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Empirical investigation of preparations produced according to the European Pharmacopoeia monograph 1038

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ABSTRACT

According to the European Pharmacopoeia monograph 1038 (*Praeparationes homoeopathicae*), homeopathic preparations are produced by successive dilution and succussion steps. Dilution levels beyond Avogadro's limit, however, render specific effects implausible according to standard scientific knowledge. Accordingly, we were interested in a critical empirical investigation of preparations produced according to this monograph.

Within a precursor study we developed a bioassay based on a fingerprint metabolomic analysis of *Lepidium sativum* seeds germinated in vitro in either homeopathic preparations or controls in a blinded and randomized assignment. Results of the precursor study were not consistent with the hypothesis that the effects of a *Stannum metallicum* 30x preparation are identical to placebo.

In the present study we investigated the reproducibility of these effects after scrutinizing and optimizing experimental procedures. Ten independent experiments were performed in a blinded and randomized assignment in two independent laboratories. Additionally, 10 systematic negative water control experiments were performed in both laboratories to critically assess the stability of the experimental set-up.

The effects of the *Stannum metallicum* 30x treatment were reproduced. The systematic negative control experiments did not yield false-positive results, indicating a stable experimental set-up.

We thus repeatedly observed biological effects conflicting with the assumption that *Stannum metallicum* 30x is identical to placebo. We therefore wish to discuss whether these findings are to be considered a scientific anomaly or whether they might stimulate further investigations to clarify whether application of the European Pharmacopoeia monograph 1038 may result in pharmaceutical preparations with specific effects.

1. Introduction

Within some branches of Complementary and Alternative Medicine, serially diluted and succussed or "homeopathic" preparations – produced according to the European Pharmacopoeia monograph 1038 (EDQM, 2017) – are being used for therapeutic purposes. In general, practitioners prescribe these preparations under the assumption that these exert specific remedy effects (Fisher, 2012). For higher dilution levels any such presumed specificity is being questioned (Pandolfi, 2010; Sehon and Stanley, 2010; Smith, 2012) due to the fact that the number of remedy-specific molecules is too low in order to induce any specific effects.

There are more than 260 randomized controlled trials comparing homeopathic preparations with placebo (Mathie et al., 2013). An older review came to the conclusion that > 75% of all clinical trials analysed yielded positive results for the homeopathic treatments (Kleijnen et al., 1991). There are meta-analyses for specific medical conditions which showed significant results in favour of specific homeopathic remedy effects (Wiesenauer and Lüdtke, 1996; Taylor et al., 2000; Jacobs et al., 2003; Boehm et al., 2014), whereas some other meta-analyses were inconclusive due to too low study quality (Barnes et al., 1997; Mathie et al., 2012). General meta-analyses – not focusing on specific medical conditions – arrive at inconsistent conclusions (Linde et al., 1997; Linde et al., 1999; Shang et al., 2005; Lüdtke and Rutten, 2008; Mathie et al., 2014; Mathie et al., 2017). Irrespective of any positive empirical evidence from clinical trials, however, clinical use of homeopathic preparations will remain to be controversially discussed as long as the mode of action is unclear (Sehon and Stanley, 2010).

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One of the aims of preclinical research into homeopathic potentisation according to Ph. Eur. monograph 1038 is to reveal whether this monograph describes procedures merely for the sake of safety, or whether these might indeed result in pharmaceutical preparations with specific biological effects. Preconditions for any such research are stable and reliable preclinical test systems that either measure defined physico-chemical properties or yield consistent biological effects of homeopathic preparations.

There are > 1000 basic research publications dealing with preclinical effects of homeopathic preparations (Clausen et al., 2011). However, we do not know a single experimental model that yielded consistent results in all replication trials (Endler et al., 2015), although there are promising candidates (Endler et al., 2015; Ücker et al., 2018). Assuming as a thought experiment, that monograph 1038 leads to pharmaceutical preparations with specific effects, the problems in setting up reproducible test-systems might be due to various reasons: (I) instabilities in the experimental set-up, (II) unknown and therefore uncontrolled critical parameters influencing the response of the experimental set-up, (III) inadequate outcome measures, (IV) underpowered replication trials or (V) inherent properties of homeopathic preparations (Baumgartner, 2009).

In a previous investigation we evaluated the potential of a fingerprint metabolomic approach integrated into a seedling test system for homeopathic preparations (Baumgartner et al., 2012). This approach is based on the generation and subsequent evaluation of dendritic crystallization patterns (i.e. the 'fingerprints'), which emerge when an aqueous dihydrate cupric chloride solution ($CuCl_2{\cdot}2H_2O$) is crystallized on a glass plate in the presence of a water soluble additive (the sample) (Gallinet and Gauthier-Manuel, 1992; Busscher et al., 2010a). Additives can be single molecules as well as complex organic matrices. The crystallization patterns are additive specific (Vester, 1960; Andersen et al., 1998; Shibata et al., 2000; Andersen et al., 2001; Schweizer et al., 2010; Szulc et al., 2010; Kahl et al., 2014; Seidel et al., 2015; Kahl et al., 2016; Fritz et al., 2017) and emerge through a self-organization process which is influenced by the properties of the additive (Busscher et al., 2014). The characteristics of the crystallization patterns are evaluated by human visual evaluation using defined criteria (Huber et al., 2010; Doesburg et al., 2015), and/or by computer-based image-analysis using texture or structure variables (Andersen et al., 1999; Doesburg and Nierop, 2013; Unluturk et al., 2013). This kind of fingerprint metabolomic analysis has been applied to a broad range of additives addressing different research questions such as the distinction between different molecular weights of polyvinylpyrrolidone (PVP) (Andersen et al., 1998; Kahl et al., 2014), the effect of enantiomer mixtures, BSA and Glycogen on the crystallization pattern formation (Vester, 1960; Gallinet and Gauthier-Manuel, 1992; Schweizer, 2007), in the diagnosis of diabetics (Shibata et al., 2000), doping (Shibata et al., 1996) and the early onset of cancer (Barth, 1990; Koopmans, 1990) and other pathologies (Piva, 1994) in human blood, but predominantly in food quality analysis. With respect to the latter, the method has been shown to reflect plant physiological processes like ripening and decomposition (Fritz et al., 2011; Fritz et al., 2017), the effect of processing, feeding regime and farming system (Kahl et al., 2009; Szulc et al., 2010; Kahl et al., 2015; Seidel et al., 2015; Kahl et al., 2016) in a broad range of agricultural products. The use of the method for decades in organic food quality supports the hypothesis of an assessment from an ontological holistic stance. This premise implies evaluating a sample as a whole, which complements analytic approaches (Doesburg et al., 2015).

In the previous investigation *Lepidium sativum* seeds germinated and grew for 4 days in vitro in either homeopathically prepared tin (*Stannum metallicum* 30x) or homeopathically prepared water (30x) in blinded and randomized assignment (Baumgartner et al., 2012). 15 experiments were performed at two laboratories. Fingerprint metabolomic analysis was performed with seedlings extract as additive, extracted in the corresponding homeopathic preparations. The resulting crystallization patterns were analysed by computerized textural image analysis (Carstensen, 1992; Andersen et al., 1999). The 15 available texture analysis variables analysed (henceforth denoted as TA variables), yielded significant results for the homeopathic treatment. This outcome was not consistent with the hypothesis that the effects of a *Stannum metallicum* 30x preparation are identical to placebo, and pointed towards specific effects of the homeopathically prepared tin in a nominal dilution of 10^{-30}.

Two main groups of texture analysis variables could be distinguished, which were closely correlated within the respective group, but not or only weakly correlated to those of the other group: group I (cluster shade and diagonal moment) and group II (all remaining 13 TA variables). In the analysis of variance (ANOVA) model applied, both groups showed main effects of the homeopathic treatment; in addition, variables of group II showed an interaction with experimental day and partially exhibited differences between the two laboratories involved. Thus, texture analysis variables of group I (cluster shade and diagonal moment) were of primary interest for follow-up studies, since they yielded the most stable results.

The conclusion of the previous investigation was that this fingerprint metabolomic analysis yielded a promising complementary outcome measure for plant-based bioassays investigating effects of homeopathic preparations (Baumgartner et al., 2012). However, this study had some methodical weaknesses: the non-sterile preparation of the homeopathic samples, a processing order effect, the lack of full systematic negative control experiments, and the excess in evaporation time in one laboratory leading to unwanted peripheral crystallizations. It seems unlikely however that any of these weaknesses should have led to false-positive results.

In the present investigation, we wanted to critically assess the reproducibility of the effects found in repeated experiments with improved methodology. Therefore, the following changes were planned and implemented: (I) sterile production of the homeopathic preparations, (II) change in person performing potentisation, (III) change in location where the homeopathic samples were prepared (Switzerland instead of Denmark), (IV) optimization of the fingerprint metabolomic analysis laboratory procedures to avoid processing order effects as well as excesses in evaporation time, and (V) full implementation of blinded systematic negative control (SNC) experiments. Two further unintentional changes concerned (VI) the location of the Dutch laboratory that had been completely dismantled and rebuilt in another city, and (vii) the *Lepidium sativum* seed batch that had to be replaced due to a decreased germination rate. Furthermore, (viii) water 30x was replaced by lactose 30x as a more appropriate control sample due to the fact that *Stannum metallicum* is triturated in lactose up to 4x.

In total, 10 independent homeopathic sample sets of *Stannum metallicum* 30x and lactose 30x were produced that were investigated in randomized and coded (blinded) experiments in two independent laboratories in the Netherlands and Denmark. In addition, ten full systematic negative control experiments were performed in each laboratory to control the stability of the experimental set-up and to test the applicability of the statistical model applied. SNC and verum experiments were coded (blinded). SNC experiments were performed with distilled H_20 1x, produced as 10 independent sample sets. We furthermore treated part of the homeopathic samples with cell phone radiation or autoclaving, in order to test the stability of the homeopathic preparations against these physical treatments. However, the evaluation of these treatments is beyond the scope of the current paper. Besides fingerprint metabolomic analysis, we also measured germination rate and length of the *Lepidium sativum* seedlings. In addition, all homeopathic and SNC samples will also be investigated with physical methods.

In the current manuscript, we restrict the data evaluation to the texture analysis variables of group I (cluster shade and diagonal moment) that had been identified in the former study as the outcome variables of predominant interest (Baumgartner et al., 2012). Presentation of the results of the texture analysis variables of group II, as

well as the *Lepidium sativum* seedling germination rate and length data, is beyond the scope of the current paper. The same applies to the presentation and discussion of the physical measurements of the homeopathic preparations.

2. Materials and methods

2.1. General experimental procedure

The general experimental procedure was according to Baumgartner et al. (2012). In short: *Lepidium sativum* seedlings were germinated and grown for 96 h in hanging plastic bags on chromatography paper that had been wetted with either aqueous homeopathic or control solutions. A watery extract was produced based on a filtrate of seedlings that were crushed and extracted in the corresponding solution. This extract was mixed with a dihydrate $CuCl_2$ solution and pipetted in levelled dishes. Evaporation under standardized conditions led to the emergence of specific $CuCl_2$ crystal patterns on the glass surface. The resulting crystallization patterns were evaluated by means of computerized image analysis. Experiments were randomized and blinded, and the code was revealed only after completion of the evaluation.

2.2. General experimental design

10 independent sets of preparations of *Stannum metallicum* 30x and lactose 30x were investigated in 10 independent verum experiments performed in parallel at 2 independent laboratories (BRAD, CL; n = 10 + 10 sub-experiments). In each experiment, the two preparations were examined untreated, cell phone treated, and upon autoclaving (data analysis of cell phone and autoclaving treatments is beyond the scope of the current paper). Simultaneously 10 independent systematic negative control (SNC) experiments were performed at both laboratories (n = 10 + 10 sub-experiments), assessing 6 preparations of distilled H_20 1x each, produced as 10 independent sample sets. The 20 experiments were performed blinded (coded) and randomized on 2 levels: regarding the experimental series (verum or SNC) as well as regarding the individual treatments within the experiments. Six *Lepidium sativum* extracts corresponding to the 6 experimental parameters or the 6 SNC preparations were examined in 6-fold glass plate replicates yielding 36 crystallization plates (Figs. 1 and 2), and in addition 7 crystallization plates of a reference compound (freeze-dried wheat), thereby applying the crystallization apparatus capacity of 43 crystallization plates per day. The experimental design, encompassing 10 SNC experiments besides the 10 verum experiments allowed controlling reproducibility as well as experimental stability of the bioassay. Reproducibility could be examined between laboratories and between experiments (by comparing the results as a function of individual experiments). The stability of the experimental set-up could be monitored throughout the entire investigation due to the incorporation of the SNC experiments.

2.3. Homeopathic preparations

All liquid potency and control solutions were prepared under sterile conditions (laminar flow cabinet HFX-180, Skan, Basel, Switzerland, situated in a class C clean room) according to the multiple glass method (for every succussion and dilution step a new glass vessel is used) by CS at Hiscia Research Institute (Arlesheim, Switzerland). For potentisation of the verum experiments, *Stannum metallicum* or lactose were repeatedly diluted and succussed. Potentisation up to the fourth potentisation step was performed in solid medium by triturating one part of *Stannum metallicum* or lactose in nine parts of lactose monohydrate as dilution medium (both 4x triturations supplied by Weleda AG, Schwäbisch Gmünd, Germany), according to manufacturing method no. 6 of the German Homeopathic Pharmacopoeia (Homöopathisches Arzneibuch Amtliche Ausgabe, 2011). Subsequent dilutions were performed in liquid medium (using distilled and autoclaved water as dilution medium). In total, the procedure of dilution at a ratio of 1:9 and trituration or succussion, respectively, was repeated 30 times yielding a nominal dilution of 10^{-30} of the original substance.

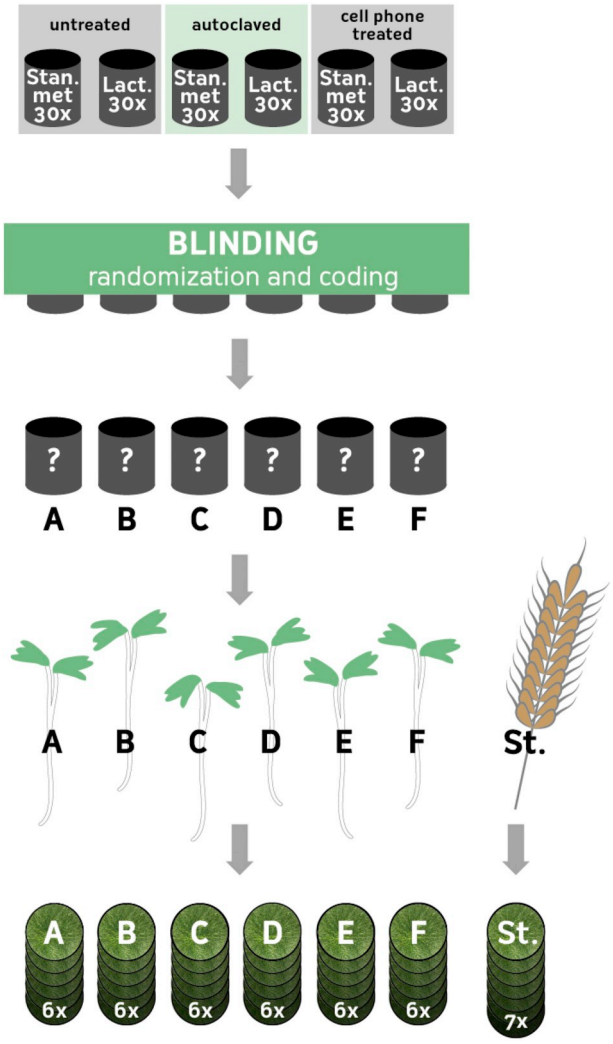

Fig. 1. General design of a verum experiment.
(1) Homeopathic potencies of *Stannum metallicum* 30x and lactose 30x were prepared and were left untreated or subjected to autoclavation or cell-phone radiation. (2) The six homeopathic potencies were randomized and coded A–F. (3) The preparations were used to cultivate the cress seedlings (wetting 10 filter papers in plastic bags for each of the six experimental conditions). (4) An aqueous extract was prepared from crushed seedlings after a 96 h growth period, extracted in the corresponding potencies. For each experimental condition, six crystallization plates were prepared. In addition, seven crystallization plates were prepared using an open (non-coded) internal standard (freeze-dried wheat).

All glassware used was individually permanently numbered. Borosilicate glassware was used for potentisation, storage of water and storage of the final potencies (Duran, hydrolytic class 1; laboratory bottles of 50 mL, 5 L, 10 L; reagent bottles with glass stoppers of 50 mL, 500 mL, 2000 mL; measuring cylinders 100 mL, 1000 mL, funnels, beakers, all Duran Group, Mainz, Germany). All glassware was thoroughly cleaned and sterilized. Cleaning was done by flushing with hot tap water, cleaning in a dishwasher (Miele Professional G7883, Miele AG, Spreitenbach, Switzerland) without detergent, cleaning twice with demineralised water (DW, final conductivity < 4.3 µS/cm; Ministil, BWT Aqua AG, Aesch, Switzerland) and once with distilled demineralised water (DDW, final conductivity < 1.1 µS/cm; Ministil/Aquatron

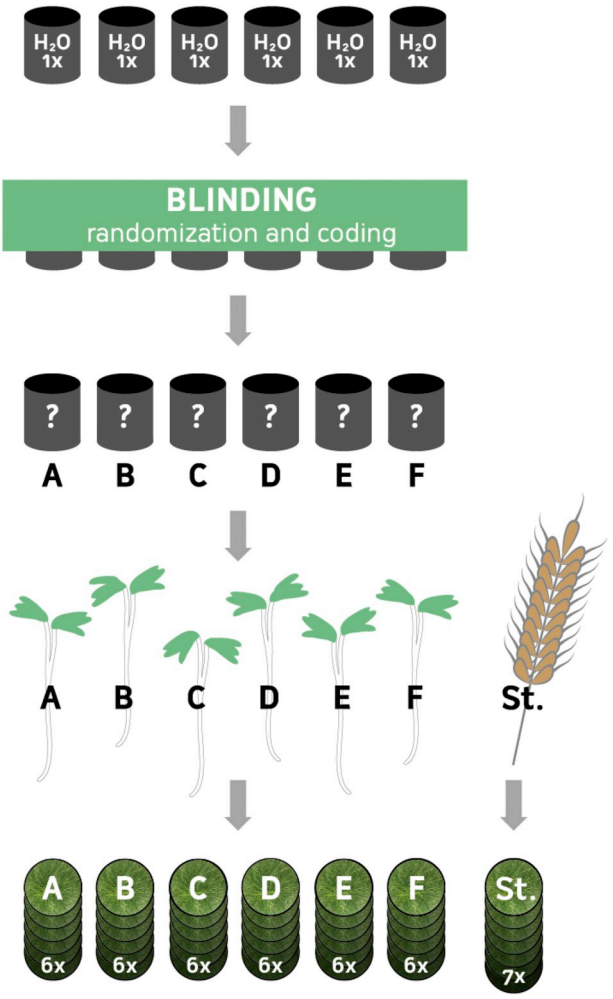

Fig. 2. General design of one systematic negative control (SNC) experiment. (1) Succussed dilutions of Water 1x were prepared, divided into six portions and left untreated. (2) The six preparations were randomized and coded A–F. Thereby obscuring whether an experiment is a verum or SNC (3) The preparations were used to cultivate the cress seedlings (wetting 10 filter papers in plastic bags for each of the six experimental conditions). (4) An aqueous extract was prepared from crushed seedlings after a 96 h growth period, extracted in succussed dilution. For each experimental condition, six crystallization plates were prepared. In addition, seven crystallization plates were prepared using an open (non-coded) internal standard (freeze-dried wheat).

water still A8000, Barloworld Scientific, Nemours, France) and drying (Salvis F400, Binder GmbH, Tuttlingen, Germany) at 80 °C for 3 h. Afterwards the glassware was welded in autoclavable bags (Sterilsop transparent tubing, Paul Hartmann, Selestat, France), autoclaved at 121 °C for 20 min and dried again at 80 °C for three hours. Other materials used (e.g. water dispenser Fortuna Optifix Basic, Poulten & Graf, Wertheim, Germany) were washed only by hand (omitting the dishwasher), dried and autoclaved as described above.

The homeopathic and control samples were prepared between September and December 2012 in a total of 10 production weeks as follows: Every week samples were prepared on two days; on one day the samples for one verum experiment (*Stannum metallicum* 30x and lactose 30x) and on the other day the samples for one SNC experiment (Water 1x only). During the first 5 weeks the lactose 30x samples were prepared in the morning, and the *Stannum metallicum* 30x samples in the afternoon; afterwards the order was reversed. Likewise, during the first five weeks the samples for one verum experiment were prepared on the first production day, and on the second production day the samples for one SNC experiment; afterwards the order was reversed. On the first day of each production week, a 10 L batch of autoclaved distilled demineralised water (ADDW) was divided into two 5 L laboratory bottles for the two production days. Thus, all liquid samples for a given verum experiment and the corresponding SNC experiment were produced from the same batch of ADDW.

On production day one of a given week, the samples for the SNC experiments were prepared. Two 2000 mL reagent bottles were filled with 1000 mL ADDW each from the first 5 L laboratory bottle. The reagent bottles were succussed to obtain a 1x potency. Succussion was performed by hand by holding the bottles horizontally in front of the body and shaking them rhythmically for 2 min each at a rate of approximately 2 Hz (all 50 mL bottles were succussed vertically). A total of 30 50 mL laboratory bottles were filled with 50 mL Water 1x using sterile funnels, the bottles were subsequently wrapped in aluminium foil, coded (see Section 2.4) and stored in cardboard boxes at 18 °C until shipment to the crystallization laboratories.

On production day 2 the samples for the verum experiments were produced using water from the second 5 L bottle of ADDW. For the verum samples of one potency series 3 g of either *Stannum metallicum* trituratio 4x or lactose trituratio 4x was transferred into a 50 mL reagent bottle and diluted in 27 mL ADDW. In order to fully dissolve the solid potency, the solution was gently heated in a water bath (42 °C) for 4 min. The 30 mL solutions were succussed vertically by hand as indicated above, thereby obtaining a 5x potency preparation. Subsequently 3 mL of the 5x potency preparation was added to the next reagent bottle containing 27 mL ADDW and was succussed analogously. To scale the volume up to a final volume of 1 L 30x potency preparation, 30 mL of the 28x potency preparation was added to a 500 mL reagent bottle containing 270 mL ADDW and succussed horizontally after which 100 mL of the 29x potency preparation was added to a 2000 mL reagent bottle containing 900 mL ADDW and succussed horizontally as described above. The two 30x verum samples (*Stannum metallicum* 30x and lactose 30x) were poured into 15 50 mL laboratory bottles each as described above for the SNCs. Subsequently, the verum samples were divided into three groups for the different treatments. One third of the verum samples were autoclaved at 121 °C for 20 min (HST 6-6-6, Zirbus technology AG, Bad Grund, Germany). Another third of the verum samples were treated with electromagnetic radiation emitted by a cell phone due to a continuous vocal phone call for 14 h (Sony Ericsson W300i, Lund, Sweden). Verum samples were put on a rotating plate (2 rpm) adjacent to the cell phone to ensure homogeneous irradiation, with an average distance of 23 cm between cell phone and samples. For autoclaving and cell phone irradiation, verum samples were temporarily removed from their aluminium foil wrapping. The last third of the verum samples were left untreated. Also, all water samples for the SNC remained untreated. Each preparation was supplied as two portions of 50 mL, intended for germination and extraction fluid, respectively, and specified as I and II. In total 150 bottles of *Stannum metallicum* 30x (50 untreated, 50 autoclaved, 50 cell phone-treated), 150 bottles of lactose 30x (50 untreated, 50 autoclaved, 50 cell phone-treated) as well as 300 bottles of Water 1x were produced. This allowed 10 verum experiments and 10 SNC experiments to be performed at the two laboratories, each consisting of three treatments of the two verum samples supplied in two bottles (2 x 10 x 3 x 2 x 2 = 240 preparations) or 240 “pseudo-treatments” (Water 1x only), respectively. 50 mL aliquots of all samples (60 verum samples, 60 SNC samples) were stored under the same conditions for further investigations with physical methods (data presentation and analysis are beyond the scope of the current publication).

2.4. Coding (blinding), randomization and decoding

The investigation was coded (blinded) and randomized on the level of type of experiments (verum or SNC) as well as on the level of investigated parameters (*Stannum metallicum* 30x or lactose 30x, both either untreated, cell phone irradiated or autoclaved). In order to obtain

a more or less uniform distribution of experiment types over time, pairs of verum and SNC experiments were randomized regarding their sequence by attributing random numbers based on a random number generator (Excel for Mac 2011, Microsoft, USA). The sequence of the six treatment parameters was based on a stratified randomization, ensuring a uniform distribution of the treatment parameters with respect to processing order. Randomization lists were made by SB, coding of the bottles containing the treatment parameters was done by CS and SB. Neither CS nor SB was involved in the conduct of the fingerprint metabolomic experiments, which were performed by PD and JOA only. Calculation of texture analysis variables was performed by PD and JOA. Only after delivery of the entire data set to SB, all codes were inserted into the data set by SB for subsequent statistical analysis by SB and PD (see below).

2.5. Seed selection

Lepidium sativum seeds (Bingenheimer Saatgut AG, Echzell-Bingenheim, Germany; article no. G250, harvest 2011) were applied as basis for the following three mechanical selection steps performed by Bingenheimer Saatgut AG: (a) size fractionation by means of an 'upper' and 'lower' sieve of 2.25 respectively 0.9 mm pore size; (b) density fractionation by means of a Gravity Separator (type LA-K, Westrup A/S, Denmark); (c) density fractionation based on the seeds' aerodynamic characteristics in an air stream (laboratory aspirator DUOASP–1101, Westrup A/S, Denmark).

The main fraction (1.5–1.75 mm) from the size fractionation represented approximately 75% of the original batch weight. Subsequent Gravity Separator density fractionation yielded 5 manually divided subfractions with comparable germination rates. The lowest density subfraction with an average thousand seed weight (TSW) of 2.47 g represented approximately 15% of the main fraction weight, i.e. approximately 11% of the original batch weight. This sub-fraction was subsequently subjected to air stream density fractionation whereupon approximately 20% of the seeds were regarded non-optimal (broken, insufficiently filled, etc.) and were discarded.

Consequently approximately 9% of the original batch remained after the 3 types of fractionation with a final average TSW of 2.48 g. Finally, a trained person (JOA) performed a manual discarding of non-optimal seeds, based on the following nine visual size, shape and colour criteria: very small, very large, mechanically damaged at harvest, insufficiently filled seed coat, semi-globular, irregularly shaped, dark-brown, grey-white, dis-coloured from fungal attack. Hereby approximately 57% of the mechanically selected seeds remained, representing approximately 5% of the original batch weight. From this final 'optimal' seed batch approximately 20,000 seeds were sent to each of the 2 laboratories for the planned experiments.

2.6. Seed germination and growth

The applied germination procedure was a modification of the procedure developed by Baumgartner and Flückiger (2001). Germination took place on filter papers (blotting paper 151B; 85 × 140 mm; 87 g/m^2; thickness 0.17 mm; Frisenette, Knebel, Denmark), placed in plastic bags (MiNiGRiP Colorline; type 11–16; 100 × 150 × 0.05 mm; JOKA Plastic- Emballage A/S, Holte, Denmark). Germination was performed on the "smooth" side, i.e. the side free of loose fibres. A volume of 3.00 mL from bottle I of a given preparation was pipetted onto each filter. A total of 10 filters were used per preparation. A code was written on each bag specifying the laboratory (NL/DK), experiment (1 − 20), preparation code (A-F), and bag replicate (1 − 10). When the solution was absorbed evenly over the filter, 16 seeds were placed on the filter. A 12-minute time-block was applied for the germination start of each preparation in order to minimize any processing order effect. A minimum access of air into the bags was arranged by manually expanding the upper closing area of the bags at both sides. The bags were placed in a germination box (polyethylene; 200 × 315 × 450 mm), hanging vertically from two stainless steel rods (diameter 3 mm) free from contact with the bottom and the lid. The bags were positioned on the rods according to the processing order of the six preparations (i.e., A-C on the first rod and D-F on the other). Bags belonging to the different preparations were separated by a plastic bag containing a filter paper covered with double layers of aluminium foil. Similarly, in the area between the two rows of plastic bags a cardboard covered with a double layer of aluminium foil was placed. The germination box was covered with a plastic plate and placed in a heating cabinet at 18 °C (± 1 °C) on a perforated plate. After approximately 2.5 h the seeds had developed a mucous sphere, allowing the 16 seeds to be aligned 9 cm from the bottom of the filter at 2-3 mm distance, followed by incubation for another 93.5 h. After this period, seedlings had typically grown to a total shoot and root length of about 9 cm.

2.7. Preparation of seedlings

After the 96 h growth period a total of five plastic bags containing the seedlings were cut open at both length sides, approximately 3 mm from the filter paper and similarly approximately 3 mm above the paper. The front side of the bag was opened, and the upper part of the paper was bent backwards whereby the seedlings were accessible for manual selection of single seedlings. Seedlings with a minimum root length of 6.5 cm were selected from each bag, generally resulting in 8–12 seedlings per bag. Seedlings that deviated in shape or colour, or showed signs of fungal growth, were discarded. The brown seed coats, which generally were separated from the seedlings after germination, were discarded. The seedlings were collected in a Petri-dish placed on a balance. The number of seedlings from each bag was noted on a standard form, together with the sum weight of the selected seedlings. A total of 1.50 g seedlings were collected, made up from on average 10 seedlings per bag, each seedling weighing approximately 0.03 g. The seedlings were placed in a mortar (porcelain; 160 mL; Ø = 90 mm; Haldenwanger, Waldkraiburg, Germany) containing 10.0 mL from bottle II of a given preparation. The mortar was covered with Parafilm M (Pechiney, Menasha, USA) until seedlings from all treatments had been prepared. This procedure was repeated with the remaining five plastic bags until each mortar contained a total of 3.00 g seedlings. This dual seedling collecting step was applied in order to further minimize a potential processing order effect. The seedlings were crushed by means of a pestle applying diagonal movements for 2 min, resulting in no intact leaf or root parts to be observed in the solution. Subsequently lemniscate movements were applied for 1 min whereby the leaf and root parts were homogenized further. Pestle and mortar were each flushed with 8.50 mL from bottle II of a given preparation, thereby generating a 10% solution on weight basis (3.00 g seedlings; 27.00 mL potency; in total 30.00 g solution). Extraction of the seedlings in a given preparation was performed to obtain a maximal differentiation between the experimental groups. The solution was transferred to a wide-necked 100 mL Erlenmeyer flask. Each flask was covered with Parafilm and left standing until all treatments were performed. The solutions were extracted on a horizontal shaker (Heidolph Unimax 2010) at 125 rpm for 45 min. The extract was filtered for 3 min by means of a nylon filter (pore size 150 µm; 03–150/38; Sefar AG, Heiden, Switzerland) placed in a glass funnel (upper diameter 70 mm, tube length 70 mm, tube diameter 10 mm) placed in a pre-weighed wide-necked 100 mL Erlenmeyer flask. The weight of the filtrate was noted.

2.8. Fingerprint metabolomic analysis

Fingerprint metabolomic analysis was performed at the two laboratories in similarly constructed crystallization chambers according to the procedures described by Busscher et al. (2010a), during the period January–June 2013. The solutions applied for fingerprint metabolomic analysis were produced on the basis of extract of 300 mg

substance (seedlings) per crystallization plate, in combination with 150 mg of dihydrate $CuCl_2$ (copper (II) chloride dihydrate; Merck, article no. 1.02733.1000; pro analysis) from a 5% aqueous solution, hereby generating a volume of 6.0 mL per plate. This yielded 6 crystallization plates (replicates) per *Lepidium sativum* extract preparation per experiment, mounting to a total of 36 crystallization plates for the 6 *Lepidium sativum* extract preparations. Plate replicates were numbered in accordance with the preparation codes: A1-A6…F1-F6. The glass plates applied were circular float glass (1st quality float glass; "air side" applied; 100.0 mm diameter; thickness 2.0 mm ± 0.2; Pfaehler GmbH & Co. KG, Gengenbach, Germany). The cleaning of the glass plates, as well as other glass utensils, was performed by means of the following procedure at BRAD: 12 h in 20 °C 5% Merck MA 01 Extran bath; thoroughly flushing with tap water; 1 h in 15% nitric acid; 4 consecutive baths of demineralised water, the 4th with maximal conductivity 1.5 µS; flushing with 96% ethanol; drying in air. At CL, glass plates were cleaned in a Miele desinfector G7735, performing the following three washing steps: neodisher FLA, neodisher Z (both VWR), and deionized water 90 °C. The plates were combined with acrylic rings (made from GS acrylic tubes Riacryl; length 35 mm, thickness 5 mm ± 0.5 mm; Broennum Plast, Rodovre, Denmark) to constitute a basin for pipetting the crystallization solution. The glass plates and the acrylic rings were assembled by means of Vaseline (Prolabo, Fontenay sous Bois, France; article no. 28908.290). The 36 numbered (A1-A6…F1-F36) crystallization plates with *Lepidium sativum* extract (corresponding to the 6 experimental parameters or the 6 SNC preparations in 6-fold glass plate replication, see Figs. 1 and 2) and the 7 plate replicates from the freeze-dried reference sample (Busscher et al., 2010a) were positioned on the same cell number in the crystallization chamber for each experiment. The 2 laboratories had different recurring distributions of the 43 available positions amongst the 6 + 1 preparations. Evaporation of the water in the dishes took place under defined conditions (29 °C; initial rH 49%, air velocity 4 cm s^{-1}; HygroClip S, Rotronic AG, Switzerland). The entire evaporation process was monitored by means of time-lapse stills at 10-minute intervals from a digital camera mounted on the ceiling of the crystallization chamber. Based on the photographs obtained, the evaporation time was determined for each crystallization plate, i.e. the time in hours/min from the onset of the experiment until initial nucleation.

2.9. Computerized textural image analysis

The crystallization plates were scanned after a minimum storage time of 48 h under controlled climatic conditions as 8 bit RGB images and converted to 8 bit grey levels with 600 dpi resolution using a Umax PowerLook III scanner in transmission scan mode. The RGB distribution was set to 33/33/33, and for preprocessing normalisation a Gaussian normalisation was applied. The crystallization centre was marked interactively. Dedicated software for pattern evaluation was used which connected each scanned image to the documented information (sample, (x,y) location of the primary nucleation centre, temperature, humidity, evaporation and crystallization process) (Busscher et al., 2010a). Circular regions of interest (ROIs) were defined around the geometrical centre of the crystallization plates, with a diameter Dx given as percentage of the total diameter D (90 mm, set to 100%). Additionally, four circular radial ROIs (CRROIs) of the entire crystallization plate were defined with the radii 0–50%, 50–70%, 70–90%, and 90–100%. For the analysis of the experiments, all scanned crystallization plates of the 20 CL and 20 BRAD experiments were used, minus those discarded due to technical errors (see below).

Texture analysis was performed as described by Carstensen (1992) and concerning the specific application in a $CuCl_2$ crystallization pattern context by Andersen et al. (1999). The applied texture analysis considers the spatial relationship of pixels in each image, for which a so-called grey level co-occurrence matrix (GLCM) is conceived. The GLCM allows a characterization of the texture of an image by calculating how often pairs of pixel with specific grey-values and in a specified spatial relationship occur in an image (Hall-Beyer, 2017). Subsequent statistical measures are extracted from this matrix by means of 15 seconds order texture analysis variables. The here applied GLCM is defined as the distribution of co-occurring grey values at a one pixel offset in a horizontal and vertical direction. Both GLCMs are summed to construct the final GLCM.

Texture analysis variable cluster shade is defined as a measure of skewness (asymmetry) and uniformity. A higher cluster shade output implies a greater degree of asymmetry. Texture analysis variable diagonal moment measures the difference in the correlation for high grey levels and for low grey levels (Carstensen, 2001). Diagonal moment will have a positive value if the bright areas (low grey levels) are rough and the dark areas (high grey levels) are smooth. If there is no difference between the correlation in the dark and bright areas then diagonal moment is close to zero.

2.10. Location of the primary nucleation centre

Crystallization plates were scanned with the primary nucleation centre oriented vertically relative to the geometric centre of the dish so that the nucleation centre would appear in the lowest position on the image. Subsequent to scanning the crystallization plates, the scanned images were cut to a rectangle, so that the top, bottom, right and left represented the framing of the crystallization plate (Ø 9.0 cm) (Andersen et al., 1999). The (x, y) location (0.0, 0.0) of the resulting rectangular image specifies the upper left corner. Consequently, the geometric-centre has an (x, y) location of (4.5, 4.5). The location of the primary nucleation centre (marked interactively during the scanning process) was determined relative to the geometrical centre of the Petri-dish as the absolute difference between the two Y-values.

2.11. Missing data

Fourteen crystallization plates (out of $40 \times 36 = 1440$ in total) were discarded due to technical errors, primarily due to leakage of solution from under the surrounding acrylic ring. Six originated from verum experiments and eight from SNC experiments. Thus 1426 crystallization plates were used for computerized texture analysis.

2.12. Analysis of variance (ANOVA)

Evaporation time and texture analysis data for the variables cluster shade and diagonal moment were analysed separately for the verum and SNC experiments by means of ANOVA F-tests with the independent parameters experimental number $\{1-10\}$, laboratory {BRAD, CL}, and treatment {*Stannum met.* 30x, lactose 30x}. For all evaluations, the software Statistica 6.0 for Windows (StatSoft Inc., Tulsa, USA) was used.

2.13. Meta-analysis

For the meta-analysis of the precursor study (Baumgartner et al., 2012) and the present study texture analysis data for the variables cluster shade and diagonal moment were analysed by means of ANOVA F-tests with the independent experimental parameters experimental day {1–35} (N° 1–7 correspond to precursor study day 1–7 from LBI, N° 8–15 correspond to precursor study day 1–8 from BRAD, N° 16–25 correspond to the present study day 1–10 from BRAD, and N° 26–35 correspond to the present study day 1–10 from CL), and treatment {*Stannum met.* 30x or water/lactose 30x}.

Laboratories involved: LBI = Louis Bolk Institute (NL), CL = Crystal Lab (NL), BRAD = Biodynamic Research Association Denmark (DK).

Table 1
Changes in the applied materials and methodological procedures compared to the precursor study (Baumgartner et al., 2012).

	Materials and methods used in the precursor study Baumgartner et al. (2012)	Materials and methods used in present study
Cress seed batch	*Lepidium sativum L.,* Bingenheimer Saatgut AG, Echzell-Bingenheim, Germany; article no. G250, harvest 2006.	*Lepidium sativum L.,* Bingenheimer Saatgut AG, Echzell-Bingenheim, Germany; article no. G250, harvest 2011.
Cress seed selection	Seeds that were damaged and/or not showing characteristics of fully developed seeds were discarded (approximately 5%).	Application of size, gravitational and air stream density fractionation (Bingenheimer Saatgut AG), and a successive visual selection applying nine criteria (approximately 95% seeds discarded).
Potency preparation	Homeopathic preparations were prepared up to 30x from aqueous 28x homeopathic preparations under semi sterile conditions (Allergica, Silkeborg, Denmark).	Homeopathic preparations were prepared up to 30x from trituratio 4x under sterile conditions (Hiscia Institute, Arlesheim, Switzerland).
Control	Comparison of *Stannum metallicum* 30x with Water 30x.	Comparison of *Stannum metallicum* 30x with lactose 30x (to control for the initial trituration in lactose).
Seed germination	The germination start of the six treatments was performed in succession.	A 12-min time-block was applied for the germination start of each treatment in order to minimize any processing order effect.
Amount of cress filtrate/ plate	310 µl filtrate	300 µl filtrate
Seedling selection	Suitable seedlings were collected from the 10 available plastic bags of each treatment in one run.	Suitable seedlings were collected in two runs, each run consisting of five plastic bags per treatment (to avoid processing order effects).
Experimental design	In total 15 single experiments were performed in 2 laboratories. In each experiment the two preparations were examined in three-fold repetition (independent replicates).	In total 20 single verum experiments were performed in 2 laboratories. In each experiment the two preparations were examined untreated, cell-phone treated, and upon autoclaving
Systematic Negative Control (SNC)	Since one of the homeopathic preparations was water 30x (prepared in triplicate), the SNC approach was incorporated into the experimental design in modified form.	In total 20 independent SNC experiments were performed in 2 laboratories consisting of 6 preparations of water 1x each.

2.14. Differences compared to the precursor study (Baumgartner et al., 2012)

Intentional and imposed changes in the applied materials and methodological procedures compared to the precursor study (Baumgartner et al., 2012) are summarized in Table 1.

3. Results

3.1. Texture analysis of the entire crystallization plate

Consistent with the precursor study by Baumgartner et al. (2012), many of the 15 texture analysis (TA) variables were correlated to each other. Within the verum data two main groups of variables can be distinguished, which were closely correlated within the respective group, but not or only weakly correlated to those of the other group:

(i) group I (cluster shade and diagonal moment, $r = -0.97$ within group), (ii) group II (all other TA variables with the exception of variable cluster prominence, $r > 0.71$ within group). The variable cluster prominence showed a medium correlation to all but one other TA variables (maximum probability) and takes an intermediate position. Additionally, the correlation of the two variable groups to evaporation time was consistent with respect to the precursor study. Variables of group II were moderately correlated to evaporation time ($r > 0.53$, at $p < 0.05$) except for maximum probability, and sum energy ($r = 0.41$, and 0.39, respectively), while variables of group I were only weakly correlated to evaporation time ($r \leq 0.37$).

Due to this consistency of the basic data structure with the precursor experiment, we restrict the evaluation in the present manuscript to the data of the texture analysis variables of group I (cluster shade and diagonal moment), which had been identified in the former study as the outcome variables of predominant interest (Baumgartner et al., 2012).

Data of the verum experiments were analysed by a 3-way analysis of variance (ANOVA) with the independent factors (1) laboratory {BRAD, CL}, (2) experimental number {n = 10}, and (3) treatment {*Stannum met.* 30x, lactose 30x}. Dependent variables were cluster shade and diagonal moment, calculated based on the entire crystallization plate diameter (Region Of Interest (ROI) 0–100%) (Table 2 and Fig. 3). Variable cluster shade yielded a significant homeopathic treatment effect ($p = 0.034$), meaning that a difference was found in the texture of crystallization patterns of *Lepidium sativum* seedlings germinated in *Stannum metallicum* 30x versus lactose 30x. Variable diagonal moment showed a tendency towards a treatment effect. Significant differences in absolute values were found for both TA variables between the single experimental numbers (significant experimental day effect), and between the two laboratories. Additionally, an interaction was observed between potency treatment and experimental number for the variable cluster shade ($p = 0.041$, Table 2, interaction 2–3), for further analysis and discussion see below.

No significant interaction was observed between the factors laboratory and potency treatment, meaning that the treatment effect was quite reproducible between the two laboratories from a statistical point of view. However, numerically, the effect of the homeopathic treatment in the BRAD experiments was about 20% of that at CL regarding the variable cluster shade (data not shown).

Concerning the systematic negative controls (SNCs), significant differences in absolute values were found for both TA variables between the single experimental numbers (significant experimental day effect), and between the two laboratories. However, no significant differences were observed between the water control samples and no interactions were found between treatment and experimental day, indicative of a stable experimental set-up (Table 2, SNC main effect 3 and interaction 2–3).

3.2. Crystallization evaporation time

One of the major influences on the dendritic growth during the crystallization process is the degree of supersaturation of the solution in the Petri-dish prior to nucleation (Busscher et al., 2010b). Under the present experimental conditions initial nucleation occurs at a mean of ≈12–13 h, but might vary considerably as it is governed by a stochastic process. At BRAD the average evaporation time (the time between the pipetting of the solution in the Petri-dish and the primary nucleation) for all experiments showed a range between 10.0 and 16.3 h (12.5 h mean). At CL the average evaporation time ranged between 10.7 and 17.4 h with a mean of 13.9 h (see Fig. 4 for verum and SNC evaporation times). We set out to determine whether the potency treatment effect could be attributed to systematic variations in the evaporation times. For this the evaporation times of the verum experiments and of the SNC experiments were analysed by a full 3-way ANOVA using (1) laboratory {BRAD, CL}, (2) experimental number {verum n = 9; SNC n = 10} (the evaporation data of one experimental day of the verum experiments at

Table 2
Results from ANOVA F-tests for the verum and systematic negative control (SNC) experiments (main effects and interactions).

Parameters	Main effects			Effect interactions			
	(1) Lab	(2) Exp. Num.	(3) Treatment	1-2	1-3	2-3	1-2-3
Verum experiments (p-value)							
Cluster shade	**<0.0001**	**0.0152**	**0.0336**	**<0.0001**	0.1363	**0.0409**	0.3801
Diagonal moment	**<0.0001**	**0.0446**	0.0622	**<0.0001**	0.0565	0.0927	0.5026
SNC experiments (p-value)							
Cluster shade	**<0.0001**	**<0.0001**	0.2397	**<0.0001**	0.8439	0.3575	0.0682
Diagonal moment	**<0.0001**	0.0022	0.2011	**<0.0001**	0.8747	0.3805	0.1258

Independent experimental parameters were (1) Lab = laboratory {BRAD, CL; n = 2}, (2) Exp. Num. = experimental number {1–10; n = 10}, (3) treatment {*Stannum met.* 30x or lactose 30x (verum experiments), or water 1x and water 1x (SNC experiments), respectively; n = 2}. Dependent parameters were texture analysis variables cluster shade and diagonal moment. Each experimental parameter combination was assessed by six crystallization plate replicates. Significant effects ($p < 0.05$) are shown in bold red font.

CL is missing due to technical failures; in order to achieve a fully balanced experimental design the corresponding experiment from BRAD was omitted from analysis too) and (3) treatment {*Stannum met.* 30x, lactose 30x (verum experiments), or water 1x and water 1x (SNC experiments), respectively} as the three independent variables.

For the verum experiments, experimental number and laboratory yielded a highly significant effect ($p < 0.0001$, Table 3), indicative of significant differences in absolute values of the evaporation time between the independent experiments and between the two laboratories. No differences ($p = 0.52$) were found for the homeopathic treatment {*Stannum met.* 30x and lactose 30x}, nor for any interaction. Thus, evaporation time did not differ between the different experimental treatments, though there were differences in absolute values from experiment to experiment and between the two laboratories (Fig. 4). Concerning the SNC experiments, results were essentially similar to the verum experiments, albeit that a highly significant interaction was found between laboratories and experimental number ($p < 0.0001$) and a mild interaction between the laboratories and the water control samples ($p = 0.03$), implying that the laboratories exhibited varying results for the different experiments and to a lesser degree for the water control samples.

3.3. Processing order effect

As can be seen in Table 2, variable cluster shade showed a weak interaction between potency treatment and experimental day. We tested the hypothesis that the observed interaction between potency treatment and experimental day in the verum experiments was due to a laboratory processing order effect as follows: for the variable cluster shade the data from all SNC experiments was normalized to the corresponding day mean and analysed with a 1-way ANOVA with the independent variable “processing order,” varying between 1 and 6. This

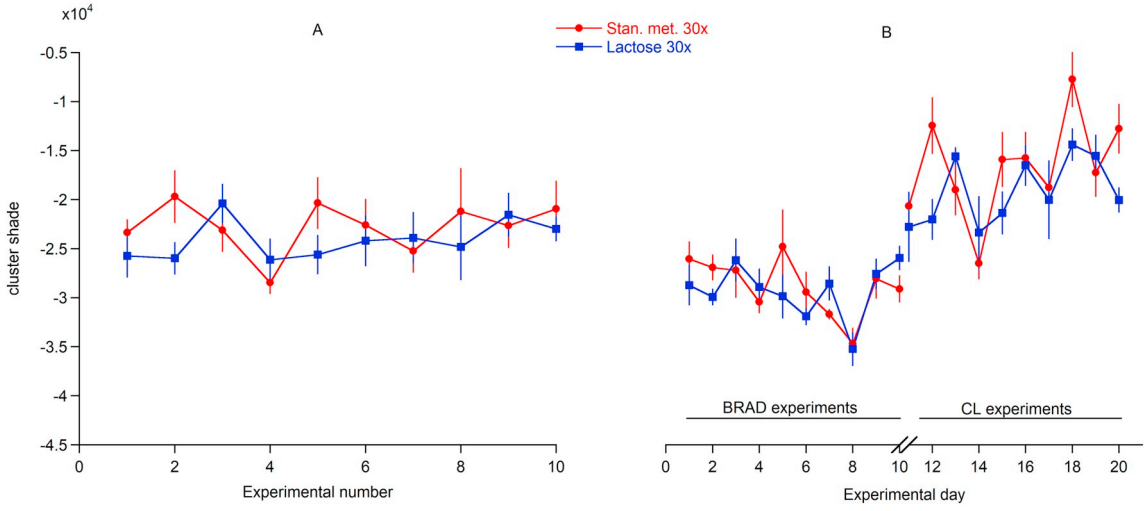

Fig. 3. Texture analysis data of the verum experiments for variable cluster shade, based on the entire crystallization plate diameter ROI 0–100%.
(A) Texture analysis variable cluster shade (mean ± SE of 12 replicates each) of the cress extract crystallization patterns for the 10 individual experimental numbers, pooling data from both labs (verum experiments). (B) Texture analysis variable cluster shade (mean ± SE of 6 replicates each) of the cress extract crystallization patterns for the 20 individual experimental days (verum experiments). Day 1–10 corresponds to verum experiments 1–10 from BRAD and day 11–20 to verum experiments 1–10 from CL.

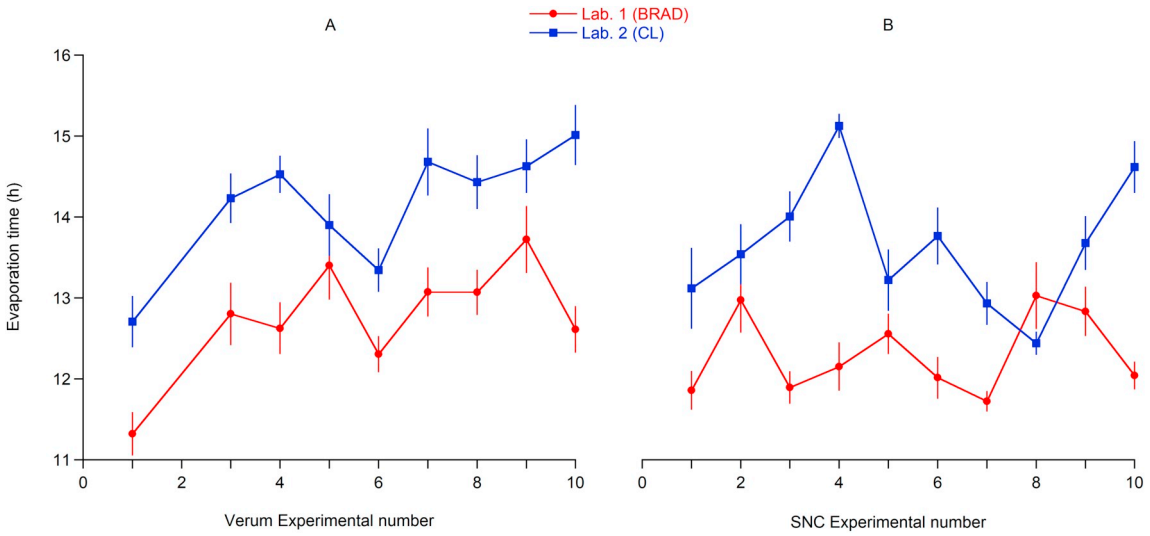

Fig. 4. Evaporation time (mean ± SE of 12 replicates each) of the cress extract crystallization patterns for the 10 individual experimental numbers. (A) Verum experiments (n = 9 as the evaporation data of experimental day 2 at CL is missing due to technical failures). (B) SNC experiments (n = 10).

Table 3
Results from ANOVA F-tests for evaporation time in the verum and SNC experiments (main effects and interactions).

Parameters	Main effects			Effect interactions			
	(1) Lab	(2) Exp. Num.	(3) Treatment	1-2	1-3	2-3	1-2-3
			Verum experiments (p-value)				
Evaporation time	**<0.0001**	**<0.0001**	0.5239	0.1877	0.3618	0.9453	0.9002
			SNC experiments (p-value)				
Evaporation time	**<0.0001**	**<0.0001**	0.5207	**<0.0001**	**0.0340**	0.2828	0.9454

Independent experimental parameters were (1) Lab = laboratory {BRAD, CL; n = 2}, (2) Exp. Num. = experimental number {verum 1, 3–10; n = 9 and SNC n = 10}, (3) treatment {*Stannum met.* 30x or lactose 30x (verum experiments), or water 1x and water 1x (SNC experiments), respectively; n = 2}. Dependent parameter was evaporation time. Each experimental parameter combination was assessed by six crystallization plate replicates. Significant effects ($p < 0.05$) are shown in bold red font.

analysis yielded highly significant differences in absolute values for the different processing-orders ($p = 0.00034$). However, there was no linear trend in the data ($r = 0.13$), indicative of an absence of a simple linear processing-order effect.

3.4. Meta-analysis: texture analysis of the entire crystallization plate

The texture analysis data from the entire crystallization diameter (ROI 0–100%) from the precursor (Baumgartner et al., 2012) and present study were pooled for analysis by full 2-way ANOVA for the texture analysis variables cluster shade and diagonal moment, using experimental day {n = 35} and treatment {*Stannum met.* 30x, water/lactose 30x; n = 2} as the two independent variables. Results showed a highly significant treatment effect ($p \leq 0.001$) for both texture analysis variables, and no interaction with experimental day (see Table 4 and Fig. 5). Moreover, the directionality and the relative homeopathic treatment effect was for both texture analysis variables comparable to the data obtained in the precursor study (0.93 and 0.92 for variable cluster shade, respectively, and 1.14 versus 1.15 for diagonal moment, respectively). The relative homeopathic treatment effect was defined as

Table 4
Results from ANOVA F-tests for the meta-analysis of the data of the present and the precursor study (Baumgartner et al., 2012) (main effects and interactions).

Parameters	Main effects		Effect interaction
	(1) Exp. day	(2) Treatment	1-2
Cluster shade	**<0.0001**	**0.0004**	0.0719
Diagonal moment	**<0.0001**	**0.0011**	0.1442

Independent experimental parameters were (1) experimental day {n = 35; day 1–15 correspond to precursor study day 1–15; day 16–35 correspond to the present study day 1–20 (BRAD 1–10, CL 11–20)}, and (2) treatment {*Stannum met.* 30x or water/lactose 30x; n = 2}. Dependent parameters were texture analysis variables cluster shade and diagonal moment. Each experimental parameter combination was assessed by 18 (precursor study) or 6 (present study) crystallization plate replicates. Significant effects ($p < 0.05$) are shown in bold red font.

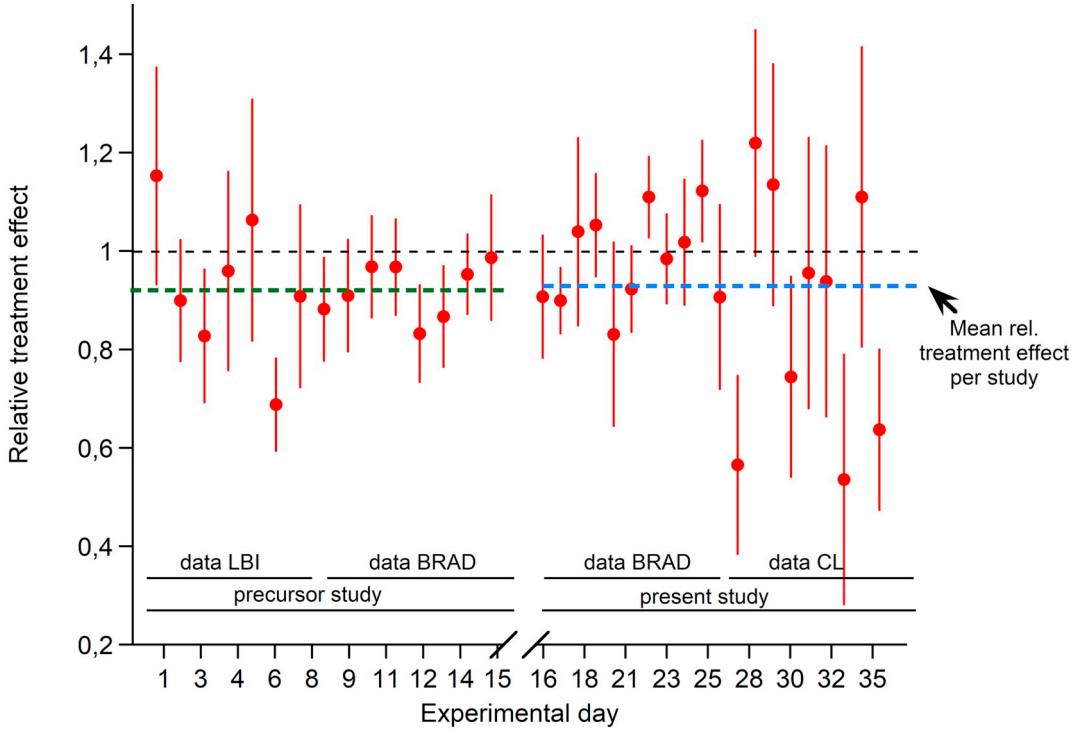

Fig. 5. Relative treatment effect of the cress extract crystallization patterns for the 35 individual experimental days for the texture analysis variable cluster shade. The relative treatment effect is determined as the *Stannum metallicum* treatment effect relative to the water/lactose 30x treatment effect (mean ± SE of 18 replicates (precursor study) or 6 replicates (present study)). Day 1–15 correspond to precursor study day 1–15; day 16–35 correspond to the present study day 1–20 (BRAD 1–10, CL 11–20). Green dashed line: mean relative treatment effect for the precursor study (0.92). Blue dashed line: mean relative treatment effect for the present study (0.93). Laboratories involved: LBI = Louis Bolk Institute (NL), CL = Crystal Lab (NL), BRAD = Biodynamic Research Association Denmark (DK). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

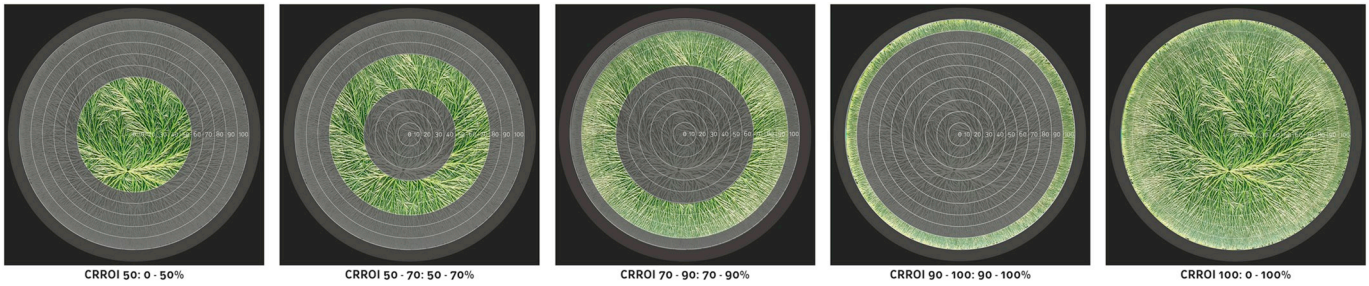

Fig. 6. Circular radial regions of interest (CRROI), defined by radius segments 0–50%, 50–70%, 70–90%, and 90–100%, relative to the entire image (CRROI 0–100%).

the relative difference between the *Stannum metallicum* 30x and water/lactose 30x groups for either of the two TA variables (cluster shade and diagonal moment).

3.5. Meta-analysis: texture analysis of segments of the crystallization plates

Comparable to the precursor study, we performed a subgroup analysis in which we aimed at identifying the spatial region of the crystallization patterns where the potency treatment effect manifests predominantly. We defined four different circular radial ROIs (CRROIs) of the entire crystallization plate with the radii 0–50%, 50–70%, 70–90%, and 90–100% (Fig. 6). Full 2-way analysis of variance was performed on the 2 TA variables, using experimental day {n = 35}, and treatment {*Stannum met.* 30x, water/lactose 30x; n = 2} as independent variables. Results for the potency treatment main effect and the interaction of potency treatment with experimental day are given in Table 5.

The signal in the TA variables cluster shade and diagonal moment manifests predominantly in the geometrical centre of the crystallization plates (ROI 0–70%), but was found numerically to be approximately a factor 20 times lower than for the entire crystallization plates at ROI 0–100% (data not shown). No interactions with experimental day were found.

3.6. Optimal evaporation time and relative location nucleation start

As the morphology of crystallization patterns is determined considerably by the evaporation time we tested whether a correlation exists between the relative homeopathic treatment effect for both texture analysis variables, and 1) the evaporation time, or 2) the localization of the primary nucleation site, for all experimental days (n = 33 for the correlation with the evaporation time and n = 34 for the correlation with the localization of the nucleation site, due to missing evaporation

Table 5
Results from ANOVA F-tests for the homeopathic treatment (main effects and interactions with experimental day) in four different CRROIs for the pooled data set of the present and precursor study.

Parameters	Main effects (p-value)				Interactions with experimental day (p-value)			
	CRROI 0 – 50%	CRROI 50 – 70%	CRROI 70 – 90%	CRROI 90 – 100%	CRROI 0 – 50%	CRROI 50 – 70%	CRROI 70 – 90%	CRROI 90 – 100%
Cluster shade	**0.0094**	**0.0060**	0.6741	0.0901	0.1648	0.3027	0.3116	0.7967
Diagonal moment	**0.0194**	**0.0074**	0.8653	**0.0433**	0.2872	0.4147	0.2210	0.8757

Independent experimental parameters were (1) experimental day {n = 35; day 1–15 corresponds to precursor study day 1–15; day 16–35 corresponds to the present study day 1–20 (BRAD 1–10, CL 11–20)}, and (2) treatment {*Stannum met.* 30x or water/lactose 30x; n = 2}. Dependent parameters were texture analysis variables cluster shade and diagonal moment. Each experimental parameter combination was assessed by 18 (precursor study) or 6 (present study) crystallization plate replicates. Significant effects ($p < 0.05$) are shown in bold red font.

data as a consequence of technical failures). The relative treatment effect is defined as the *Stannum metallicum* 30x treatment effect relative to the water/lactose 30x treatment effect. The location of the primary nucleation centre was determined relative to the geometrical centre of the Petri-dish as the absolute difference between their vertical positions (see Section 2.10 for details).

The treatment effect was not significantly correlated to neither the evaporation time, nor the localization of the primary nucleation site. However, a significant correlation was found between the evaporation time and the localization of the primary nucleation site ($r = 0.83$, $p < 0.01$, Table 6).

4. Discussion

4.1. Reproducibility of the effect of Stannum metallicum 30x

In a precursor project, we developed a bioassay which yielded statistically significant effects of a *Stannum metallicum* 30x preparation compared to water 30x, based on 15 independent randomized and blinded experiments performed at 2 independent laboratories. In the present project we investigated the reproducibility of the effects found in repeated experiments. To control for the trituration of *Stannum metallicum*, water 30x was replaced by lactose 30x as more adequate control sample. In total 10 independent, randomized, coded experiments were performed in 2 independent laboratories in the Netherlands and Denmark. In addition, 10 systematic negative control SNC experiments were performed in each laboratory to control experimental stability and to test the applicability of the statistical model applied. The in total 20 verum and 20 SNC experiments were performed blinded (coded) and randomized on two levels: regarding the level of type of experiments (verum or SNC) as well as on the level of investigated parameters (*Stannum met.* 30x, lactose 30x).

Consistent with the precursor study two main groups of texture analysis variables could be distinguished, which were closely correlated within the respective group, but not or only weakly correlated to those of the other group. Relevant for this study was the $r = -0.97$ correlation between cluster shade and diagonal moment, which had been identified in the former study as the outcome variables of predominant interest (Baumgartner et al., 2012). Also consistent with the precursor study was the weak correlation between these two variables and the evaporation time (the time between the pipetting of the solution in the Petri-dish and the initial nucleation).

Evaluation of the resulting crystallization patterns with computerized texture analysis yielded significant differences between *Stannum metallicum* 30x and lactose 30x ($p = 0.034$ for variable cluster shade), or a tendency towards such an effect (variable diagonal moment $p = 0.062$). The less pronounced statistical significance of the treatment effect for the TA variable cluster shade in the present compared to the precursor study ($p = 0.034$ and $p = 0.0004$, respectively) is a consequence of the experimental set-up. Whereas the precursor experiment consisted of 15 experimental days with 18 replicates per treatment group per experimental day (i.e. a total of 270 independent

Table 6
Correlation matrix of the relative treatment effect and 1) the evaporation time and 2) the geometrical location of the primary nucleation centre.

	Treatment effect (Cluster shade)	Treatment effect (Diagonal moment)	Evaporation time	Location primary nucleation centre
Treatment effect (Cluster shade)	1.00			
Treatment effect (Diagonal moment)	**0.98**	1.00		
Evaporation time	-0.07	-0.14	1.00	
Location primary nucleation centre	-0.07	-0.10	**0.83**	1.00

The relative treatment effect is determined as the *Stannum metallicum* treatment effect relative to the water/lactose 30x treatment effect for the texture parameters cluster shade and diagonal moment. Significant effects ($p < 0.01$) are shown in bold red font.

crystallization replicates), the present experimental set-up consisted of 20 experimental days which were however limited to 6 replicates per treatment group per experimental day (i.e. 120 independent crystallization replicates in total). This is also reflected in a higher degree of data scatter for the different experimental days in the present study (see Fig. 5).

The replacement of water 30x in the precursor study by lactose 30x in the present study to control for the trituration of *Stannum metallicum* was performed under the assumption that lactose is an inert carrier for homeopathic remedies. Considering the confirmation of the treatment effect found in the precursor study this assumption seems justified, although Scherr et al. (2007) reported a treatment effect of potentised lactose in a *Lemna gibba* L. bioassay. Consequently, the inert nature of lactose remains under debate. Concerning the SNCs, no significant differences were observed between the water control samples and no interactions were found between SNC water control and experimental day, indicative of a stable experimental set-up, and a reliable and specific treatment effect.

In summary, the main results of the precursor study could be confirmed concerning 1) the treatment effect (*Stannum met.* 30x versus lactose 30x) with the TA variable cluster shade, 2) directionality and effect-size of the homeopathic treatment, and 3) reliability and specificity of the homeopathic treatment effect.

4.2. Meta-analysis

The data from the precursor experiment and present study were pooled for a meta-analysis. This revealed a highly significant treatment effect for both TA variables, and no interaction with experimental day. Moreover, the directionality and relative effect-size of the homeopathic treatment effect was comparable to the data obtained with the precursor study (0.93 and 0.92 for variable cluster shade, and 1.14 versus 1.15 for diagonal moment, respectively).

In a subgroup analysis we identified that the spatial region of the crystallization patterns where the homeopathic treatment effect manifests predominantly is confined to the geometrical centre of the crystallization plates (ROI 0–70%), which is in agreement with the precursor study. However, numerically the potency treatment effect in this spatial region was approximately a factor 20 times lower than for the entire crystallization plate (ROI 0–100%).

The treatment effect was not significantly correlated to neither evaporation time nor localization of the primary nucleation site. Consequently, this rules out any further improvement of the bioassay by decreasing the variation of the crystallization starting time. A significant correlation was found between the evaporation time and the localization of the primary nucleation site ($r = 0.83$, $p < 0.01$, Table 6). This can be understood from the observation that prior to nucleation and crystal growth an area with a homogeneous degree of supersaturation will emerge in a radius of about 0–40% around the geometric centre of the Petri-dish (Busscher et al., 2010b). Consequently, the nucleation probability will be constant at each position in this area and increase proportional to the squared radius of the supersaturated area.

Summarized, we observed biological effects conflicting with the assumption that *Stannum metallicum* 30x is identical to placebo when replicated independently.

4.3. Evaporation time

Studies focussing on the reproducibility of this fingerprint metabolomic analysis indicate that the overall morphology of the crystallization patterns is influenced primarily by the additive and to a lesser though significant extent by the evaporation time (Busscher et al., 2010a). A prerequisite for nucleation is that the $CuCl_2$ concentration in the Petri-dish exceeds the solubility threshold of 56 w%. This process is driven by the evaporation of water from the Petri-dish by heating the dishes from below to a constant temperature. The actual onset of crystallization is governed by a *stochastic* process, and as such, is somewhat unpredictable. Consequently, crystallization can occur at different degrees of $CuCl_2$ supersaturation. A later onset of crystallization implies a higher degree of supersaturation and consequently a higher crystal growth rate. At low supersaturation levels the crystals are growing alongside an axis whereas high supersaturation levels alter the energetically favorable directions of crystal-growth towards more dendritic structures (Leray, 1968; Sunagawa, 2005; Reiter and Barth, 2010). Therefore, standardization of the main factors of influence governing the evaporation rate has been a major point of focus in the development of this fingerprint metabolomic analysis (Busscher et al., 2010a, 2010b), as this will increase the reproducibility of the outcome.

The evaporation times of the 43 Petri-dish positions exhibit a non-random distribution which most likely is a consequence of constructional limitations of the crystallization apparatus and due to the positioning of heating and humidifying apparatuses (outside the chamber) relative to the Petri-dish positions. To obtain a better balanced distribution of the evaporation times we characterized the evaporation rates for the 43 positions prior to this study and allocated the 7 experimental groups accordingly, to obtain as homogeneous as possible evaporation times. Not-withstanding the above, we analysed whether systematic variations in evaporation times occurred during the time span of this project, and if so, whether this biased the treatment effect. Analysis of variance revealed that the evaporation times for the verum experiments did not differ between the different treatments, though there were differences in absolute values from experiment to experiment and between the two laboratories. Concerning the SNC experiments, results were essentially similar to the verum experiments, albeit that a highly significant interaction was found between laboratories and experimental number ($p < 0.0001$) and a mild interaction between the laboratories and the water control samples (Table 3, interaction 1–3; $p = 0.03$). This most likely points towards an instability regarding the mean evaporation times between the two laboratories as it conflicts with the variance analysis performed on the verum data of the present and precursor study. Visual inspection of the corresponding scatter plots revealed outliers regarding the evaporation times between the 2 laboratories for SNC experimental numbers 4 and 10, which didn't occur in the verum experiments (see Fig. 4). Furthermore, SNC experimental number 8 showed an inversion of the mean evaporation times between the 2 laboratories. Re-analysis upon exclusion of experimental numbers 4, 8, and 10 indeed nullified both interactions.

4.4. Processing order effect

In the present project we investigated the reproducibility of the effects found in repeated experiments based on improved methodology compared to the precursor experiment. Methodological improvements covered, amongst others, the optimization of the laboratory procedures to avoid any processing order effects which were present in the precursor study. To this end the harvesting of suitable seedlings was performed in two runs, each run consisting of half the amount of the available germination bags per treatment. Furthermore a 12-minute time-block was applied for the germination start of each treatment to control for the time required for the harvesting of the seedlings and thereby further minimizing a potential processing order effect. The randomization procedure incorporated in the experimental set-up resulted on average in an equal processing order for all treatments. Consequently, any processing order effect would not show up in the mean, but only as an interaction between treatment and experimental day.

Variable cluster shade showed such an interaction between potency treatment and experimental number, albeit weak (Table 2, interaction 2–3). This seemed to point towards a persistent processing order effect presumably as a consequence of time variations inadvertently introduced while producing the six *Lepidium sativum* extracts. Analysis

yielded highly significant differences in absolute values for the different laboratory processing-orders in the SNCs, albeit that there was no approximate linear trend in the data, indicative of an absence of a simple linear processing-order effect.

Apparently the introduced methodological improvements were insufficient regarding the eradication of the processing order effect, necessitating further methodological optimisations.

4.5. Preclinical research into potentized substances

Replication trials of preclinical research into potentized substances by means of in vitro assays were last addressed in a review by Endler et al. (2015). Of the 28 experimental models which underwent replication, 5 models were externally reproduced with comparable results. These five models consist of plant-based bio assays, cell cultures, models with cells from donated blood, and animal models. Plant-based bio assays are of high interest for preclinical research into potentized substances due to their comparative simplicity, sensitivity, and cost-effectiveness.

One of the most promising plant-based bioassays is the study of the effect of *Argentum nitricum* 24x (nominal dilution of 10^{-24} of the original substance) on the development of wheat seedling coleoptiles. Scherer-Pongratz et al. (2015) performed meta-analyses on pooled data from multi-centre experiments performed between 1989 and 1995, and from 1998 to 2014 on statistical significance and average effect size. For the experiments from 1989 to 1995 verum stalk length was 21.9% bigger as compared to water control ($p < 0.01$). For the experiments 1998–2014 verum stalk length was 21.7% bigger than control ($p < 0.01$), thereby confirming the previous findings and presenting empirical evidence of specific effects of *Argentum nitricum* 24x. However, external replication studies did not show a statistical significant treatment effect in all cases (Pongratz et al., 1998).

A similar model with wheat seedlings and gibberellic acid 30x showed inconsistent results. Upon meta-analysis a season related effect-inversion was detected. Re-analysis of autumn experiments only (pooled data of 10 individual experiments) revealed a verum stalk length reduction of 7.3% compared to water control (Endler et al., 2015).

A major challenge in basic research into homeopathic potentisation is to develop bioassays that yield consistent results. Difficulties reproducing the results could be caused by the fact that the experimental systems and/or parameters chosen to document the nature of the effect of potentized substances are inadequate (Baumgartner, 2016). Nani et al. (2007) postulated a 'systemic' mode of action of potentized substances, which is thought to promote homeostasis, and consequently reduce the scatter of the system. In this light, the addition of this fingerprint metabolomic analysis to the *Lepidium sativum* bioassay seems a logical choice. The resulting crystallization patterns are additive specific and emerge through a self-organization process which is influenced by the properties of the additive (Busscher et al., 2014).

It seems likely that the complex and multidimensional characteristics of crystallization patterns relate better to the nature of homeopathic remedies than uni-dimensional (e.g. growth) parameters. Clinical evaluation of the effects of homeopathic remedies in healthy volunteers yielded a multitude of specific symptoms of homeopathic remedies (Möllinger et al., 2009) pointing towards a mode of action involving a multidimensional information content.

4.6. Methodical strengths and weaknesses

Methodical strengths of our study are the parallel investigations in two independent laboratories, the use of independent homeopathic sample batches (*Stannum met.* 30x, lactose 30x) for each experimental day, the randomization and blinding procedures applied, the objective outcome measure, the sterile preparation of the homeopathic samples, the use of lactose 30x as control sample to control for the trituration of *Stannum metallicum* in lactose up to 4x, and a full implementation of blinded systematic negative control (SNC) experiments to control experimental stability.

The methodical weakness is the persistent processing order effect which was also present in the precursor study, although this should not lead to false-positive results. Apparently the introduced methodological improvements were insufficient regarding the eradication of the processing order effect, necessitating further methodological optimisations. Considered are 1) harvesting the seedlings under non-saturating, photosynthesis-limiting light conditions, thereby standardizing the light source and any additional metabolic activity during the harvesting procedure 2) performing the entire procedure of harvesting, crushing, extracting and pipetting into the $CuCl_2$ solution one sample at a time to limit metabolic activity during the time-lag otherwise introduced at the different procedural steps by the processing of successive samples. Furthermore, the high salt concentration ($0.15\ mol\ L^{-1}$) of the final *Lepidium sativum* extract-$CuCl_2$ mixture is estimated to minimize any further metabolic activity by acting as a thiol oxidizing agent. Concentrations of 40 µM $CuCl_2$ were shown to almost completely inhibit protein import into chloroplasts (Seedorf and Soll, 1995), and 3) inhibiting any further metabolic activity directly after harvesting the seedlings by crushing the seedlings in liquid nitrogen.

Limitations or disadvantages of the experimental setup consist of the need for a careful manual selection of *Lepidium sativum* seeds prior to germination, and the relatively time-intense bioassay. It would be interesting to determine whether homeopathic preparations could directly be characterized with this fingerprint metabolomic analysis (i.e. omitting the *Lepidium sativum* seedling bioassay). The crystallization of $CuCl_2$ on a glass surface gives rise to complex dendritic crystallization patterns (needles with branching and sub-branching) only if the glass surface is wettable (Gallinet and Gauthier-Manuel, 1992; Busscher et al., 2010b; Busscher et al., 2014). To induce a more wettable (hydrophilic) condition, the adsorption of minimally one monolayer of protein is required, which normally occurs during the evaporation phase as a consequence of the increase in the $CuCl_2$ salt concentration and the resulting desalting of proteins. According to Busscher et al. (2018), 100 mg cupric chloride per crystallization plate (float glass, Ø = 90 mm) in a total volume of 6.0 ml requires a minimal of approximately 1–3 calculated monolayers of BSA to prevent dewetting. Possibly the combination of a homeopathic potency together with 1–3 calculated mono-layers of BSA would be sufficient to obtain sample specific crystallization patterns. Thereby significantly easing the procedure and surpassing the time-intense and variation-prone *Lepidium sativum* bioassay.

4.7. Conclusion and outlook

In this manuscript we present empirical evidence of specific biological effects of a homeopathic preparation produced according to Ph. Eur. monograph 1038 when replicated independently. The test system is based on *Lepidium sativum* seed germination, fingerprint metabolomic analysis and subsequent computerized textural image analysis of the resulting crystallization patterns. These results must either be considered as a scientific anomaly or they are in line with the hypothesis that application of the European Pharmacopoeia monograph 1038 for the production of homeopathic preparations may lead to pharmaceutical preparations with specific biological effects.

Further practical questions that could be addressed might include assessing of whether or not the practice of sterilizing ophthalmic solutions (according to Good Manufacturing Practices in Homeopathy by autoclaving at 121 °C for 20 min (Homöopathisches Arzneibuch Amtliche Ausgabe, 2011)) has any effect on the efficacy of the homeopathic remedies. The same question applies for cell phones, operating at radio-frequencies between 450 and 2700 MHz. The European Pharmacopoeia (EDQM, 2017) states that the quality standards applied to homeopathic remedies are similar to those applied to all other

medicinal products. This concerns amongst others the identification and assay of all the toxicologically relevant constituents and demonstrating the stability of the finished medicinal product, although considerations are made as identification and assay of source materials may not be feasible at high potencies. Consequently a bioassay yielding specific effects for different homeopathic remedies would be invaluable for quality control. This *Lepidium sativum* seedling fingerprint metabolomic analysis test-system might develop into a potential candidate to respond to these challenges if developed further.

CRediT authorship contribution statement

P. Doesburg: Conceptualization, Investigation, Formal analysis, Validation, Writing - original draft. **J.-O. Andersen**: Conceptualization, Resources, Investigation, Writing - review & editing. **C. Scherr**: Conceptualization, Resources, Writing - review & editing. **S. Baumgartner**: Conceptualization, Formal analysis, Validation, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare no competing interests.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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