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## Statistical evaluation of the effect of ultrasound on the synthesis of calcium phosphates

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## ABSTRACT

Calcium phosphates are widely used materials in tissue engineering due to their biocompativility and bioactivity. They are present in the bone along with the hydroxyapatite, which facilitates its use as a bioceramic. The mechanical and biological properties of the material obtained in the synthesis process are determined by the size of the particles. There are different ways to decrease the particle size of the materials. Among the methodologies used is the application of ultrasonic radiation. The objective of this work was to statistically evaluate the influence of the ultrasonic application during the synthesis of different phases of calcium phosphates, as well as the type of drying. The particle size determination was performed by scanning electron microscopy. The statistical comparison was made using one way ANOVA. Previously, the hypotheses necessary to validate the model were evaluated. The results were interpreted through its implementation in a statistical software InfoStat (2017 version). The results allow us to conclude that the application of ultrasound during the synthesis process significantly influences the particle size. The type of drying had no significant influence on the particle size.
**Keywords:** *Calcium phosphates, Particle size, Ultrasonic method, Statistical evaluation.*

## 1. INTRODUCTION

Calcium phosphates are materials widely used in tissue engineering due to their biocompatibility and bioactivity. They are present in the bone together with hydroxyapatite, which facilitates its use as a bioceramics [1-6]. Amorphous calcium phosphates (ACP), octacalcium phosphate (OCP) and tricalcium phosphate (TCP) are the main phosphates present in biological systems [7, 8].

The particle size of the material obtained in the synthesis process has a great influence on the surface area; as well as on the absorption, proliferation and differentiation of osteoblastic cells, which influences the bone formation process [9-12]. Dorozhkin [9] noted that the main advantages of using calcium phosphate in a nanometric structure were that they caused an increase in the adhesion of osteoblastic cells with the consequent proliferation and osseointegration. This process will accelerate bone formation [10-12].

Ultrasound has been one of the techniques studied in the development of the process for obtaining nanometric particles, based on dispersing effect of the ultrasound process. Sound waves are mechanical waves produced by deformations caused by the pressure difference in an elastic or deformable medium. These waves need a means to spread causing a phenomenon known as cavitation. This process allows the production of small particles in the form of nanoparticles [8, 13, 14]. The chemical physical characteristics of the calcium phosphate nanoparticles synthesized by wet chemistry method allow their use in the development of Scaffolds for bone regeneration [9-12]. The objective of this work was to statistically evaluate the influence of the ultrasonic application during the synthesis of different phases of calcium phosphates, as well as the type of drying.

## 2. MATERIALS AND METHODS

**Synthesis of different phases of calcium phosphate.** The amorphous calcium phosphate (ACP) and octacalcium phosphate (OCP) were synthesized using the methodology reported by Rodriguez-Chanfrau *et al*. [8]. In both syntheses, after completion of the reaction, the suspension obtained was concentrated by evaporation and then dried at 80 °C for 12 h. To obtain tricalcium phosphate (TCP) a sample of the ACP batch was subjected to a sintering process at 800 °C for 3 hours.

In parallel, tray drying was evaluated. For this, the same synthesis methodology was applied with the modification that the samples were placed in trays and dried at 80 °C for 24 hours.
**Use of ultrasound in the synthesis process.** To evaluate the influence of the ultrasound application on particle size, two

methodologies were used. In one, ultrasound was applied at the end of the synthesis (US1); while, in the other case, ultrasound was applied during the synthesis process (US2). A Sonus Vibra Cell, USA was used, set on Pulse for 15s (on time) and 3s (off time) and 30% of amplitude.
**Scanning Electron Microscopy (SEM).** Scanning electron microscopy (SEM) imaging of samples was carried out using a FEG-MEV; JEOL 7500F scanning electron microscope (Germany). The equipment was operated at an acceleration voltage of 2 kV. The samples were coated by carbon evaporation (Baltec SCD 050 Spputer Coater, USA). Particle size measurements were made according to Rodriguez-Chanfrau *et al*. [8].

**Statistical analysis.** The statistical analysis was evaluated using one-way ANOVA. Previously an exploratory data study was performed. Subsequently, normality, homocedasticity and independence, using SHAPIRO WILKS test, LEVENE test and data dispersion graphs, respectively, was evaluated. From these results a classic ANOVA or a non-parametric ANOVA was used for the statistical comparison of the data [15-17].

# 3. RESULTS

Table 1 presents the results of the particle size measured at 2000X for each of the treatments and type of drying. Meanwhile, table 2 shows the results for the measurement at 100000X.

**Table 1.** Average particle size for measurements at 2000X.

| Drying type | SEM | 2000X (microns) | | |
|---|---|---|---|---|
| | Treatment | SUS | US1 | US2 |
| Evaporation | ACP | 8,3/1,1 | 10,1/1,1 | 3,1/0,9 |
| | TCP | 8,8/1,0 | 6,7/0,9 | 4,0/0,8 |
| | OCP | 2,2/0,6 | 1,3/1,2 | 1,3/0,5 |
| Tray-dried | ACP | 7,8/3,0 | 6,5/2,9 | 2,7/0,2 |
| | TCP | 7,4/3,9 | 6,0/1,6 | 5,0/1,9 |
| | OCP | 2,0/0,2 | 1,0/0,3 | 1,8/0,4 |

The normality test evaluation for the ACP sample dried by evaporation showed a normal distribution (p = 0.6457 and p = 0.0687 for measurements at 2000X and 100000X, respectively). The independence of the data was verified. Meanwhile, the homoscedasticity evaluation showed that the variances of the populations were homogeneous (p = 0.4659 and p = 0.0582 for measurements at 2000X and 100000X, respectively).

**Table 2.** Average particle size for measurements at 100000X.

| Drying type | SEM | 100000X (nano) | | |
|---|---|---|---|---|
| | Treatment | SUS | US1 | US2 |
| Evaporation | ACP | 31,7/1,6 | 25,6/2,4 | 20,7/1,7 |
| | TCP | 39,8/2,3 | 32,1/2,6 | 29,8/2,3 |
| | OCP | 70,4/5,0 | 69,2/4,5 | 64,2/6,1 |
| Tray-dried | ACP | 24,4/1,9 | 24,5/1,6 | 17,2/1,6 |
| | TCP | 40,1/5,3 | 36,4/3,6 | 24,5/2,4 |
| | OCP | 72,8/4,4 | 69,9/4,7 | 66,2/5,5 |

Based on these results a classic ANOVA was performed. The results of this analysis (p = 0.0003 and p = 0.0002 for measurements at 2000X and 100000X, respectively) showed that the average particle size after applying the different ultrasonic treatments was significantly different. The TURKEY test showed that there were no significant differences between SUS and US1; and if there were significant differences between US2 and the rest of the treatments for the measurements made at 2000X and 100000X, respectively (Figure 1a and d).

Similar results were observed in the statistical analysis of the tray dried ACP sample, where the data come from a normal subpopulation (p = 0.3430 and p = 0.6390 for measurements at 2000X and 100000X, respectively), verifying that are independent. However, the evaluation of the homoscedasticity test showed that the variances of the populations were not homogeneous (p = 0.0001 and p = 0.0176 for measurements at 2000X and 100000X, respectively).

Based on these results a nonparametric ANOVA was applied. The KRUSKAL WALLI test showed that there were no significant differences between SUS and US1; and if there were significant differences between US2 and the rest of the treatments

In parallel, the types of drying were statistically compared by applying a comparison of means in independent samples using t Student,

In all cases, a confidence level of (1-α) = 95% and a significance of α = 5% were used. All data was processed using the statistical software InfoStat (2017 version).

for the measurements made at 2000X and 100000X, respectively (Figure 2a and d).
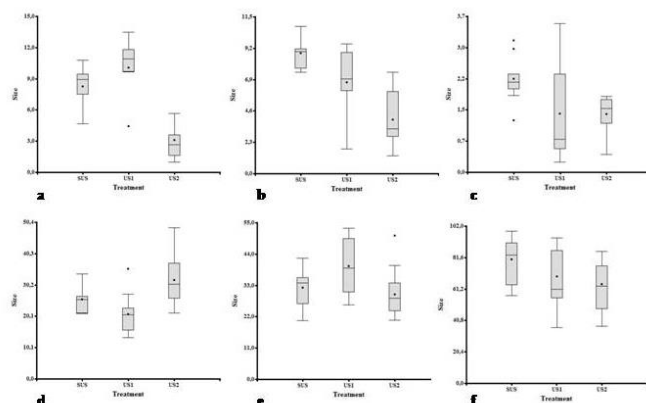


**Figure 1.** Box plot diagram of the comparison between the different treatments dried by evaporation (a: ACP; b: TCP; c: OCP in the 2000X measurement and d: ACP; e: TCP; f: OCP in the 100000X measurement).
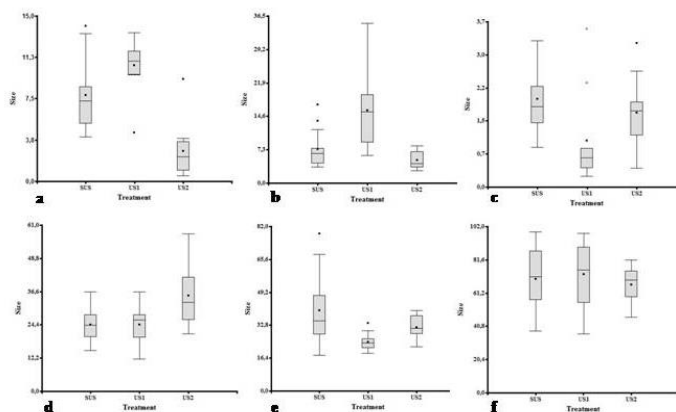


**Figure 2.** Box plot diagram of the comparison between the different tray-dried treatments (a: ACP; b: TCP; c: OCP in the 2000X measurement and d: ACP; e: TCP; f: OCP in the 100000X measurement).

The normality test evaluation for the TCP sample dried by evaporation showed a normal distribution (p = 0.7870 and p = 0.2971 for measurements at 2000X and 100000X, respectively), proving that are independent. The homocedasticity test showed that the variances of the populations were homogeneous (p = 0.9990 and p = 0.1106 for measurements at 2000X and 100000X, respectively).

Based on these results a classic ANOVA was applied. The results of this analysis (p = 0.0055 and p = 0.0107 for measurements at 2000X and 100000X, respectively) showed that the average size of the particles after applying the different ultrasonic treatments was significantly different. The TURKEY test showed that between SUS and US1 there were no significant differences; and if there were significant differences between US2 and the rest of the treatments for the measurements made at 2000X and 100000X, respectively (Figure 1b and e).

The statistical analysis of the tray-dried TCP sample showed that the data also comes from a normal population (p = 0.1380 and p = 0.2200 for measurements at 2000X and 100000X, respectively), verifying that they are independent. However, the evaluation of the homoscedasticity test showed that the variances of the subpopulations were not homogeneous (p = 0.0105 and p = 0.0006 for measurements at 2000X and 100000X, respectively) Based on these results a nonparametric ANOVA was applied. However, like the analysis performed on the sample dried by evaporation, the KRUSKAL WALLI test showed that there were no significant differences between SUS and US1; and if there were significant differences between US2 and the rest of the treatments for the measurements made at 2000X and 100000X, respectively (Figure 2 b and e).

Finally, in the statistical analysis of the OCP samples dried by evaporation the results showed that the data come from a normal population (p = 0.1900 and p = 0.1640 for measurements at 2000X and 100000X, respectively), proving that are independent. The homoscedasticity test showed that the variances of the populations were not homogeneous (p = 0.0179 and p = 0.0006 for measurements at 2000X and 100000X, respectively), so a nonparametric ANOVA was applied.

The results of this analysis (p = 0.0430 and p = 0.1034 for measurements at 2000X and 100000X, respectively) showed that the average particle size after applying the different ultrasonic treatments was significantly different for the samples evaluated at 2000X (micro structure). However, for the samples evaluated at 100000X (nano structure) there were no significant differences between the ultrasonic treatments, which indicates that when applying ultrasound the particles in micro structures decreased significantly, being smaller when applying the US2 treatment, but in the case of particles in nano structures, there was no significant decrease in particle size when applying the different treatments (Figure 1c and f).

The result of the statistical analysis of the tray-dried OCP sample showed that, as in the case of the sample dried by evaporation, the data come from a normal population (p = 0.1006 and p = 0.2460 for measurements at 2000X and 100000X, respectively), verifying that are independent. The evaluation of the

homoscedasticity test showed that the variances of the populations, unlike the evaporative drying, were homogeneous (p = 0.4240 and p = 0.0720 for measurements at 2000X and 100000X, respectively).

Based on these results a classic ANOVA was applied. Like the samples dried by evaporation, the results of this analysis (p = 0.0330 and p = 0.6512 for measurements at 2000X and 100000X, respectively) showed that the average size of the particles after applying the different ultrasonic treatments were significantly different for samples evaluated at 2000X and not significantly different for samples evaluated at 100000X (Figure 2c and f).

It is noteworthy that in both cases (drying evaporation and tray drying) although statistically there is no significant difference in the average size of the particles measured at 100000X, the tendency is to decrease the size as ultrasound is applied, being smaller when US2 is applied. In our opinion, the structural and morphological characteristics of the starting material in the synthesis process can have an influence on the results obtained.

The statistical comparison between the drying methods studied (at 2000X) showed that there were no significant differences (Table 3, Figure 3). This result indicates that the types of drying used in this study do not significantly influence the size of the particles. It is more economical to use tray drying, especially when working at a scale larger than the laboratory.

**Table 3.** Results of the statistical comparison between the methods for the different samples measured at 2000X.

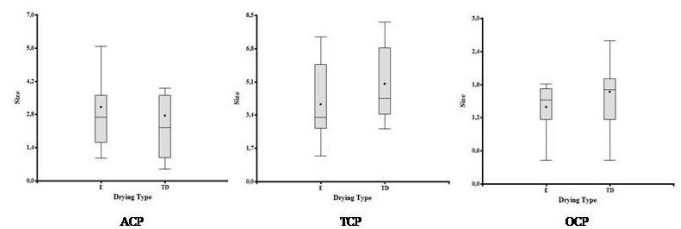| Sample | Media/DE | | p |
|---|---|---|---|
| | Evaporation | Tray dried | |
| ACP | 3.11/1.7 | 2.75/2.4 | 0.7150 |
| TCP | 3.95/2.1 | 5.02/1.9 | 0.2748 |
| OCP | 1.39/0.5 | 1.67/0.7 | 0.2911 |



**Figure 3.** Box plot diagram of the comparison of drying methods.

## 4. CONCLUSIONS

The results of this work allow us to conclude that for the synthesis processes of amorphous calcium phosphates and tricalcium phosphate, the application of ultrasound significantly influences the size of the particles (micro and nano), being more significant when applying the variant in which ultrasound is applied during the synthesis process. In the case of calcium octophosphate, the influence of the application of ultrasound was

significant only for the microparticles, being also superior when applying the ultrasonic variant US2. The application of ultrasound does not influence the size of the particles in nanometric structure. It can be concluded that there are no significant differences in the size of the particles when comparing the type of drying, however, tray drying is much more economical and feasible to execute, specifically at work scales higher than the laboratory scale.

## 5. REFERENCES

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## 6. ACKNOWLEDGEMENTS