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1 Title: Spherical depth-sensing nanoindentation of human anterior skull base bones: establishment of
2 a test protocol

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4

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28

29 **Abstract**

30 The mechanical properties of anterior skull base (ASB) bones are not well understood due to their
31 complex geometry and deep location. However, it is of particular interest for skull base surgeons to
32 appraise the force range they can apply during procedures and know what kind of haptic feedback a
33 simulation device should produce in order to be realistic for trainees. **The aim of this study was to**
34 **establish a measurement protocol to set the level of hydration state, temperature and curve analysis**
35 **method for spherical depth sensing nanoindentation of ASB bones.** A definitive screening design
36 method was used to test the different possible combinations of these factors. Two samples of ASB
37 bones from the heads of two human body donors (two specimens) were selected according to their
38 microstructure as assessed by micro-CT (microtomography): **low-porosity (16.87%**, sphenoid bone)
39 **and high-porosity (79.85%**, ethmoid bone). Depth measurement series of 36 nanoindentations
40 (n=288) were performed on specimen 1 according to the L8 Taguchi orthogonal array to study the
41 effect of temperature (two levels: 20 or 37°C), hydration state (dry or immersed in physiological
42 saline sodium chloride), and loading curve analysis according to the Hertzian contact theory (fitting at
43 the start or at the end). The mean values of reduced Young's (E*) modulus varied significantly
44 depending on the hydration status and bone microstructure. In order to obtain the physiological
45 properties of ASB bones, we thus propose performing immersion tests. To simplify the
46 experimentation protocol, future experiments must include a room temperature level and a fit of the
47 curve at the end of the load. A validation series was performed on the second specimen to assess the
48 set of parameters. The E* in dry bone gave mean values of 994.68 MPa, versus 409.79 MPa in
49 immersed bones (p <0.00001). This is the first time a study has been carried out on ASB bones,
50 defining the experimental parameters related to physiological conditions.

51

52 **Keywords:** Anterior skull base, bone, spherical depth sensing nanoindentation, temperature,
53 hydration, **experimental design**, mechanical properties

54

55 **1. Introduction**

56 Anterior skull base (ASB) bones, comprising the frontal, ethmoid, and sphenoid bones, must be
57 partially broken and resected in many endoscopic endonasal skull-base operations to access tumor
58 or inflammatory lesions. These bone structures are close to vital anatomical structures, such as the
59 internal carotid arteries, brain, and optic nerves (Hosemann and Draf, 2013). Therefore, an
60 understanding of their mechanical properties may help surgeons analyze and prevent surgical
61 complications, such as uneventful perforation of the ethmoid roof (White *et al.*, 2004). It could also
62 be useful to understand what type of haptic feedback a simulation device should give to be realistic
63 for trainees (Favier *et al.*, 2019).

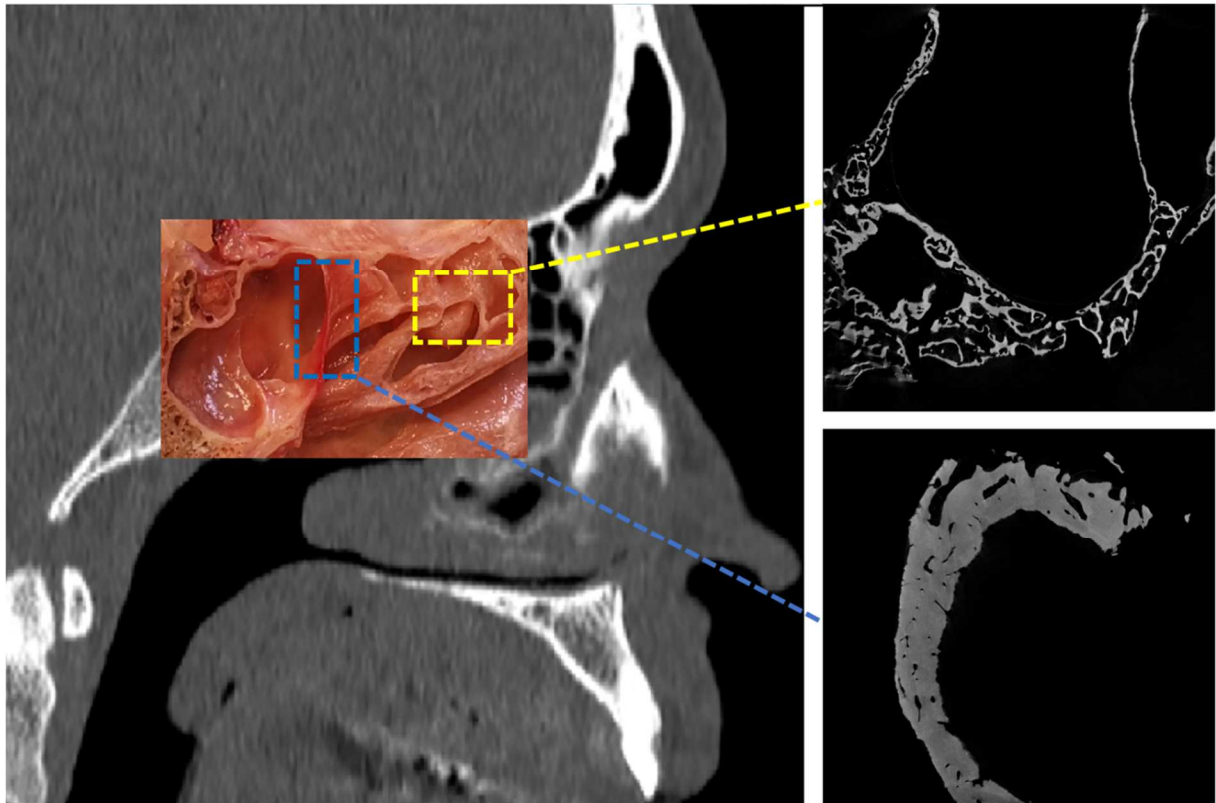
64 Most biomechanical studies on skull bones report on the behavior of the calvaria, which protects the
65 brain from external injuries, to understand the mechanisms of skull fractures in forensics (Raul *et al.*,
66 2008) and traumatology (Alexandre *et al.*, 2019). In this perspective, authors have studied the whole
67 bone without distinguishing between cortical and trabecular bone to better understand the
68 mechanisms of skull fractures. The ASB bones, located at the interface between the brain and
69 paranasal sinuses, are of particular interest because of their unusual anatomy, which has no
70 equivalent anywhere in the body. They comprise sections featuring thin bone walls (approximately
71 100 micrometers' thick) that encompass the paranasal sinuses and are mainly cortical in nature, with
72 no or little trabecular bone (Berger *et al.*, 2013). Due to this unique, complex, and variable anatomy
73 (see geometry, Figure 1), it is impossible to collect standardized specimens to perform common
74 material tests. Therefore, to test the mechanical properties of ASB bones one must consider the
75 microstructure rather than the macroscopic geometry of bone samples. Spherical depth-sensing
76 nanoindentation is a localized test that studies mechanical properties at the tissue level, related to a
77 microscopic structure. It has never before been used to characterize ASB bones. According to a study
78 by Rodriguez-Florez (Rodriguez-Florez *et al.*, 2013), the tissue-level mechanical properties of bone

79 measured by nanoindentation depends on the hydration state and data analysis method, among
80 other factors.

81 Thus, the optimal parameters for studying ASB bones using nanoindentation are unknown. According
82 to our previous experiment in sampling thin walls of ASB bones (Favier *et al.*, 2017), the consistency
83 of ASB bones seems to be affected by the hydration state: the more exposed to ambient air the
84 samples were, the more brittle they became. Other parameters may interfere with the results of
85 experiments. For example, samples are usually at room temperature (20°C) during tests, while the
86 body temperature is 37°C. Different analysis methods on loading curves may also influence the final
87 value of the reduced Young's modulus (E^*) (Fischer-Cripps, 2001). This modulus is a characteristic
88 that can define the localized mechanical behavior of a material. Subsequently, using this E^* is a way
89 to compare different structures of bones.

90 An experimental design was carried out i) to evaluate and define the level of experimental
91 parameters (temperature, hydration and curve analysis) influencing the nanoindentation tests of ASB
92 bones and ii) to analyze the interactions or dependencies between these variables (factors).

93



94

95 *Figure 1: Sample extract sites and micro-CT imaging (Specimen 1). Yellow: ethmoid bone (high-*
 96 *porosity level shown in micro-CT), acquisition with 5 μm voxel size; blue: sphenoid bone (low-porosity*
 97 *level in micro-CT), acquisition with 3.75 μm voxel size*

98

99 **2. Material and methods**

100 2.1 ASB samples

101 The samples were extracted from two human heads (two men, 81 and 84 years-old, without known
 102 disease related to their skeleton), each from bodies donated to the anatomy laboratory of Nancy,
 103 France.

104 Each head was preserved frozen at -18°C , without any chemical fixation. For the sampling, the head
 105 underwent parasagittal hemisection, and two samples were meticulously extracted from the left
 106 side: one from the ethmoid cell walls and one from the anterior wall of the sphenoid sinus (Figure 1).
 107 Each bone sample was separated from its surrounding mucosa and periosteum.

108 In order to avoid the samples deteriorating, they were immediately refrozen following a common
109 procedure (Bakhach et al., 2009, Dall'Ara *et al.*, 2012) for bone samples conservation. The samples
110 were not stored in saline, but frozen without medium, just after extraction from the heads. All
111 samples were stored in airtight boxes.

112 The indent area of 1 mm² was selected without visible porosity using the microscope integrated in
113 the system (Rho *et al.*, 1997). A roughness test (Taylor-Hobson, Leicester, United Kingdom) was then
114 performed on this surface. The surface was eligible for nanoindentation when the local roughness
115 defect (Rt) was less than or equal to 0.02 μm for a sampling length (Lc) of 250 μm. Before the test,
116 the sample was placed on a sample holder (Petri dish), and fixed with cyanoacrylate and sand grains
117 allowing the adjustment of the horizontality of the surface to be indented (by microscopy integrated
118 in the system). This made it possible to obtain perpendicularity between the tested surface and the
119 axis of the indenter.

120

121 2.2 Micro-CT imaging

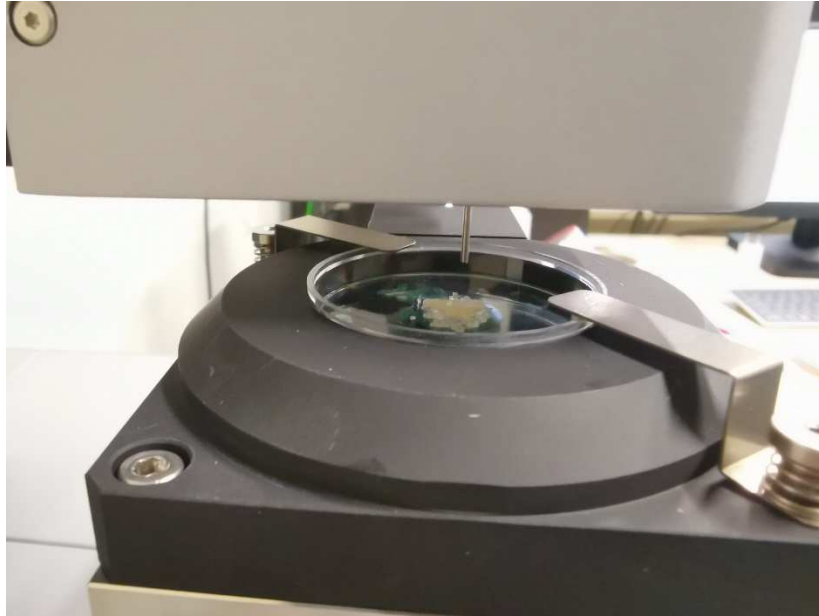
122 Each sample was scanned using X-ray micro-CT (microtomography) (Figure 1) with the Nanotom S
123 Phoenix X-ray (Baker Hughes GE, Boston, Massachusetts, USA, 180 kV - 150 μA, 25 W) at maximum
124 accuracy according to the size of the sample. Before starting experiments, a blank of detector noise
125 was recorded, then subtracted of radiography data set. Samples were placed frozen in the micro-CT
126 to be thawed in ambient air atmosphere (constant temperature during micro-CT acquisition,
127 approximately 20°C) and put on a polyethylene rod holder with a polystyrene home-made holder
128 stuck on it, to avoid samples movement during the acquisition process. Scanning in air affords the
129 highest contrast between the specimen and surrounding medium (Bouxsein *et al.*, 2010). Total scan
130 time was 144 minutes per sample (0.25° step, 1440 images, 6 second exposure, 80 KVp, 6.5W,
131 microfocus beam 2 μm). The distance between the detector and the source was set to 200 mm and
132 sample-source distance was set to 20 mm, with a constant magnification factor of 13.3. No dilatation

133 was observed during experiments. Voxel size was 3.75 μm in each dimension (isometric) for “low-
134 porosity” bone (LPB) and 5 μm for “high-porosity” bone (HPB). For 3D-imaging reconstruction, the
135 cone-beam algorithm of Feldkamp was applied using Phoenix datos|x software (GE, Boston,
136 Massachusetts, USA). The 3D qualitative representation of the porosity (LPB or HPB, see Figure 1)
137 was obtained by importing the “.raw” file (16 bits) in the 3D software (VGStudio Max 3.3 material
138 package, Volume graphics, Heidelberg, Germany). To accurately distinguish voids from bone
139 material, some filters were applied (adaptive gauss and opacity mapping filters) on each volume.
140 Finally, the porosity was calculated as the total pore volume divided by the total sample volume
141 (Boughton et al., 2019), using the porosity analysis VGDefX algorithm of VGStudio Max. Porosity rate
142 (obtained from bone volume fraction, BV/TV) and wall thickness were calculated directly from the
143 numerical model of the specimen (Bouxsein *et al.*, 2010).

144

145 2.3 Spherical depth-sensing nanoindentation

146 An ultra nanoindenter (UNHT³ Bio) from ANTON-PAAR, Peseux, Switzerland (Figure 2) was used. This
147 system enables testing at penetration depths varying from just a few nanometers to 100 μm , using
148 loads of up to 20 mN. The depth resolution was 0.006 nm and the load resolution 0.001 μN . The
149 radius of the spherical indenter (diamond tip) was 100 μm . The contact surface disc generated by
150 indentations did not exceed a radius of 30 μm . The load and unload speeds were intentionally kept
151 low (40 mN/min), enabling us to generate bone elasticity as well as reversible deformations, avoiding
152 any interference from neighboring indentations. The tool was adapted to enable testing on biological
153 materials in liquid medium. Raw data curves were obtained using OriginPro 9 software (OriginLab,
154 Northampton, Massachusetts, USA).



155

156 *Figure 2: Bone samples placed in the Anton Paar ultra nanoindenter (dense bone, dry environment)*

157

158 2.4 Experimental design

159 Due to the nature of ASB bones (different microstructures and organizations), how the
160 environmental factors of the test and their inter-factor interactions influenced the results should be
161 assessed in order to define the optimal test configuration. The following parameters were studied:
162 temperature (two levels: 20°C; 37°C) and hydration (two levels: testing in liquid environment with
163 physiological saline sodium chloride after one hour of immersion; in ambient air). To ensure this
164 protocol would be usable for all bone structures, we introduced two “extreme” bone
165 microstructures: one **LPB**, the other **HPB** (Figure 1). Furthermore, we studied two analysis methods
166 for characterizing bone structures: one where the loading curve was fitted at the start, the other
167 applying it at the end, to separate any influence of potential residual soft tissues (periosteum) or
168 surface irregularities.

169 One way of addressing this issue of fixed parameters is to conduct an experimental approach testing
 170 the different possible combinations of these factors. We used the “definitive screening design”
 171 method according to the following principles (Lundstedt *et al.*, 1998).

172 Consider an experiment of which the outcome depends on p parameters. Assume that the range of
 173 variations of each parameter can be subdivided into n levels. The basic experimental design requires
 174 p^n experiments. This is evidently not a feasible approach for problems that depend on a high number
 175 of parameters, however, such as those encountered in living organisms. In order to overcome this
 176 obstacle, one should use the fractional factorial designs introduced by Genichi Taguchi (Taguchi,
 177 1990]). Then, one can perform a simplified analysis of interactions and evaluate the impact of each
 178 parameter.

179 Each sample was defrosted at room temperature (20°C) for the micro-CT acquisition and fixed to a
 180 support with glue before being tested with the nanoindenter. A series of 36 indentations was
 181 performed for each step of the experimental design, using the same load on each sample analyzed.
 182 According to an L8 Taguchi orthogonal array (Taguchi, 1990), a total of eight series were performed
 183 to analyze all the parameters mentioned above (*i.e.*, 288 indentations, [Table 1](#)).

184

Tests	Bone microstructure	Hydration status	Temperature	Analysis
# 1	Low-porosity	Dry	20°C	Start
# 2	Low-porosity	Dry	37°C	End
# 3	Low-porosity	Hydrated	20°C	End
# 4	Low-porosity	Hydrated	37°C	Start
# 5	High-porosity	Dry	20°C	End
# 6	High-porosity	Dry	37°C	Start
# 7	High-porosity	Hydrated	20°C	Start
# 8	High-porosity	Hydrated	37°C	End

185 [Table 1: L8 Taguchi orthogonal array for indentation tests configuration](#)

186

187

188 2.5 Data analysis

189 Bone tissue has a viscoelastic behavior. In this study, we focused only on its elastic behavior thanks
190 to low speed loading (quasi-static) and low load (low displacement). It is thus possible that, at very
191 shallow indenter penetration depths, the surface of contact between the sample and indenter takes
192 a similar form to a disc. Given these properties, one can apply an analysis of elastic contact (Jehl *et*
193 *al.*, 2016). Thus, a strategy enabling to define the range of values on commencing the load was
194 designed, for which the Hertzian contact relation (Hertz, 1882) can be applied. As a brief reminder,
195 this formula establishes the relationship between the force applied (F), the relative approach
196 distance between the two elements (h), and the radius of the disc contact area (a) (formulae (1) and
197 (2)). These formulae also implicate the radius of the indenter's curve (R) and the mechanical
198 characteristics of the sample and indenter, *i.e.* their Young's moduli and Poisson's ratios, using the
199 reduced elasticity modulus, defined by formula (3).

200

201
$$h^3 = \left(\frac{9.F^2}{16 \times R \times E^{*2}} \right) \quad (1)$$

202

203
$$a = (R \times h)^{1/2} \quad (2)$$

204

205
$$\frac{1}{E^*} = \frac{1-\nu^2}{E} + \frac{1-\nu_p^2}{E_p} \quad (3)$$

206

207 In formula (3), E_p and ν_p represent, respectively, the Young's modulus and Poisson's ratio of the
208 indenter. When the rigidity modulus of the tested material is very weak compared to that of the
209 indenter, it is calculated as $E^* = E/(1 - \nu^2)$.

210

211 During the indentation testing, the penetration depth **was continually recorded** according to the
212 applied load. After each test, a series of data pairs (F, h) **was generated**, from which **one** would be
213 able to identify the characteristics of the material tested using formula (1). In log-log plotting, this
214 formula becomes:

215

$$\ln(F) = \frac{3 \cdot \ln(h)}{2} + \frac{1}{2} \ln\left(\frac{9}{16 \cdot R \cdot E^{*2}}\right) \quad (4)$$

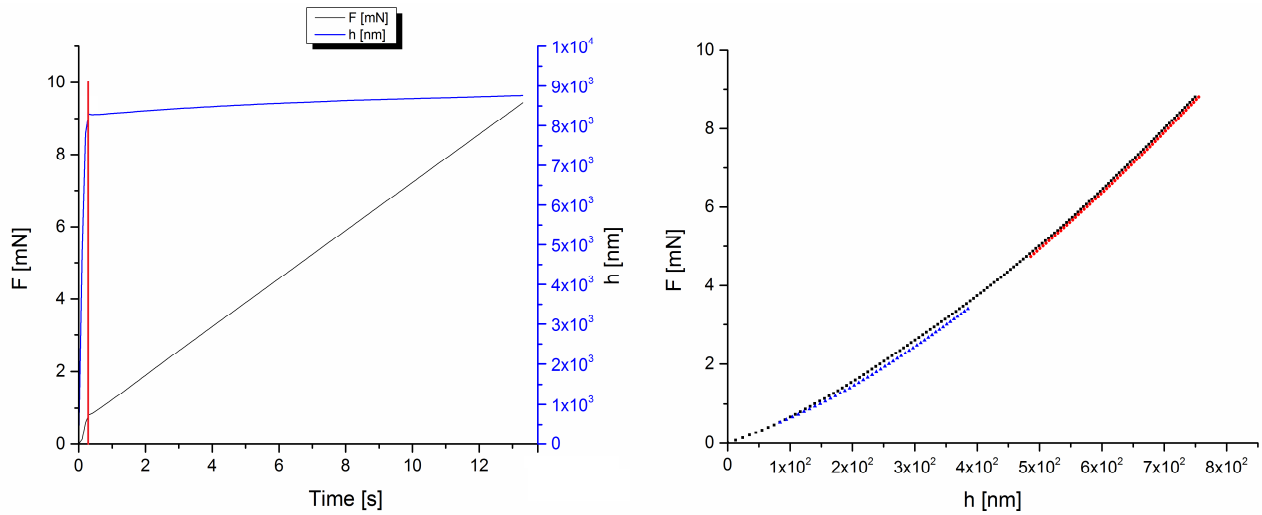
217

218 The aim was thus to identify the parameters of a linear function.

219

220 Figure 3 presents a test **on a calibration sample: the determination of the contact point (Figure 3A)**
221 **allows shifting the origin of the loading curve (Figure 3B)**. The reduced Young's modulus (E^*) was
222 calculated both at the start and end of the loading curve to evaluate the influence of data analysis on
223 measurement. The fittings produced similar scores (E^*). Thus, there was no significant difference
224 between the two fittings, but the "end" part of the curve provided a better fit of the slope for the
225 reduced Young's modulus calculation ($R=1$ for all tests), since surface irregularities **have less effect**.

226



227 A.

B.

228 *Figure 3: A: Graphical determination of the contact point (red line) from the curves $h(t)$ and $F(t)$. B:*
 229 *Comparison of curve analysis on a calibration sample. Black dots: loading curve; blue dots: fit at the*
 230 *start of the curve; red dots: fit at the end of the curve*

231

232 2.6 Ethical considerations

233 This study is in accordance with the Helsinki Convention (1964), the French legislation governing the
 234 donation of bodies and the relevant guidelines and regulations from the laboratory of anatomy of
 235 Nancy, France. Neither donor was from a vulnerable population, and written informed consent was
 236 obtained for all anatomical and surgical studies.

237

238 **3. Results**

239 Bone volume fraction was 20.15 % (porosity of 79.85 %) on HPB and 83.13% % (porosity of 16.87%)
 240 on LPB. The minimum thickness of the indented zone was 48 μm for HPB and 105 μm for LPB.

241 The main results are summarized in Table 2 and categorized by the four parameters of analysis:
 242 density of bones, hydration of the environment, temperature of samples, curve analysis. Figure 4
 243 presents a box-plot graph illustrating the mean values and dispersion of E^* according to the different
 244 parameters.

245

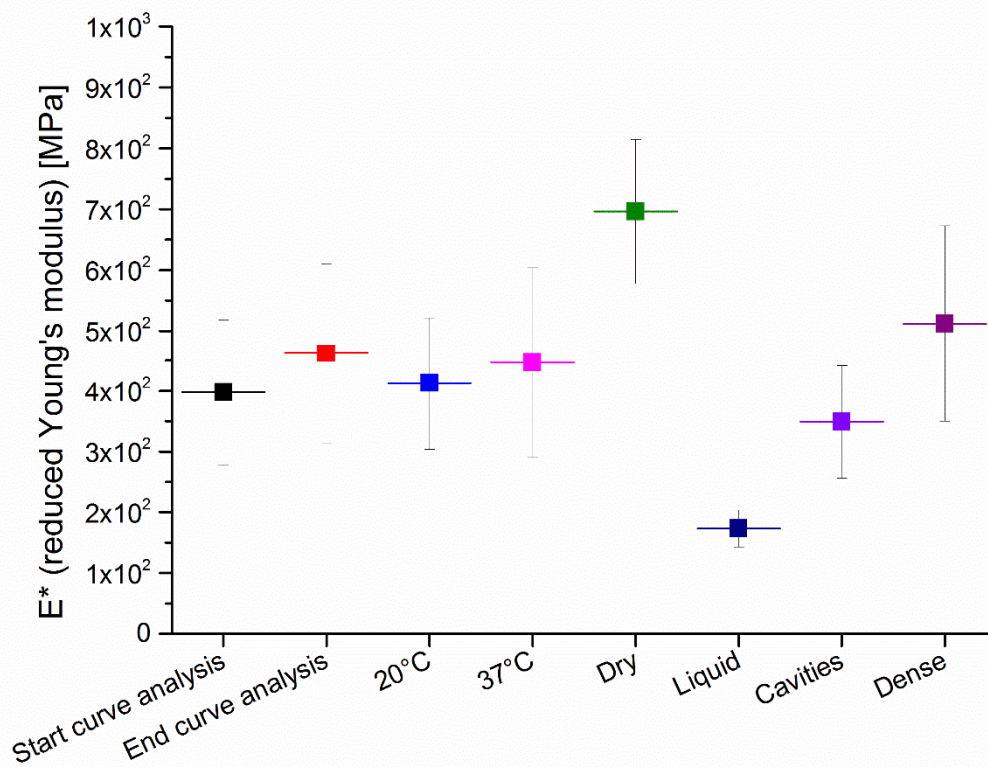
<i>Characterization</i>	Reduced Young's Modulus E^* (mean)	Reduced Young's Modulus E^* (median)	Standard deviation E^*	Range	P-value (test)
<i>Low-Porosity Bone</i>	519.66 MPa	491.23 MPa	182.82 MPa	157.61 to 934.08 MPa	< 0.00001 (Welch's t-test)
<i>High-Porosity Bone</i>	350.68 MPa	351.68 MPa	104.65 MPa	147.42 to 545.33 MPa	
<i>Environment: dry</i>	697.13 MPa	665.56 MPa	221.32 MPa	545.33 to 934.08 MPa	< 0.00001 (Wilcoxon)
<i>Environment: liquid</i>	173.21 MPa	177.34 MPa	66.16 MPa	147.42 to 230.2 MPa	
<i>Temperature: 20°C</i>	419.92 MPa	422.2 MPa	109.88 MPa	147.42 to 756.74 MPa	0.0836249 (Welch's t-test)
<i>Temperature: 37°C</i>	450.42 MPa	420.7 MPa	177.60 MPa	157.59 to 934.08 MPa	
<i>Curve analysis: start</i>	403.54 MPa	401.09 MPa	141.48 MPa	147.42 to 756.74 MPa	0.000285377 (Welch's t-test)
<i>Curve analysis: end</i>	466.80 MPa	441.81 MPa	145.99 MPa	157.59 to 934.08 MPa	

246 *Table 2: Main results of characterization according to studied parameters (Specimen 1)*

247

248 A statistical comparison was performed using Welch's t-test for normal distribution variables
 249 (density, temperature, and curve analysis) due to the unequal variances in the series. The distribution
 250 of values in the "temperature: 20°C" record was not normal, so a Wilcoxon test was performed to

251 compare the effect of porosity on measured mechanical properties. There was no statistical
 252 difference between the temperature parameters, yet the bone porosity and environmental factors
 253 were found to effect significant changes in the measured mechanical properties. A comparison of the
 254 loading curve fitting parameters revealed a statistical difference, although the absolute difference
 255 was 63 MPa. On the other hand, hydration seemed to have a huge impact on measured properties,
 256 varying by up to 400%. Finally, spherical depth-sensing indentation was proven able to distinguish
 257 between different porosity levels, leading to different mechanical properties.



258

259 *Figure 4: Comparison of in vitro properties of anterior skull base bone following different testing*
 260 *parameters (see L8 Taguchi orthogonal array, Table 1). Means and dispersion of reduced Young's*
 261 *moduli (E*) for a series of 36 indentations per sample (288 indentations)*

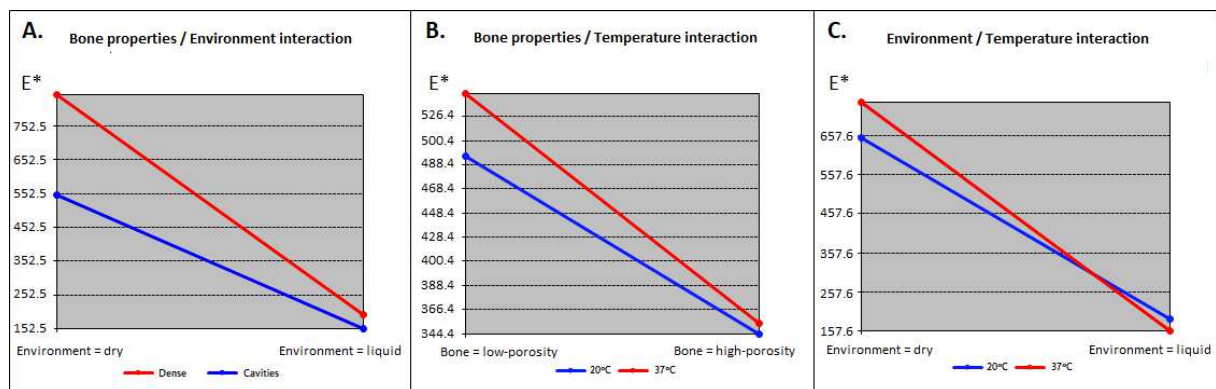
262 To complete the analysis of the impact of individual factors, the presence or absence of interactions
 263 between these factors should be verified. Three plots (Figure 5) allow this verification. They are “x-y
 264 line plots of cell (level combination) means on the response variable for each level of one factor over

265 all the levels of a second factor" (Lovie, 2005). The title of each plot provides information on the
 266 interaction tested. E* is represented on the y-axis. On the x-axis, the two levels of a studied
 267 parameter are represented (discrete variable). Thus, if the lines or profiles corresponding to the
 268 separate factor levels are parallel, that is, no differential effect over different combinations of the
 269 levels of the factors is revealed, then there is no interaction. If they are not parallel, then an
 270 interaction is present.

271

272 Figure 5A shows a decreasing trend in E* for both bone microstructures (red and blue curves) when
 273 the environment changes (dry then liquid). An interaction between bone microstructure and
 274 hydration status led to a difference in elasticity between the two bones: greater in a dry environment
 275 (approx. 300 MPa) than in a liquid medium (approx. 50 MPa), i.e., a difference of 250 MPa. The
 276 slopes of Figures 5.B and 5.C are similar, and the differences are lower than 50 MPa, thus inducing
 277 only slight interactions that are negligible.

278



279

280 *Figure 5: Interaction plot: representation of interactions according to the experimental design method*
 281 *(Lovie, 2005).*

282

283

284 4. Discussion

285 To the best of our knowledge, this is the first study on the mechanical characterization of the thin
286 bone walls of the ASB. Biological tissues are known for their viscoelasticity behavior (Oyen *et al.*,
287 2012). The Hertzian load-displacement relation (Hertz, 1882) focuses on the elastic behavior (quasi-
288 static loading at low load). It was used to define the measurement environment of spherical depth
289 sensing nanoindentation.

290 Waridel *et al.* studied the stress resistance of ethmoid and sphenoid bones using dynamometer
291 measurement (Waridel *et al.*, 1997) to understand the pressure required to cause bone breakage at
292 11 anatomical sites (bones conserved in 40% ethanol). They identified that highly fragile sites
293 correlated with the location of surgical complications relating to endonasal endoscopic sinus surgery,
294 although did not include characterization of mechanical properties. This finding is of particular
295 interest for Ear Nose and Throat surgeons and neurosurgeons who usually perform endoscopic
296 endonasal surgical procedures. Surgeons need to safely break and remove these walls to access the
297 boundaries of the nasal fossae and sinuses in order to treat infectious diseases or tumors of the ASB.
298 Thus, understanding the mechanical properties of these bones is essential for surgeons at different
299 levels. First of all, it is important for a better design of surgical simulators. Indeed, surgical simulators
300 (i.e., virtual simulator with haptic feedback and/or physical simulators) have to replicate the complex
301 anatomy and mechanical properties of ASB bones. This is, of course, important for improving the
302 visual and haptic realism of the simulation, but also mandatory for safety reasons. Ethmoid and
303 sphenoid bones are surrounded by vital structures such as the forebrain, orbits, optic nerves and
304 internal carotid arteries. By practicing with a simulator with inadequate feedback, trainees could
305 learn to apply aberrant and potentially dangerous forces in the operating room (Yamauchi *et al.*,
306 2002). Furthermore, it could induce negative training transfer (Våpenstad *et al.*, 2017). Second, these
307 data are required for the objective assessment of these simulators. Currently, the “mechanical
308 fidelity” of simulators is only assessed by subjective ratings provided by users, as there are no data

309 on the mechanical behavior of these bones. Through biomechanical characterization, simulators may
310 be more “realistic”, by selecting appropriate materials (Favier *et al.*, 2017) or improving haptic
311 feedback (Favier *et al.*, 2019). Third, biomechanical data may help design surgical robots by
312 appraising the range of forces that an automated device may apply on these structures. Finally,
313 knowing mechanical properties is helpful to better understand the mechanisms of ASB bone fracture.

314 The reason this haptic feedback is still poorly understood may be explained by the complex geometry
315 presented by ASB bones (Figure 1), which are highly resistant to the use of conventional mechanical
316 tests (bending, strain or torsion tests). Such tests require standardized geometrical samples of the
317 material, impossible in this case due to these bones’ anatomical properties. Furthermore,
318 conventional tests do not take into account the variable microstructure that could be related to
319 different mechanical behaviors.

320

321 Spherical depth-sensing indentation is an engineering technique involving pressing a hard-tipped
322 material into another material with a known force, which has already been used to characterize the
323 mechanical properties of human femoral cortical bone (Boughton *et al.*, 2018). The elastic modulus
324 of a material is calculated from the loading (Hertz relation (Hertz, 1882)) or unloading curve (Oliver
325 and Pharr theory (Oliver and Pharr, 2004)). The advantage of spherical rather than sharp-tip
326 indentation is that, at small loads and displacements, the deformation is almost entirely elastic using
327 the former (Oliver and Pharr, 2004). The viscoelastic behavior of biological materials can generate
328 unloading curves that are difficult to interpret using the Oliver and Pharr method (Malgat *et al.*,
329 2016). In addition, the indentation in a liquid medium makes it difficult to detect the point of contact.
330 This difficulty affects the results from the Oliver and Pharr method, but has no influence on the Hertz
331 relationship (Jehl *et al.*, 2016). To illustrate the impact of the analysis method, an experiment on a
332 well-studied bone (femur cortical bone) is presented in supplementary material (Supplementary
333 material S1). A finite element simulation confirms the validity of the developed method

334 (Supplementary material S2). Arnold and colleagues (Arnold *et al.*, 2017) conducted a systematic
335 review on micro-indentation used to measure cortical bone stiffness. They concluded that “due to
336 the porous nature of cortical bone at the millimeter scale, the apparent elastic modulus at the
337 millimeter scale will be less than the modulus at a smaller material scale, where pores will not affect
338 its stiffness” and recommended combining indentation with high-resolution imaging. In the study
339 presented herein, a nanoindenter associated with micro-CT imaging were therefore used.

340 The reduced Young’s moduli reported in this study are lower than data reported on other cortical
341 bones. The method described here was not intended to provide explanations of such low moduli.
342 However, several hypotheses can be drawn. First, in ASB cortical bones, the orientation of
343 osteons/lamellae is poorly studied but seems to be randomly distributed (Dempster, 1967)
344 (supplementary material S3) and may lead to different mechanical properties (Smit *et al.*, 2002). It is
345 radically different from long bones, where osteons are oriented along the loading force axis in long
346 bone and vertebrae (Roy *et al.*, 1998). Second, the bone mineral density may be different in ASB
347 bones, and will be study in further works. Finally, ASB bones have an embryological origin different
348 from long bones: the former develop from the neural crest, the latter from the mesoderm
349 (McBratney-Owen *et al.*, 2008). A flaw in the measurement method could also have been discussed,
350 but this method was validated on a femur cortical bone (supplementary material S1) and found
351 similar results than previous study using indentation tests (Boughton *et al.*, 2019). Nevertheless, it
352 will be interesting to evaluate the impact of the liquid medium (saline solution) on the
353 nanoindentation results, compared to other liquid medium (Hank’s balanced saline solution, HBSS)
354 (Habelitz *et al.*, 2002).

355 To establish a measurement protocol, environmental parameters must be set to characterize the
356 bone’s behavior in a physiological way. Dehydration (Rodriguez-Florez *et al.*, 2013) and temperature
357 are known to increase the stiffness and strength of cortical bone (Nyman *et al.*, 2006). An
358 experimental design was thus created according to the L8 Taguchi orthogonal array (Taguchi, 1990)

359 fixing four parameters: **i)** temperature, **ii)** hydration, **iii)** loading curve fitting method, and **iv)** bone
360 microstructure.

361 **i)** Our results demonstrate that the two temperature levels studied (20 and 37°C) had no impact on
362 the mechanical properties of ASB bones (Figure 4). Furthermore, temperature had only a weak
363 influence on the environment (Figure 5.C.). Similarly, while there was a slight difference in
364 mechanical behavior when comparing the bone structures according to temperature, the effect of
365 this interaction is related to measurement dispersion, and is not significant (Figure 5.B). Thus, to
366 simplify the experimental protocol, we chose to perform future tests at ambient temperature (20°C).

367 **ii)** In contrast, the hydration status was found to be important, since the results demonstrate a
368 reduced Young's modulus (E^*) **four times higher in the dry samples** (Figure 4). In the human body,
369 water resides within the bone canals and also within the extracellular mineralized matrix (Nyman *et*
370 *al.*, 2006). We therefore chose to set a liquid environment to replicate the human bone physiology.
371 Furthermore, interaction between bone and hydration status (Figure 5.A) revealed greater difference
372 in the dry environment. We hypothesize that drying improves the difference between low and high-
373 porosity bones in a non-physiological manner (denaturation).

374 **iii)** The Hertzian contact theory (Hertz, 1882) enabled us to study part (or all) of the loading curve of
375 the nanoindentation tests (Figure 3). The mechanical characterization obtained by curve indentation
376 fitting requires one to hand-select the contact point (beginning of the test) in order to overcome the
377 bone roughness and complex geometric surfaces. This is why we chose to compare tests with the
378 curve indentation fitting at the start (10% to 50% of the load) as well as at the end (50% to 98%) of
379 the load, the latter not requiring human selection. Our results reveal a significant difference of
380 63 MPa for E^* (Table 1). This can be explained by the residual soft tissues at the bone surface
381 (periosteum). Curve indentation fitting at the end of the learning curve would appear to be the
382 optimal solution to avoid interpretation bias.

383 **iv)** The results show the different mechanical properties presented by LPB compared to HPB
 384 (Figure 4). HPB cause reduced stiffness consistent with the literature (Cooper *et al.*, 2016). Thus,
 385 nanoindentation would appear a suitable technique for studying the mechanical behavior of these
 386 bones according to the variation of the internal microstructure.

387 To confirm the protocol based on these parameters, two new bone samples were scanned and
 388 tested. The results are summarized in Table 2. This protocol enables the differentiation of bone
 389 microstructures, using an environment close to physiological conditions. A thorough analysis and
 390 quantification of the microstructure via micro-CT will be performed in future experiments, which
 391 could explain the standard deviation values (Boruah *et al.*, 2017).

392

393

<i>Characterization of specimen 2</i>	Reduced Young's Modulus E* (mean)	Reduced Young's Modulus E* (median)	Standard deviation E*	Range	P-value (test)
<i>Low-porosity bone (10.8%)</i>	994.68 MPa	991.94 MPa	342.52 MPa	434 to 1745.2 MPa	< 0.0001 (Welch's t-test)
<i>High-porosity bone (60%)</i>	409.79 MPa	379.07 MPa	175.14 MPa	206.09 to 755.56 MPa	

394 *Table 3: Main results of characterization in protocol validation (specimen 2)*

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401 **5. Conclusion**

402 The aim of the study was to define the environment for ASB bones measurements. The choice of
403 comparison from E^* , associated with suitable measurement conditions (low load, low loading speed),
404 represents a first step in the characterization of these bones.

405 Nanoindentation associated with micro-CT imaging would appear to be the optimal technique to
406 characterize the mechanical properties of thin ASB bone walls, capable of differentiating between
407 samples according to microstructure. Furthermore, to obtain the physiological properties of ASB, the
408 tests must be performed involving immersion. Temperature during testing does not interfere with
409 the mechanical properties, and future experiments should ideally be performed in 20°C temperature
410 and a liquid (physiological saline sodium chloride) environment to measure ASB mechanical
411 properties. Finally, it is recommended to perform curve fitting at the end of the load.

412 Future experiments will use the integrated Sinus Mode, which enables to perform Dynamic
413 Mechanical Analysis for depth profiling of mechanical properties (HIT, EIT vs. depth) and
414 measurement of viscoelastic properties (E' , E'' : storage and loss moduli, $\tan \delta$) on samples ranging.
415 Viscoelastic indentation responses will be examined based on an approach using elastic–viscoelastic
416 correspondence. These experiments will be coupled with a precise analysis of the microstructure
417 (bone mineral density, porosity and orientation of the osteons) using micro-CT. Furthermore, other
418 mechanical tests will be performed (uniaxial and biaxial microtraction) to study the anisotropy of ASB
419 bones.

420

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425

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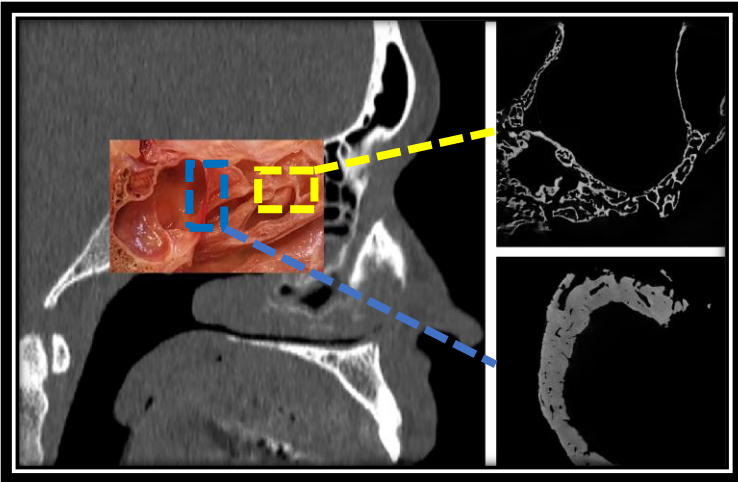
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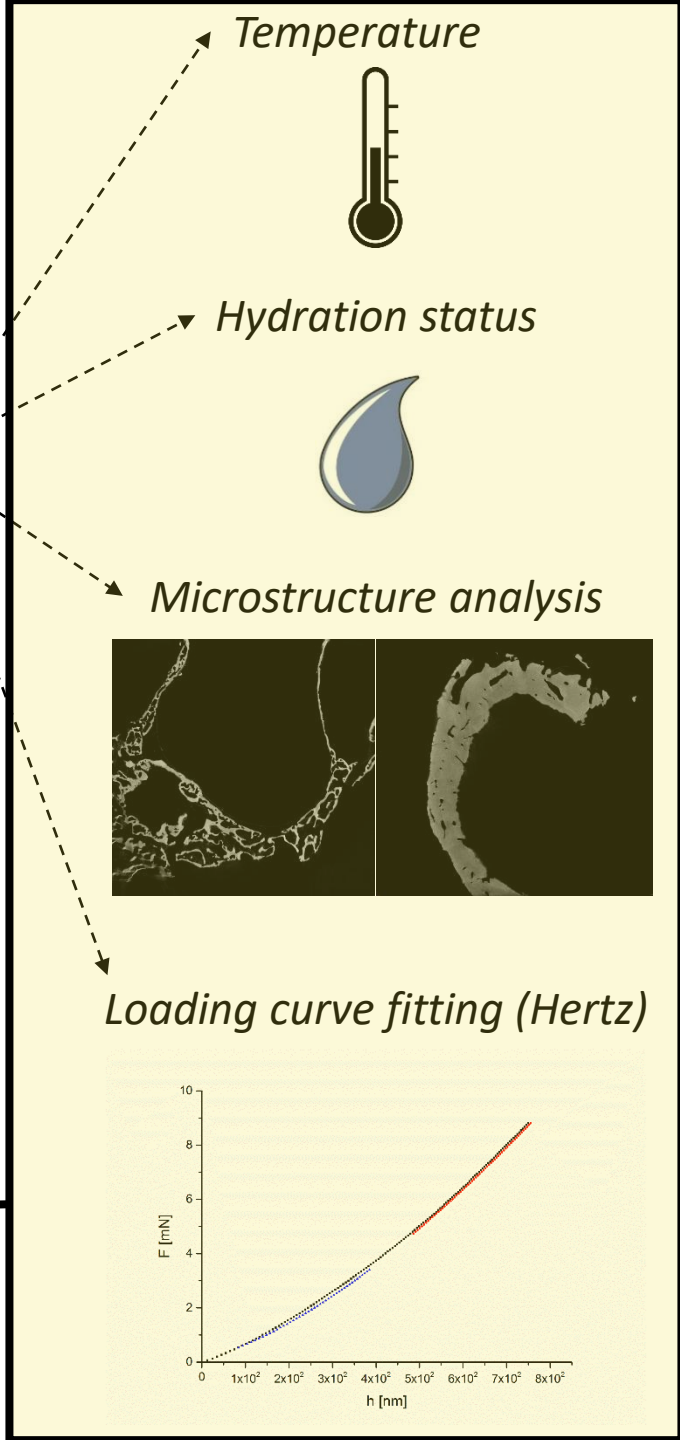
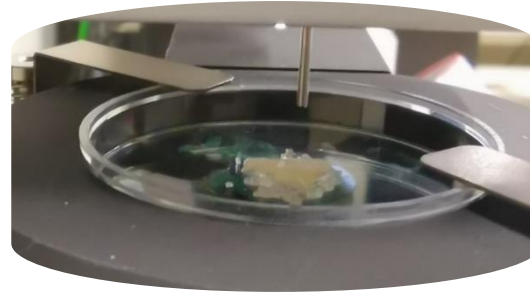
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Nano-indentation experimental design

Skull base bone samples



Tests	Bone microstructure	Hydration status	Temperature	Analysis
# 1	Low-Porosity	Dry	20°C	Start
# 2	Low-Porosity	Dry	37°C	End
# 3	Low-Porosity	Hydrated	20°C	End
# 4	Low-Porosity	Hydrated	37°C	Start
# 5	High-Porosity	Dry	20°C	End
# 6	High-Porosity	Dry	37°C	Start
# 7	High-Porosity	Hydrated	20°C	Start
# 8	High-Porosity	Hydrated	37°C	End

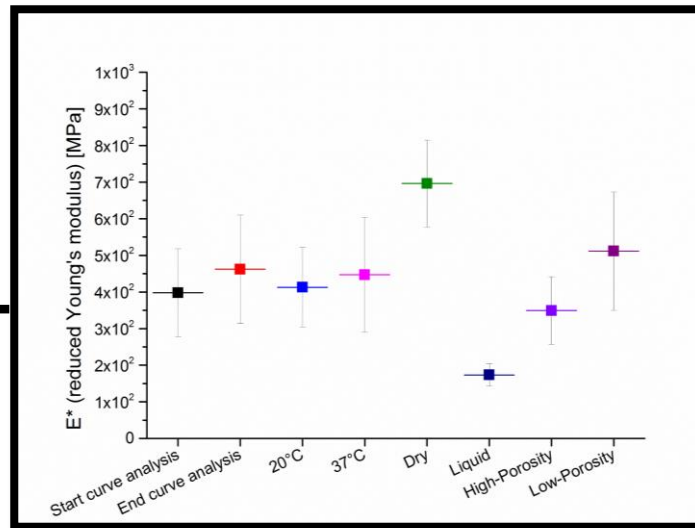


Final setting of parameters

Temperature: 20°C

Environment: liquid

Loading curve fitting: end



Loading curve fitting (Hertz)

