10 | 115 | 0.18 a |

The graphs represent the relevant differences found in the image evaluation parameters in pharmaceutical preparations prepared with varying numbers of succussion strokes NS = 100, 10, or 0. Different letters (a, b, c) indicate significant differences at p < 0.05. LEGEND: GLD – grey level distribution; FP – foreground pixels; LCDF – local connected fractal dimension; LAC – lacunarity.
The analysis of the systematic positive control experiments by the F-test of analysis of variance with independent factors row and column put in evidence that the factor row significantly influenced 14/25 parameters (Table 7). In most cases (13/14) this systematic error could be successfully eliminated (Tables 1–5) by the application of a quasi-randomization design, consisting in the randomization of the samples only within the columns, keeping simultaneously an even distribution of the samples within the rows. In future experiments, however, a better isolation of the inner-chamber should be aimed at to improve the homogeneity of evaporating conditions.

The influence of the factor day was significant in most of the here presented experiments (24/25 differentiation and 23/25 control experiments) (Tables 1–5). A significant influence of the experimentation day has been reported in many previous studies concerning methods based on phase-transition-induced pattern formation11,13,14,19–22. This fact might be due to some day-to-day variations in the experiment performance or experimental conditions; or to other yet unknown and uncontrolled influences.

To conclude, we observed that the impact of agitation on solutions, which has great importance for fabrication and distribution of pharmaceutical preparations in general and which is addressed in many recent investigations. In particular, it might serve to compare the role of several factors known for being critical for the solution properties, like for instance the kind of induced flow (e.g. chaotic vs. ordered, vortex-like)2,3,23, different surfaces and coatings of the recipient’s walls24,25, different volumes also their intensity and type of movement.

The here presented experimental protocol might constitute a fairly economic and quick tool to investigate the impact of agitation on solutions, which has great importance for fabrication and distribution of pharmaceutical preparations in general and which is addressed in many recent investigations. In particular, it might serve to compare the role of several factors known for being critical for the solution properties, like for instance the kind of induced flow (e.g. chaotic vs. ordered, vortex-like)2,3,23, different surfaces and coatings of the recipient’s walls24,25, different volumes also their intensity and type of movement.

Table 7. F-test results of the analysis of variance with independent factors row and column of the systematic positive control experiments for *Echinacea* 10⁻², *Baptisia* 10⁻², *Baptisia* 10⁻⁴, *Luffa* 10⁻⁴, and *Spongia* 10⁻⁶ prepared by applying 10 succussion strokes in-between the dilution steps. LEGEND: GLD – grey level distribution; FP – foreground pixels; LCFD – local connected fractal dimension; LAC – lacunarity; * p < 0.05; ** p < 0.01; *** p < 0.001; ns – not significant.

| Factor | *Echinacea* 10⁻² | *Baptisia* 10⁻² | *Baptisia* 10⁻⁴ | *Luffa* 10⁻⁴ | *Spongia* 10⁻⁶ |
|--------|----------------|----------------|----------------|-------------|--------------|
|        | F   | p   | F   | p   | F   | p   | F   | p   | F   | p   |
| GLD Row | 3.35 | 0.0191* | 3.52 | 0.0152* | 3.29 | 0.0028* | 1.58 | 0.1934 ns | 2.38 | 0.0691 ns |
| Column | 0.97 | 0.0364 ns | 2.49 | 0.0083 ns | 2.46 | 0.0238* | 1.01 | 0.1496 ns | 1.35 | 0.2592 ns |
| Interaction | 0.97 | 0.4443 ns | 2.49 | 0.0223* | 1.22 | 0.2968 ns | 0.81 | 0.0910 ns | 0.42 | 0.8625 ns |
| Entropy Row | 2.08 | 0.0101 ns | 1.80 | 0.1457 ns | 4.39 | 0.0047** | 0.71 | 0.5460 ns | 5.20 | 0.0016** |
| Column | 0.97 | 0.3787 ns | 0.85 | 0.4269 ns | 0.75 | 0.4737 ns | 1.60 | 0.2039 ns | 2.03 | 0.1324 ns |
| Interaction | 0.52 | 0.7958 ns | 3.14 | 0.0051** | 2.29 | 0.0351* | 1.83 | 0.0902 ns | 3.33 | 0.0034** |
| FP Row | 4.79 | 0.0027** | 2.33 | 0.00570 ns | 6.20 | 0.0094** | 0.75 | 0.5250 ns | 0.88 | 0.4515 ns |
| Column | 1.06 | 0.3839 ns | 0.90 | 0.4053 ns | 0.15 | 0.8633 ns | 0.21 | 0.8121 ns | 1.05 | 0.3501 ns |
| Interaction | 1.06 | 0.3839 ns | 2.07 | 0.0564 ns | 3.67 | 0.0015* | 0.51 | 0.7990 ns | 0.59 | 0.3732 ns |
| LCFD Row | 2.95 | 0.0032* | 2.34 | 0.00729 ns | 5.15 | 0.0017** | 2.65 | 0.0485* | 0.15 | 0.9315 ns |
| Column | 1.24 | 0.2914 ns | 1.47 | 0.2301 ns | 1.08 | 0.3417 ns | 1.54 | 0.2148 ns | 1.46 | 0.2335 ns |
| Interaction | 1.55 | 0.1607 ns | 1.47 | 0.1888 ns | 3.50 | 0.0022** | 1.08 | 0.3757 ns | 1.39 | 0.1218 ns |
| LAC Row | 0.35 | <0.0001*** | 3.95 | 0.0086** | 5.69 | 0.0088*** | 3.90 | 0.0091** | 0.34 | 0.7994 ns |
| Column | 0.77 | 0.5902 ns | 2.09 | 0.1245 ns | 2.02 | 0.1403* | 3.69 | 0.0258* | 1.37 | 0.2549 ns |
| Interaction | 0.77 | 0.5902 ns | 2.03 | 0.0605 ns | 2.23 | 0.0240* | 0.24 | 0.9640 ns | 2.01 | 0.0639 ns |
Study design. The experimentation took place in the laboratories of Society for Cancer Research (Arlesheim, Switzerland). As shown in Fig. 2 the study consisted of main experiments and full systematic positive control experiments. The main experiments were performed on five pharmaceutical preparations (Echinacea $10^{-2}$, Baptisia $10^{-3}$, Baptisia $10^{-4}$, Luffa $10^{-4}$, and Spongia $10^{-6}$) prepared from the $10^{-1}$ dilutions by applying different numbers of succussion strokes ($N_s = 100, 10, 0$). These three variations of a given homeopathic preparation were analyzed in one experimental run, consisting of twelve slides with droplets deposited on them (Fig. 3). Four slides were used for each pharmaceutical preparation. The slides were distributed in a climatized chamber following a quasi-randomization design. Each main experiment had a corresponding systematic positive control experiment where the analyzed sample was prepared three times with $N_s = 10$ and analyzed following the same quasi-randomization design. All experiments were independently repeated three times.

Preparation of pharmaceutical preparations for analysis. 0.8 g of a pharmaceutical preparation in dilution $10^{-1}$ was weighed and placed in a sterile glass cylinder (SBR-ET, Mix Cyl. 10 ml, B; Brand GmbH + CO KG, Wertheim, Germany) with stopper (untargeted volume 13 ml); subsequently 7.2 ml purified water according to Pharm. Eur. 9.412 (“purified water in bulk”, X-SEPTRON LINE 10 VAL, BWT AQUA AG, Aesch, Switzerland) was added in order to reach a dilution of 1:9. The cylinder was closed tightly; 10 or 100 succussion strokes were applied by hand. The movement to achieve succussion was performed in the air without hitting against a firm base. For the unsuccussed samples, the content of the cylinder was mixed with a glass stirrer by performing circular movements in order to not create any foam. After the settling of any foam in preparations $N_s = 10$ and 100, the cylinders were re-opened and 0.8 ml of the solution were taken for the preparation of the next dilution, as described previously. In this way three variants ($N_s = 100, 10, 0$) of each preparation (Echinacea $10^{-2}$, Baptisia $10^{-3}$, Baptisia $10^{-4}$, Luffa $10^{-4}$, and Spongia $10^{-6}$) were produced. All samples were prepared fresh for each experiment. The samples were not blinded.

Droplet evaporation method. Microscope slides ($76 \times 26$ mm, pre-cleaned, cut edges; Thermo Scientific, Gerhard Menzel B.V. & Co. KG, Braunschweig, Germany) were degreased by washing them with a dishwasher liquid, then thoroughly rinsed with hot tap water, and placed in 4 consecutive purified water baths. Each slide was wiped dry with a laboratory wiper (KIMTECH science, Kimberly-Clark Professional, Roswell, Canada) just before droplet deposition. 3 μl droplets of the tested pharmaceutical preparation were deposited on the slides in two parallel rows, 7 droplets per row, by the use of a micro-pipette of 20 μl capacity (Eppendorf Research Plus, Eppendorf, Hamburg, Germany).

Evaporation took place in an incubator (KBF 720, cooled incubator with controlled humidity system, WTB Binder Labortechnik GmbH, Tuttingen, Germany) with an inner plexi-glass-chamber with a semi-permeable cover placed on a vibration absorbing basis. The microscope slides with droplets were placed in the inner-chamber and left for evaporation in 26°C and 44% rH for 1 hour. The slide distribution inside the chamber followed a quasi-randomization design in order to provide a uniform arrangement of the samples within the rows (Fig. 2).
false-negative conclusions. Results of the transformed data sets were back-transformed for presentation.

gives a good safeguard against type I as well as type II errors, and thus balances well between false-positive and

test (pairwise comparisons were evaluated only if the global F-test was significant at p < 0.05). This procedure
gives a good safeguard against type I as well as type II errors, and thus balances well between false-positive and
false-negative conclusions. Results of the transformed data sets were back-transformed for presentation.

**Statistical analysis.** The data deriving from the computerized image analysis were analyzed by means of a
two-way analysis of variance (CoStat, v. 6.311) (CoHort Software, Monterey, USA) at alpha = 0.05 with independent
factors number of succussion strokes (N), day or row and column. An interaction term between the independent
factors was included in the statistical model in order to assess stability and reproducibility. Distribution of data was
checked by visual inspection. Slight deviations from normality were irrelevant due to the central limit theorem.
Data-sets with larger deviations from normality were logarithmically transformed (log10); in total 18 data sets were
checked by visual inspection. Slight deviations from normality were irrelevant due to the central limit theorem.

Photographing of patterns. The droplet residues were examined and photographed in dark field in magnification
100× by use of an optical microscope (Zeiss Lab.A1; Carl Zeiss Microscopy GmbH, Jena, Germany)
with an attached camera (Motimic 5.0 MP; CMOS; Motic Electric Group Co., Ltd, Xiamen, China). Droplets
with disturbed crystallization due to presence of contaminating particles or due to edge effects on the slide were
not considered. Per experiment (one chamber-run, Fig. 3), 168 droplets were prepared (14 droplets x 12 slides).

For *Echinacea* 10−2 the three main experiments yielded 399 evaluable droplet residue images and the three
positive control experiments 406 images (399/406); for *Baptisia* 10−2 415/387; for *Baptisia* 10−3 461/386; for *Luffa*
10−3 410/413; and for *Spongia* 10−3 395/354, giving in total 4'026 images. Images were saved in jpeg-format
(2592 × 1944 pixel).

In case of *Echinacea* 10−2, *Baptisia* 10−2, *Luffa* 10−4, and *Spongia* 10−6, the 100X images included the whole
structure formed inside the droplet; whereas, in case of *Baptisia* 10−3, only selected parts of the structure were
included, chosen by the experimenter on the basis of density and intensity of forms.

Computerized pattern evaluation. Image analysis was performed with the software ImageJ (v. 1.50b)27
with the plug-ins GLCM Texture28 and Frac-Lac29. All 100× images were subjected to a background extraction by
means of the sliding paraboloid with rolling ball radius set at 50 pixels ensuring same background throughout the
image database. Consecutively the images were analyzed (i) for their grey-level distribution, (ii) after conversion
into 8-bit type, by running the GLCM algorithm (considering distances between pixel pairs of 4 pixels and angles
of 90°), for their texture (parameter entropy), and (iii) after conversion into binary, by means of Frac-Lac’s DLC tool
with odd sizes scaling method and size limits for the grid caliber series of minimum 4 and maximum 40
pixels, for the size of the structures (parameter foreground pixels), complexity (parameter local connected fractal
dimension), and characterization of the gaps between the structure elements (parameter lacunarity). After conver-
sion into binary, 68 *Echinacea* 10−2 images could not be used due to a too dense ramification-network, and were
excluded from fractality analysis. Whereas, in case of *Baptisia* 10−3 and *Luffa* 10−4, fractal analysis was performed
on images reduced in size to 500 × 375 pixel.

Data availability
The datasets generated and analyzed during the current study are available from the corresponding author on
reasonable request.

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Author contributions
M.O.K., S.W., and S.B. designed the experimental set-up of the study. Experiments were performed by M.O.K. Data were extracted and statistically analyzed by M.O.K. Statistical analysis was independently cross-checked by S.B. The manuscript was written by M.O.K., S.B. and S.W. All authors approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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