Original Article

Particle Characterisation of Traditional Homeopathically Manufactured Medicine Cuprum metallicum and Controls

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Abstract

Background: Homeopathy is highly controversial. The main reason for this is the highly diluted medicines (high homeopathic potencies, HHP), diluted beyond the Avogadro/Loschmidt limit. Research using several different methods has demonstrated the presence of particles, including nanoparticles of source material, in HHPs. This study aims to verify the results of a previous publication that detected the presence of particles in all dilutions. **Methods:** We used the Nano Tracking Analyzer (NTA) to examine dilutions of a commonly used homeopathic medicine, an insoluble metal, Cuprum metallicum, for the presence of particles. The homeopathic medicines tested were specially prepared according to the European pharmacopeia standards. We compared the homeopathic dilutions/dynamizations with simple dilutions and controls, including a soluble medicine. **Results:** We observed the presence of solid material in all preparations, including HHPs (except for pure water). The measurements showed significant differences in particle sizes distribution between homeopathic manufacturing lines and controls. The probability that the observed differences could have occurred just by random chance can be rejected $p < 0,001$ (even above Avogadro limit $p < 0,01$). **Conclusion:** The homeopathic medicines Cuprum metallicum contain material with a specific size distribution even in HHPs diluted beyond the Avogadro/Loschmidt limit. This specificity can be attributed to the manufacturing and potentization process. This material demonstrates that the step-by-step process (dynamized or not) does not match the theoretical expectations in a dilution process. The starting material and dilution/dynamization method influence the nature and concentration of these NPs. Further measurements are needed on other raw materials using the same controls (solvent and simply diluted manufacturing lines) to support these findings.

Keywords: Nanoparticles; Particles; Cuprum metallicum, Copper; Lactose; Kalium muriaticum; Potassium chloride; Homeopathy; Potentization; Dynamization; Pharmacology; Nanoparticle tracking analysis (NTA).

Introduction

Homeopathic practitioners daily prescribe homeopathic medicines, and patients take these regularly. As a result, there is an increasing need for a clear explanation of the nature of these medicines. Homeopathy is a traditional medicine that has been used worldwide for more than 200 years. Recent studies indicate that homeopathically-prepared medicines (HMs) contain source nanoparticles (NPs) [1-7], silicates [4,8], and other, less well-characterized structures [9-15]. In addition, a recent RCT-

literature audit [16-17] once more confirmed a specific effect of homeopathic treatments. Nevertheless, skeptics insist that HMs are mere placebos containing no active material in any form [18].

This debate about plausibility and evidence [19-22] can only be settled by fundamental research. Skeptics have focused on the dilution of bulk source material beyond Avogadro's number and have ignored the fact that the actual manufacturing process is more than simple dilution – it involves step by step potentization (for water-soluble material), also called a “dilution-dynamization” process which is described in the European Pharmacopoeia [23]. The potentization process is performed using a certified machine which normally provides 100 calibrated vertical shocks at each dilution. The dilution process may be 1 part of the material for 9 parts of solvent (D or X potency) or 1 part of the material for 99 parts of solvent (C or Korsakov potency). The containers are always pharmaceutical soda-lime-silicate glass ISO-719, ISO4802-1, Ph-Eur 3.2.1.; USP <660>: = < 0,85 ml 0,02N HCL/g. A new container is used for C and D potencies at each step.

Nanotechnology researchers have begun to recognize similarities between the traditional manufacturing techniques of homeopathy using crude, mechanical abrasion and the top-down approaches for making nanostructures from insoluble source materials used in modern nanotechnology [1-3,5-7]. Using transmission electron microscopy (TEM) and inductively-coupled plasma atomic emission spectroscopy (ICP-AES), the authors found irregularly-shaped poly-dispersed source metal particles in concentrations ranging from picograms to nanograms per ml, in six different commercially-made metal HMs including copper.

Previous publications [13,24-27] using NMR relaxation have revealed the involvement of nanobubbles and/or nanoparticles and/or nanometric superstructures in high potentizations. The purpose of the present exploratory study was to replicate and extend the characterization of colloidal particles in homeopathically-made *Cuprum metallicum* (copper source) following good pharmaceutical practice (GPP) in 6 different manufacturing lines up to 30C potency, comparing these with solvent control, potentized lactose control, diluted copper control, and potentized soluble potassium chloride control.

Materials and Methods

Rationale: Anticipating a need to detect low concentrations of poly-disperse particles of different sizes, shapes, surface properties, and composition [1-2], we used Nano tracking analysis (NTA) [6]. We chose copper as the initial source material for HMs because it has already been characterized by previous authors [1-3], because of the well-known role of copper in the mitochondrial enzyme cytochrome c oxidase [28], and because there is an extensive homeopathic literature published on this homeopathic medicine including a randomized controlled trial [29-42].

Homeopathically-manufactured medicines

The medicines and controls were made in our laboratory located in Wepion (Belgium), under a validated (ISO 5) laminar flow (“Thermo Heragard Eco 1.2 horizontal” B75/180). Our pharmacist followed the European pharmacopoeias, which describe precisely how the manufacturing process must be done according to the homeopathic tradition. The triturations were performed manually following the standardized rules and controls of good pharmaceutical practice (GPP). We used monohydrated, moderately fine, lactose (particle x 50 = 240µm) ABC Chemicals. Aut.84GIR05797. Ph. Eur.+ Monogr. Lot 19I04-B02-194990 Exp. 30-04-2022. Cond. 30-10-2019. The water used is deionized water drawn directly from the tap after releasing some water first. The tip of the tap goes

directly into a flask containing ethanol to avoid ambient contamination. Purifying apparatus is Millipore Milli-RX 45 serial number FSDM 96292D. Remedy Bank provides 50 grams of copper powder: Sigma 203122 lot #MKBS 4830 Pcode 1001 89 73 74 containing 99.999% copper. The brown pharmaceutical 30cc flasks used are soda-lime-silicate glass ISO-719, ISO4802-1 closed by tight plastic drop-caps with an under-screwed polypropylene closure system PhEur 3.1.3 "Polyolefines"; PN*18*K*S1 0,6 of PPH.

The pharmacist's equipment included a mask (possible toxic effect on respiratory tract), protective glasses (possible irritating effect), shoe protections, a white apron, no perfume, a head cap, and gloves (during triturations). In addition, hands were washed meticulously before carrying out any operation. 100mg of copper was placed on a paper, and 9900mg of lactose was weighed for the trituration. According to the method PhEur 4.1.2., the vehicle was added carefully in small quantities while grinding until the entire vehicle was incorporated. The duration of trituration was one hour to obtain a 1C potentization. The intensity of trituration is sufficient to ensure homogeneity, with a particle size after trituration not exceeding 100 µm. The result is an ultra-fine, dry powder with a final mass between 9317mg and 9638mg (6 separate manufacturing lines were started after the trituration processes); the weight loss is due to adhesion onto the pestle and mortar.

After washing and passivating the mortar, pestle, and spatula, the same process was performed, starting with 100mg of the 1C and 9900mg of lactose. Following the same procedure with 100mg of 2C and 9900mg of lactose, we produced the 3C. From this step onward, the mixture becomes soluble in water. Therefore, we decided to avoid using an alcohol-water mixture lest the alcohol alters the measurements, adding unwanted contaminants to the solution. All further steps were performed in washed and passivated 30cc new glass containers. First, 0.2g of 3C was added to 19.8g of water and dynamized (100 +/-2 shocks in 2 seconds using a Labotics© certified and validated dynamizer "Dynamat©") to obtain a 4C potency. Then, the exact process produced further dynamizations, each time in a new container, adding 0.2g of the previous dynamization in 19.8g of water to produce 5C, 6C, up to 30C.

The potentized lactose controls were produced the same way, including the 3 first trituration steps but starting from 10g of pure lactose to produce the 1C. The simply diluted copper control was prepared following the same successive steps after 3 triturations. Further dilutions were prepared in water but without the dynamization process. Our fourth control was potentized Kalium muriaticum (soluble raw material); the manufacturing process is the same as copper (dilution and dynamization). Since this substance is directly soluble in water, a prior trituration process in lactose is unnecessary. Without trituration in sugar, any impact of lactose on the preparation is removed. We also prepared one additional pre-filtered (filter of 0,1µm) pure water control and one simply diluted lactose in pure water 1/100 control. Each flask was labeled with an abbreviated code for raw material, including the level of dilution or potentization, line number, and manufacturing date.

Randomization and blinding

Since the measurements were performed on a total of 648 samples, repeated 5 times, representing 3,240 NTA measurements, the risk of errors using a double-blind approach is very considerable in practice. The final goal of this study is to determine whether the presence of particles can be systematically confirmed to observe these later with SEM-EDX. At this point, a complete double-blind procedure will be implemented. For this reason, we preferred to use a more reliable approach here, with each bottle labeled with the abbreviated code given by the pharmacist. Samples were brought to the measurement laboratory for NTA measures. Full labeling only occurred when the final results were entered into an Excel file.

Nanoparticle tracking analysis (NTA)

Nanoparticle Tracking Analysis (NTA) utilizes the properties of both light scattering and Brownian motion in order to obtain the particle size distribution of samples in liquid suspension. This technology can only detect particles larger than 20nm. This technology has already been used by other authors [5]. The wavelength used for these measurements was 488nm. A Syringe Pump provides a continuous flow of new particles into the sample chamber. The system is used in Light Scatter mode, allowing more robust data due to improved sampling statistics. The camera captures a video file of the particles pushed through the chamber at a continuous speed. Particles are counted and their sizes measured (1500 frames analyzed per minute).

The NanoSight Malvern instrument and software were provided by SYSMEX Company and installed in our measurement laboratory in Chastre (Belgium). The mathematical analysis of the results was undertaken by the first author (MVW), supervised by the team and external experts.

The different measurements obtained are the number of particles, size (volume), and size distribution (SPAN). For a given sample, each measurement is repeated 5 times.

SPAN is a particle size (volume) distribution parameter. The NTA measurements calculate the standard distribution (SD), the LD90, LD10, and LD50, which are respectively the 90th percentile, the 10th percentile, and the median of particle sizes. The SPAN formula $LD90-LD10/LD50$ indicates the distance between the 10th and 90th percentile, normalized concerning the midpoint.

Statistical analysis

We used SigmaStat version 4, allowing multiple statistical procedures [43]. A Kruskal-Wallis test is used to determine whether or not there is a statistically significant difference between the medians of three or more independent groups. It is considered to be the non-parametric equivalent of the One-Way ANOVA. If the results of a Kruskal-Wallis test are statistically significant, then it is appropriate to conduct Dunn's Test to determine exactly which groups are different. Dunn's Test performs pairwise comparisons between each independent group and tells which groups are statistically significantly different at some level of α . For the comparison between copper and potassium chloride values, we used the Mann-Whitney *U* test (also called the Mann-Whitney-Wilcoxon), it is a nonparametric test of the null hypothesis that, for randomly selected values *X* and *Y* from two populations, the probability of *X* being more significant than *Y* is equal to the probability of *Y* being greater than *X*.

Results

Nanoparticle tracking analysis (NTA) or Nano-sight

NTA revealed the presence of particles in all diluted or potentized samples. No significant numbers of particles were found in the pure water control. Lactose, triturated and diluted 1/100, contains 3.3 times more particles than pure lactose at the same concentration. The amount of particles observed in dynamized preparations is much more stable than in simply diluted preparations. The particle size distribution (SPAN) is specific to the raw material, allowing us to differentiate a potentized raw material from another in full manufacturing lines or a simple dilution. (figure 1-8 & table 1-5).

The simple dilutions of Cuprum metallicum are prepared from the third trituration of copper in lactose, i.e., 3C, which is the same starting point as for the potentized copper. Except for the first dilution (10^{-8}) comparable with 4C potency (100 times more concentrated than the next dilution/potency 5C) and 10^{-10} comparable with 5C, which contains 1.5×10^8 particles/ml, the number of particles oscillates around 3 million particles/ml for the simple dilutions and 6 million

particles/ml for dynamized dilutions. At first glance, the particle size distribution appears unstable and chaotic for each sample (5 different measurements) and from one production line to another for the simple dilutions.

Figure 1: Comparison of number of particles in diluted 1/100 pure pharmaceutical Lactose and Lactose 4C (in this case lactose concentration is also 1/100 after 3 successive triturations).

The graphs above represent 5 successive measurements of the samples. Each measurement is derived from 1,500 successive frames of the camera. There are more heterogeneity between the 5 measurements and wider dispersion of particle size (up to 400nm) in Lactose without trituration (left curves), and more than 3 times more particles/ml (in million/ml, y-axis of the graph) after trituration and dynamization (right curves, 184 million particles/ml versus 54,2 million particles/ml).

Pooling the results obtained from 6 different manufacturing lines of each starting material (Fig. 2). the validity of these results is reinforced. Each graph comes from the recording of 45,000 successive frames of the camera. Mainly for Cuprum metallicum, there is great similarity in the number (y-axis) and size of particles (x-axis) in the 6 pooled production lines.

Figure 2: Comparison of number and size of particle in pure Lactose 4C and Cuprum metallicum 4C.

For this study we worked with 6 identical manufacturing lines of each raw material (Fig. 3). For both number of particles detected and their size it is necessary to verify if the obtained results are similar in each production line. Linear fits of the quantities of particles are overlapping for the 6 manufacturing lines of a same starting material as shown in this example for lactose CH; pooling these values appears valid. It is the same for the other starting material/manufacturing processes, even though for simple dilutions the values below 10^{-40} (corresponding to a dilution 20C but without dynamization) fluctuate more from one production line to another than showed in this example.

Figure 3: Example of control of validity of pooling measurements of number of particles in 6 Lactose CH manufacturing lines.

For this study we worked with 6 identical manufacturing lines of each raw material (Fig. 4). Also for the particles' size it is necessary to verify if the obtained results are similar in each production line. Linear fits of particle sizes are overlapping for the 6 manufacturing lines of the same starting material, so pooling these values appears valid too. However, there is variability in particle size for each isolated potentiation.

Figure 4: Example of control of validity of pooling measurements of particle size in 6 manufacturing lines of Cuprum metallicum.

Pooling the results obtained from 6 different manufacturing lines of each starting material (Fig. 5). Each graph comes from the recording of 45,000 successive frames of the camera. Even in the highest potentizations (30C of dynamized lactose and Cuprum), particles persist in fairly stable quantities, around 8 million particles per millilitre (y-axis). The distribution of particles of different sizes in nm (from small to large, x-axis), however, is specific and increases more for Cuprum throughout the dynamization process. For lactose the majority of particles sizes in behind 100nm, for Cuprum above 100nm.

Figure 5: Comparison of number of particles in pure Lactose 30C and Cuprum metallicum 30C.

The simple dilutions of Cuprum metallicum are prepared from the third trituration of copper in lactose, i.e., 3C, which is the same starting point as for the potentized copper. Except for the first dilution (10^{-8}) comparable with 4C potency (100 times more concentrated than the next dilution/potency 5C) and 10^{-10} comparable with 5C, which contains 1.5×10^8 particles/ml, the number of particles oscillates around 3 million particles/ml for the simple dilutions and 6 million particles/ml for dynamized dilutions. At first glance, the particle size distribution appears unstable and chaotic for each sample (5 different measurements) and from one production line to another for the simple dilutions.

For each sample, five 60-second films recording the passage of particles injected in a controlled and regular way into the cells are made (Fig 6). Here you can see two frames from these films. Each film contains 1,500 successive frames from 2 Lactose preparations. Each particle is illuminated by a laser beam. Frame by frame, a computer program counts the number of particles and measures their sizes. In a final report we obtain the result of $5 \times 1,500$ (7,500) measurements presented in numbers and in graphs such as the ones presented in previous figures. The computer calculates also the distribution in size of the particles (SPAN) including the standard deviation or possible margin of error.

A camera frame of pure Lactose simply diluted 1/100

A camera frame of Lactose 4C production line (a)

Figure 6 : Examples of particles passing in front of the NTA laser camera (pure lactose versus 4C).

The presence of particles above 20nm in successive dilutions/potentizations of Cuprum metallicum (Mean number of particles/frame; adding the Standard Deviation or margin of error '+SD' and subtracting the Standard Deviation (-SD) showing the reliability of the measurements) are shown in Table 1. Values below 1 are questionable but seldom. The quantities of particles per frame are quite stable in potentized preparations. However, with a simple dilution the number of particles becomes extremely unstable from 10^{-40} corresponding to a 20C. The measured numbers of particles do not allow discrimination between manufacturing lines. In pure water control we found 0,5 particles per frame with a very high standard error of 0,4.

Table 1: Comparison of pooled number of particles in Lactose C, Cuprum metallicum C & simply diluted after 5 successive measurements of each samples from 6 manufacturing lines.

Dil/Dyn	Potentized lactose			Potentized Cuprum			Simple diluted cuprum		
	+SD	-SD	Particles per frame	+SD	-SD	Particles per frame	+SD	-SD	Particles per frame
4Ch	30.68	28.72	29.7	29.82	28.58	29.2	28.28	26.82	27.55
5Ch	1.65	1.25	1.45	2.27	1.97	2.12	3.82	3.42	3.62
6Ch	2.43	2.17	2.3	2.58	1.82	2.2	1.01	0.85	0.93
7Ch	2.5	2.1	2.3	1.92	1.32	1.62	0.64	0.48	0.56
8Ch	1.92	1.48	1.7	1.83	1.17	1.5	0.74	0.62	0.68
9Ch	2.3	2.1	2.2	2.18	1.42	1.8	1.3	1.1	1.2
10Ch	3.26	3.06	3.16	1.96	1.5	1.73	0.7	0.4	0.55
11Ch	2.4	2.2	2.3	1.29	0.87	1.08	0.68	0.42	0.55
12Ch	2.99	2.73	2.86	1.17	1.15	1.16	1.25	0.95	1.1
13Ch	2.28	2.08	2.18	2.21	1.55	1.88	1.33	1.07	1.2
14Ch	2.52	1.3	1.91	0.9	0.5	0.7	1.05	0.89	0.97
15Ch	1.63	0.83	1.23	1.26	0.94	1.15	1.17	0.93	1.05
16Ch	2.21	1.81	2.01	3.78	1.52	2.65	0.52	0.38	0.45
17Ch	2.38	2.18	2.28	2.06	1.24	1.65	1.56	1.16	1.36
18Ch	2.23	2.03	2.13	2.36	1.4	1.88	1.82	1	1.41
19Ch	2.27	2.27	2.27	1.58	1.52	1.55	1.14	0.72	0.93
20Ch	2.1	1.64	1.87	2.39	1.53	1.96	8.39	4.03	6.22
21Ch	2.9	2.3	2.6	1.37	1.03	1.2	3.49	1.35	2.42
22Ch	2.59	2.07	2.33	0.78	0.62	0.7	4.37	1.61	2.99
23Ch	2.71	2.51	2.61	0.83	0.63	0.73	2.13	0.63	1.38
24Ch	1.9	1.5	1.7	1.71	1.45	1.58	2.16	0.86	1.51
25Ch	1.72	1.32	1.52	1.63	1.33	1.48	3.86	0.64	2.25
26Ch	2.12	1.72	1.92	1.3	1.1	1.2	8.98	2.02	5.5
27Ch	1.83	1.23	1.53	1.34	1.1	1.22	6.62	1.38	3.95
28Ch	1.6	1.1	1.35	1.89	1.57	1.73	5.55	1.55	3.55
29Ch	1.62	1.42	1.52	1.54	1.32	1.43	2.2	0.8	1.5
30Ch	1.85	1.45	1.65	1.29	1.03	1.16	4.49	0.97	2.73
Mean 4/9Ch			6.61			6.41			5.76
Mean 5/9CH			1,99			1,40			1,85
Mean 10/19Ch			2.23			1.54			0.96
Mean 20/30Ch			1.87			1.31			3.09
Mean 12/30Ch			1,97			2.24			2,24

For the particle size in nm, there is a tendency to differentiate each raw material without reaching a significant level of distinction (Table 2). (Mean size of particles; + Standard Deviation and – Standard Deviation are the error margins). Particles mean size of pure water control is 106nm (error size +/- 9,7nm), but the very few particles in this water (only 0,5 particles/frame) is below the limit of

accuracy of the NTA measurements). The measured sizes does not allow discrimination between manufacturing lines.

Table 2: Comparison of pooled particles size in nm for Lactose C, Cuprum metallicum C & simply diluted copper after 5 successive measurements of each samples from 6 manufacturing lines.

	+SD	-SD	Lactose C	+SD	-SD	Cuprum C	+SD	-SD	Cuprum dil.
4Ch	95.3	93.8	94.7	84.65	83.62	84.2	88.87	87.13	88
5Ch	112.5	101.9	107.2	90.68	81.32	86	108.67	100.93	104.8
6Ch	98.8	92.6	95.7	106.38	93.62	100	102.56	91.1	96.83
7Ch	92.1	85.5	89.3	104.02	94.78	99.3	147.93	121.07	134.5
8Ch	110.1	97.3	103.7	109.41	100.19	104.8	106.5	93.9	100.2
9Ch	117.5	107.5	112.5	105.6	93.4	99.5	104.75	93.85	99.3
10Ch	97.2	91.8	94.5	104.4	95.6	100	119.45	97.55	108.5
11Ch	99.7	92.7	96.2	109.23	97.77	103.5	119.2	98.2	108.7
12Ch	100.5	93.1	96.8	146.9	122.1	134.5	110.8	99.2	105
13Ch	107.2	99.4	103.3	125.68	112.52	119.1	115.38	102.02	108.7
14Ch	110.6	105	107.8	144.88	127.12	136	141.4	109.2	125.3
15Ch	145.8	127.4	136.6	143.1	123.1	133.1	111.4	102.6	107
16Ch	116.6	108.1	112.3	123.5	109.5	116.5	147.18	98.42	122.8
17Ch	123.9	113.1	118	127.6	112.4	120	117.18	105.42	111.3
18Ch	127.1	115.9	121.5	125.4	115.8	120.6	113.3	102.9	108.1
19Ch	120.1	110.9	115.5	136	123.6	129.8	132.65	108.35	120.5
20Ch	130.8	117.2	124	124.73	115.27	120	115.95	95.05	105.5
21Ch	108	101	104.5	150.28	130.32	140.3	126.73	97.27	112
22Ch	112.5	102.5	107.5	120.5	106.9	113.7	113.63	97.37	104.5
23Ch	104	97	100.5	133.4	117.8	125.6	125.3	104.9	115.1
24Ch	100.1	92.1	96.1	111.2	100.1	105.8	161.25	128.35	144.8
25Ch	106.2	98.6	102.7	108.93	100.67	104.8	117.28	96.32	106.8
26Ch	113.7	97.7	105.7	115.1	104.9	110	130.3	99.9	115.1
27Ch	106.5	95.4	101.1	110.5	101.9	106.2	113.85	85.49	99.57
28Ch	103.2	88.9	96	122.4	108.6	115.5	112.05	87.95	100
29Ch	99.1	92.4	95.7	117.9	104.1	111	114.35	84.25	99.3
30Ch	95.9	87.2	91.5	115.08	103.02	109.5	100.56	81.64	91.1

Figure 7: Comparison of pooled particles volumes' distribution (SPAN) for Lactose C, Cuprum metallicum C & simply diluted copper.

Each number in Fig. 7 is the mean of 5 consecutive measurements on 6 pooled manufacturing lines from 4 to 30C of potentized Lactose/ potentized Cuprum metallicum/ Simply diluted Cuprum metallicum. It is the result of 30 successive counts. SPAN is a particle size (volume) distribution parameter. The DL90, DL10 and DL50, which are respectively the 90th percentile, the 10th percentile

and the median of particle sizes. The SPAN formula $(DL_{90}-DL_{10})/DL_{50}$ is an indication of the distance between the 10th and 90th percentile, normalized with respect to the midpoint. The SPAN value is between 0,8 and 2,8 (Y axis) is these measurements. Linear fits (dotted lines) gradually diverge from each other during the dilution/dynamization process. Pure water contains only a few particles but of very different sizes explaining a high SPAN value. From 4C up to 9C we cannot see differences between manufacturing lines, above 9C a discrimination (specificity) became possible. Further statistical analyse is needed.

For SPAN, a discrimination of complete manufacturing lines can be made during the dynamization process of the different used raw materials and the simple dilution (Cuprum CH, Lactose CH, and Copper diluted after trituration), reaching a statistically significant level (see statistical results).

SPAN Statistical results

The SPAN values of 6 identical manufacturing lines of the same starting material are similar and, therefore, can they be pooled. The values were grouped into low (4 to 9 C), medium (10 to 19 C), and high dynamizations (20 to 30 C). The scientific literature states that a molecular effect is no longer possible above a concentration of 10^{-18} (9C). [44].

In homeopathy, practitioners also distinguish very high dynamizations. For example, most modern provings today are made with 30 C dynamizations [45].

For the SPAN calculations, linear particle size fits are comparable to the 6 manufacturing lines of the same starting material, so pooling these values is valid. Furthermore, there is no statistically significant difference between the different production lines, even though some differences are apparent from one production line to another when each dilution/dynamization is considered in isolation from the previous or following ones.

For the SPAN, there is significant discrimination between the pooled manufacturing lines. This discrimination is also evident if the highest concentrations of lactose (4 to 9 C) are excluded (see tables 3-4).

Figure 8 : pooled SPAN values for the different manufacturing lines regroupped in low, medium and high dilutions/dynamizations.

Fig. 8 presents pooled Data on SPAN values of potentized (diluted and dynamized) Lactose, Copper simply diluted and potentized Copper. On the left the values obtained from 4C to 9C (10^{-8} to 10^{-18}); in the middle the values obtained from 10C to 19C (10^{-20} to 10^{-38}); on the right values obtained from 20C to 30C (10^{-40} to 10^{-60}).

SPAN values are very robust since they originate from multiple successive measurements (Table 3). Since they have been aggregated into means, the normality test fails. For the lowest dynamizations, the particle size distribution measured in NTA cannot be differentiated by the nature of the production line (pure lactose, dynamized copper or simply diluted copper). For medium and high dynamizations, the particle size distribution measured in NTA is clearly distinct depending on the production line.

Table 3: NTA-SPAN SigmaStats 1-way ANOVA on means.

Dyn/Dil	SPAN Lactose Ch	SPAN Cuprum Ch	SPAN Cup Dil	Normality Shapiro-Wilk	Kruskal-Wallis One Way	Multiple comparison	Dunn's Method
CH4	0.81	0.77	0.75				
CH5	1.08	0.99	0.97				
CH6	1.10	1.03	1.06	$p < 0.050$	$p = 0.352$		
CH7	1.06	1.08	1.17				
CH8	1.25	0.99	1.16				
CH9	1.03	1.02	1.01				
CH10	0.80	1.05	1.26				
CH11	0.87	1.22	0.87				
CH12	0.91	1.74	1.11				
CH13	0.82	1.09	0.90				
CH14	0.83	1.02	1.40			3 vs 1	$p < 0.001$
CH15	1.13	1.28	0.96	$p < 0.050$	$p < 0.001$	3 vs 2	$p < 0.001$
CH16	0.84	1.33	1.20			2 vs 1	$p = 0.033$
CH17	0.94	1.20	0.92				
CH18	1.15	1.26	1.02				
CH19	0.92	1.17	1.03				
CH20	0.96	1.43	1.49				
CH21	0.96	1.13	2.92				
CH22	0.92	0.94	1.66				
CH23	0.86	1.10	1.46				
CH24	0.82	1.05	1.27			2 vs 1	$p < 0.001$
CH25	0.80	1.06	1.25	$p < 0.050$	$p < 0.001$	2 vs 3	$p = 0.077$
CH26	0.96	1.11	1.98			3 vs 1	$p < 0.001$
CH27	0.92	1.08	1.15				
CH28	0.95	1.20	1.83				
CH29	0.87	1.18	1.46				
CH30	0.90	1.04	1.9				

SPAN values for dilutions/dynamizations above 10^{-23} (Avogadro) are statistically significantly discriminated between copper potentization (12C up to 30C) and diluted copper (10^{-24} to 10^{-60}) $p = 0.01063$ (Table 4). The same is true between potentized lactose and the simply diluted copper $p < 0.001$ and between copper and potentized lactose $p = 0.0255$ in the same dilution range.

The difference in the median SPAN values between the two groups is greater than would be expected by chance (Table 5); there is a statistically significant difference ($p < 0.001$). Soluble material and no trituration in lactose was necessary.

Table 4: NTA-SPAN 1-way ANOVA on means above 10^{-23} (group 2) and 12c up to 30C (group 1 is below Avogadro).

ANOVA test	Df	Sum Sq	Mean Sq	F value	Pr(>F)
group	2	17.54	8.77	14.98	0.0000
Residuals	339	198.43	0.59		
Data Summary	Group	Size	Mean	Standard deviation	
	Cuprum_DIL	114	1.4776	1.2742	
	Cuprum C DYN	114	1.1823	0.2849	
	Lactose C DYN	114	0.9233	0.2265	
Paire-wise t-tests		Mean	Standard error	t-value	p-value
	Cup_Dyn-Cup_Dil == 0	-0.2952	0.1013	-2.9134	0.01063
	Lactose_Dyn-Cup_Dil = 0	-0.5543	0.1013	-5.4695	2.837e-07
	Lactose_Dyn-Cup_Dyn=0	-0.259	0.1013	-2.5561	0.02955

Table 5: Additional Control of SPAN calculations comparing Cuprum C versus Kalium muriaticum C.

Dyn/Dil	Kalium mur Ch	Cuprum Ch	Mann-Whitney Rank Sum Test
CH4	1.68	0.77	p < 0.001
CH5	1.41	0.99	
CH6	2.30	1.03	
CH7	1.97	1.08	
CH8	1.51	0.99	
CH9	1.12	1.02	
CH10	1.49	1.05	
CH11	1.33	1.22	
CH12	1.14	1.74	
CH13	1.31	1.09	
CH14	1.48	1.02	
CH15	2.08	1.28	
CH16	1.28	1.33	
CH17	1.83	1.20	
CH18	1.77	1.26	
CH19	1.76	1.17	
CH20	1.65	1.43	
CH21	1.47	1.13	
CH22	1.71	0.94	
CH23	2.36	1.10	
CH24	2.38	1.05	
CH25	1.06	1.06	
CH26	1.43	1.11	
CH27	1.49	1.08	
CH28	2.73	1.20	
CH29	1.57	1.18	
CH30	1.67	1.04	

Discussion

The volume distribution of the particles from the smallest to the largest (SPAN) is different and specific to the raw material and the manufacturing process (with or without dynamization). The used manufacturing process is not a strict dilution, as such, any reference to Avogadro's number should be prohibited when dealing with homeopathic medicines. SPAN values for dilutions/dynamizations above 10^{-23} (Avogadro) are statistically significantly discriminated between copper potentization (12C up to 30C) and simply diluted copper (10^{-24} to 10^{-60}) $p = 0.01063$. The same is true between potentized lactose and the simply diluted copper $p < 0.001$ and between copper and potentized lactose $p = 0.0255$ in the same dilution range. Comparing these values between two different drugs (full manufacturing lines of potentized Cuprum metallicum and Kalium muriaticum) also allows an obvious discrimination $p < 0.001$. For triturated potentized manufacturing lines, the first potentizations of copper and lactose (4C up to 9C) cannot be discriminated by NTA $p = 0.352$. This is probably due to the amount of residual lactose in these preparations. Above 9C (10^{-18}) statistics are significant $p < 0.001$.

The raw material impacts the formation of particles of various sizes throughout the following manufacturing process. This phenomenon also implies dynamization at each stage. Without dynamization, the number of particles and their size distribution are no longer coherent.

Following the European Pharmacopoeia [23], to avoid precipitates, first dilutions of soluble MT (Mother Tincture) must be prepared using the same alcohol concentration as the MT. However, alcohol is not required for higher dilutions when producing homeopathic medicines. When a homeopathic medicine is made for bulk storage in a pharmacy, 62% w/w alcohol is added as a preservative, but intermediate dilutions are always prepared in pure water and discarded. All Korsakov intermediate preparations are similarly prepared using pure water (Hahnemann approved this process in a letter to Korsakov), and alcohol is added only for the final dynamizations. The homeopathic information is understood to be carried by the water solvent and not by the alcohol. The NTA measuring cell would not survive solutions containing alcohol.

Given these findings, the idea that homeopathic medicines are non-material, propounded by both opponents of homeopathy and by traditional homeopathic practitioners, cannot be maintained. Science is based upon measurement; measurements are facts that cannot be disputed. Nevertheless, the presence of these particles does not allow us to conclude that the effect of homeopathy is due only to a classical molecule-receptor interaction. Given that the final product (impregnated pills, globules, granules) contains only a minimal quantity of the medicine in question, a different pathway is much more likely. To verify this assertion, other methodologies, such as Scanning Electron Microscopy & Energy Dispersive X-Ray Spectroscopy; Nuclear Magnetic Resonance; ph. Infrared measurements and Electro-photonics analysis are needed [1, 26-27].

It is essential to recognize a significant difference between the quantities of material obtained for different stocks. The hypothesis of nanobubble formation during the trituration process is plausible for insoluble material such as metals (e.g., copper), although this hypothesis has yet to be verified. There is also a very great dispersion of particle sizes; this may be because the laser can measure large particles in front or profile view, and so for a flat particle, the calculated size may vary considerably. Throughout these measurements, all manufacturing factors were tightly controlled: same environment, same water, same glass, same machines, same stock, same staff for each step of fabrication and measurement. While these controls strengthen the results, one must be careful with any generalization from these conclusions; so many different homeopathic medicines exist in different concentrations. In the future, it would be possible to study the impact of specific changes of parameters such as the use of different types of glass containers, number of dynamizations.

All measurements were carried out using samples prepared without alcohol, and previous measurements of NMR relaxation times also required preparations made with pure water. However, with electro-photonic analysis, it will be possible to use commercial homeopathic medicines maintained in a mix of water and alcohol.

NTA alone does not permit significant discrimination between single C potentization at different levels, and only the combination of results allowed the discrimination of complete manufacturing lines. The great variability in the size of particles indicates the very complex shapes of particles in homeopathic medicines, and this complexity must be taken into account when any mechanism of action of these medicines is studied.

Dynamization versus dilution

While the dilution factor is part of the potentization process, the dynamization itself produces detectable changes in the size and shape of the produced particles. It is essential to emphasize that we recovered relatively stable material concentrations in diluted/dynamized manufacturing lines. However, when the dynamization process is not applied (simple dilution process), the number of particles and their size distribution become extremely unstable from 10-40. Furthermore, the dynamization process affects the amount of material present and creates specific shapes and sizes. These crucial differences justify further studies into the behavior of the solvent surrounding these specific particles.

The simple dilution started after three triturations in lactose (1C, 2C, 3C). From this moment, the simple dilution process is done step by step without shaking. We observed that the numbers of particles are much more heterogeneous than during a dynamization process. The variations in the number of particles at each step may likely buffer or not the aggressiveness of the deionized water on the pharmaceutical glass to some extent. This effect would become more and more noticeable during the simple dilution process, causing larger and larger variabilities in particle size distribution. Dynamisation homogenizes each preparation's step, allowing the particle size distributions to become increasingly stable. These results must be confirmed on other manufacturing lines and other raw materials.

Limitations of the current study

This study was carried out according to Good Pharmaceutical Practice (GPP). It would be helpful to compare these results concerning Good Manufacturing Practice (GMP) and compare them with results obtained using other batches and other stocks. Reproducibility by other laboratories using the same framework also needs to be demonstrated. Most of the measurements were carried out on preparations without alcohol to ensure accurate results. Other techniques are required for which alcohol does not compromise the accuracy of measurement so that a comprehensive picture of the nature of homeopathic medicines can be developed. Even though our measurements identified differences between homeopathic medicines and controls, many puzzles remain, such as the specificity of the signature between different homeopathic stocks of the same family (plant/metal/others) or between each dynamization level, which this single study cannot address.

Implications for future research

More pharmacological studies are needed to address the plausibility [18] of homeopathic medicines. The present measurements justify further research and demonstrate the importance of GPP and

excellent quality control for MT. It also opens new perspectives for further research areas and alternative explanations of the mechanism of action of homeopathic medicines. Research on homeopathic medicines must not remain anecdotal but should be integrated into the pharmaceutical training of future pharmacists. The DynHom project must continue carrying out more systematic particle measurements, as has already taken place [1] using other approaches such as NMR measurements [26-27].

Conclusions

Using a step-by-step dilution or potentization process, NTA findings revealed the presence of particles in all samples (except for pure water in a PET container), even at the highest level of dilution. Furthermore, the size and the dispersion of particles increase with each step. For simply diluted samples, the number of particles had become unstable as well as their size distribution which is not the case when a dynamization process was involved.

Potentized medicines made in glass containers contained particles that can only be explained by a reaction between the deionized water and the glass because the numbers and sizes of particles were very stable and consistent at each step of the potentization process. In addition, the particles' size distributions were broad, indicating a broadly diverse but specific nature. If the dynamization process is applied, the initially concentrated raw material drives the observed particle size distribution.

We conclude that at each step of manufacturing a homeopathic medicine, a different but coherent and specific particle size distribution is generated, beginning from the specificity of the starting material, which will react with the solvent and glass container uniquely. Further measurements are needed on other raw materials using the same controls (solvent and simply diluted manufacturing lines) to support these findings.

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