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Analysis of avian eggshell microstructure using X-ray area detectors

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Abstract: Avian eggshell is a relatively simple model of biomineralization processes. This biomaterial consists mainly of a mineral part made of columnar calcite crystals and a pervading organic matrix. The organization of eggshell microstructure is determined by genetic, physiological and external factors. Eggshell microstructural characteristics can inform us about biological and physicochemical processes affecting its formation. Quantitative microstructure information can be efficiently determined from two-dimensional diffraction patterns of polycrystalline samples. The present paper analyses the potential of this methodology as applied to microstructure characterization of fully dense polycrystalline materials such as avian eggshell. High correlation was found between parameters determined by X-ray diffraction, such as the number, size and intensity of reflection spots in Debye rings, and crystal size dimensions determined by means of optical microscopy. Crystal sizes can be calculated from these parameters following calibration using samples of the same material whose sizes are already known. Estimated error in crystal size measurements was within 5 %. In comparison with traditional methodologies (*e.g.*, optical microscopy), this technique enabled much faster and more precise determination of microstructural information.

Key-words: biomineralization, calcite, eggshell, microtexture, CCD detector, Debye-Scherrer ring.

Introduction

Avian eggshell formation is an interesting and relatively simple model of biomineralization processes. Eggshell deposition is very fast being completed in less than 20 h while the egg is residing in the hen's uterus. The resulting biomaterial consists mainly of a mineral part (> 95 %) made of columnar calcite crystals and a pervading organic matrix (1–3.5 %), resulting in a composite structure which has excellent mechanical properties (Simkiss & Wilbur 1989; Arias *et al.* 1993; Nys 1999). The organization of eggshell is determined by genetic factors as different avian species develops eggshells with specific microstructure characteristics. Eggshell microstructural characteristics are also modified by different physiological conditions (i.e., age, diet, disease). For instance, as hens age there are notable changes in eggshell microstructure as well as a marked decrease of its mechanical properties (Rodriguez-Navarro *et al.*, 2002; Ahmed *et al.*, 2005). Old hens laid eggs with shells constituted of larger crystals than those laid by young hens. Also, shells of the older hens contain abnormally large and highly oriented crystals with an unusual fan-like shape. These changes are associated with modifications of the quantity and composition of organic matrix which in turn controls crystal growth (Nys *et al.*, 1999; Ahmed *et al.*, 2005). Thus, microstructural characteristics can inform us about biological and physicochemical processes affecting biominerals formation and properties (Silyn-Robert & Sharp, 1986; Berman *et al.*, 1994; Rodriguez-Navarro *et al.*, 2002; Ahmed *et al.*, 2005). Understanding these processes is of great interest to mineralogists and materials scientists. However, microstructure analysis of polycrystalline materials is a highly tedious process when using traditional techniques (*i.e.*, optical or scanning electron microscopy and image analyses; Kurzydlowski & Ralph, 1995). It is especially impractical in the study of a large number of samples necessary for statistical analyses. This paper describes an alternative methodology which based on two-dimensional (2D) X-ray diffraction allows microstructure quantification of polycrystalline materials (*e.g.*, crystal size and orientation) much more efficiently than using traditional techniques. 2D

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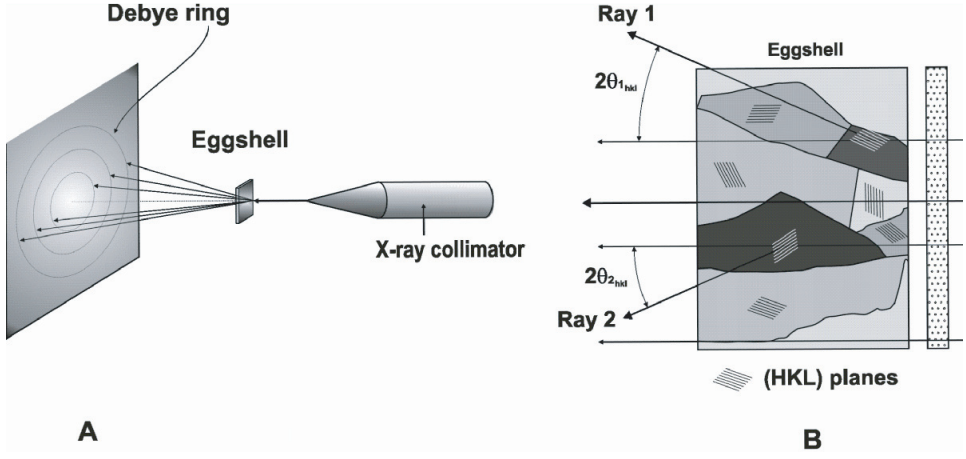

Fig. 1. (**A**) Experimental set-up for diffraction analysis of an eggshell sample. (**B**) Schematic drawing of the eggshell crystals contributing to the diffraction pattern. Only those crystals whose (*hkl*) planes are oriented in Bragg condition diffract. Only crystals 1 and 2 comply with these conditions in this figure. The intensity of the hkl reflection or spots is proportional to the size of the diffracting crystal. Crystal 2 would for this reason display a more intense reflection than crystal 1.

diffraction patterns can be collected with any diffractometer equipped with a 2D or area detector (*i.e.*, CCD, Image Plate or a photographic film) and contain much more information than conventional linear scans (*i.e.*, θ–2θ scans) collected using standard powder diffractometers (Sulyanov *et al.*, 1994; Bunge *et al.*, 2002; He, 2003; Rodriguez-Navarro *et al.*, 2006). These patterns typically consist of concentric [Debye-Scherrer] rings produced by the superposition of reflections from crystals illuminated by the X-ray beam and that are oriented with a set of {*hkl*} crystallographic planes oriented fulfilling Bragg condition (Cullity, 1977). Depending on sample characteristics, these rings might be continuous or spotty and display specific variation in the intensities along them. These features proportionate important information about the microstructure of the sample: grain size, preferential orientation, mosaicity, stress, etc. (Bunge *et al.*, 2002; He, 2003; Rodriguez-Navarro *et al.*, 2006; Rodriguez-Navarro, 2006). This paper shows how quantitative microstructure information (*e.g.*, crystal size and shape) can be automatically extracted from these patterns using specialized software. Previously, this methodology was applied to quantifying crystal sizes of abrasive powders in a sister paper (Rodriguez-Navarro *et al.*, 2006). The abrasives come in graded sizes and were especially useful to validate the methodology in particular its use to determine crystal sizes. In this previous study, the use and range of applicability of the methodology is described in detail. The main objective of the present study was to extend this characterization methodology to natural samples in which crystals are aggregated and display varying sizes, shapes and degrees of preferential orientation. A set of eggshells of different avian species with varying microstructure characteristics was chosen in order to compare microstructure information obtained by optical microscopy (mainly size and shape of crystals) with parameters determined by X-ray diffraction and to determine calibration curves relating both sets of parameters. As stated before, eggshells are very interesting materials and are also particularly suitable for XRD analyses because of their small thicknesses allows them to be analysed by transmission without a significant loss in X-ray intensity due to absorption.

Materials and methods

Samples: Fourteen eggshell samples were selected from different bird species displaying various microstructure characteristics: laying hen (8), pheasant (2), guinea fowl (1), pekins falcon (1), ostrich (1), muscovy duck (1), partridge (1).

Optical microscopy: Thin-sections (< 30 μm) of radial cut eggshells were prepared for microstructure analysis by optical microscopy (OM). Crystal size distributions were determined from microphotographs taken with cross-polarized light (×10; Olympus SZ, Japan). Crystal size measurements were done using ImageJ, an image analysis program (National Institute of Health, USA). Maximum width and length were determined for each crystal in pictures taken at three different locations within each eggshell. Eggshell thickness was considered as that of the mineral part of the eggshells and measured from the abovementioned microphotographs.

X-ray diffraction: X-ray diffraction analyses were done at the Centro de Instrumentación Científica (U. Granada) using a single-crystal diffractometer equipped with a CCD area detector (D8 Smart APEX, Bruker, Germany). Pieces (about 1 × 1 cm) were cut from each eggshell sample, mounted using clay-dough and set on a sample holder of the diffractometer. Samples were mounted so that their outer shell surface faced the area detector and the inner surface faced to the incident X-ray beam (Fig. 1). The X-ray beam goes through the sample and a transmission diffraction pattern is recorded on the area detector. Table 1 summarizes experimental conditions used for diffraction analyses. Figure 2 shows typical diffraction patterns from

Table 1. Experimental conditions for X-ray diffraction experiments.

	Value
Radiation	Mo Kα
Acceleration voltage	50 KV
Filament Current	30 mA
Eggshell sample	~ 1 cm^2
Collimator diameter	0.5 mm
Exposure time	20 s
Size of detector	512 × 512 pixels
Distance to detector	60 mm

eggshells which consist of concentric spotty rings (Debye-Scherrer rings). Each spot within a ring corresponds to a *hkl* reflection of a calcite crystal whose (*hkl*) planes are now oriented in diffraction conditions. The number of crystals contributing to diffraction patterns varies with the X-ray beam diameter, the size of crystals and sample thickness (Cullity, 1977; Ichikawa *et al.*, 1996). A large number of low intensity reflections indicates that the crystal size is very small, relative to the beam diameter, while a small number of high intensity reflections indicates that the crystal size is large. Thus, crystal sizes can be estimated from the intensity of the reflection spots in Debye-Scherrer rings (Rodriguez-Navarro *et al.*, 2006). XRD2DScan, a specially designed Windows application software (Rodríguez-Navarro, 2006), was used to analyze the 2D diffraction patterns and which automatically measures the intensity of reflection spots in selected Debye-Scherrer rings as well as other parameters such as the number of reflection spots in the ring and their breadth. Table 2 lists the value of several parameters that needs to be set for these analyses using XRD2DScan software. The software basically reads pixel intensities recorded on the detector output data file. It finds pixels within a selected 2θ range and belonging to a particular Debye-Scherrer ring to be analysed. Search for peaks in the ring with intensities above a certain threshold value, count the number of peaks and calculate their maximum and integrated peak intensity as well as their angular breadth. Sample microstructure and, specifically crystal size, are characterized by the above mentioned parameters. For each eggshell sample these parameters were determined using five different rings, associated with the strongest calcite reflections: 104, 110, 202, 113, and 108. More than one ring was used in order to minimise the influence of any preferential orientation of crystals and to be able to test which reflection was more informative regarding crystal size. The effect of absorption was corrected in peak intensities[1]. The results obtained for the five strongest calcite reflections were combined to lower data variability. Eggshell samples were measured in three locations to improve representativity.

[1] Corrected intensity was calculated as: $I_{corrected} = I_o \exp(\mu t)$, where μ is the linear absorption coefficient of eggshell and t is its thickness. Linear absorption coefficient for eggshell ($CaCO_3$) was calculated considering a density of 2.7 g/cm^3 and Mo Kα radiation. The value of μ was 0.0022 μm^{-1}. For more details about calculations see Buerger (1942).

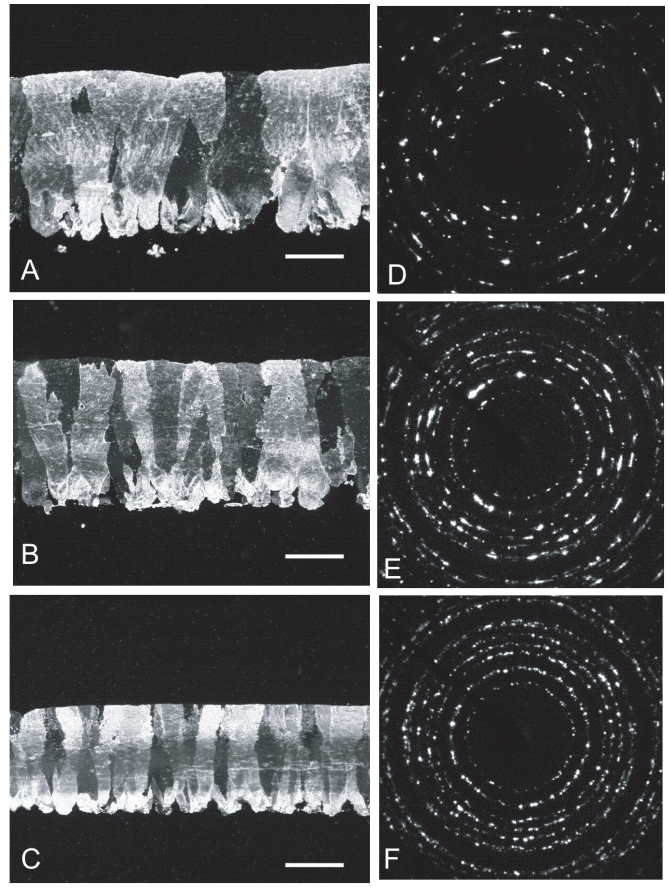

Fig. 2. Microphotographic views under cross-polarised light of a cross-section of hen eggshells, (**a**) and (**b**), and a partridge eggshell (**c**). The mineral part of the eggshells is constituted by columnar calcite crystal units (palisades). These units arise from spherulitic aggregates of smaller calcite crystals (mamillary cores) anchored onto the eggshell membranes. Crystals show varying degrees of light extinction due to differences in their orientation. Scale bar is 100 μm. (**d**)–(**f**) are the X-ray diffraction patterns of the same eggshells. It should be noted that the number of spots increases and their intensity decreases following the same sequence: hen (**a**), hen (**b**) and partridge (**c**).

Additionally, the software calculates the equivalent powder diffractogram by radially integrating the intensity of pixels equidistant to the pattern center and plotting the resulting intensity versus 2-θ values. Information regarding mineral composition and preferential orientation can be inferred from the calculated powder diffraction pattern.

Statistical analyses: Pearson correlation analyses were carried out among different microstructural parameters determined by OM and XRD. Linear regression models were calculated among significantly correlated parameters. The statistical software package SPSS 12.0 (SPSS Inc., USA) was used for all statistical analyses.

Results

Figure 2 illustrate differences in eggshell microstructure as detected by optical microscopy and X-ray diffraction.

Table 2. Values of parameters set to calculate peak intensities in the Debye-Scherrer rings using XRD2DScan software.

Parameters	Value
2 Theta integration range (deg)	1.2
Background substraction	True
2 Theta step (deg)	0.1
Psi step (deg)	0.5
Roll width (deg)	2.0
Peak width (deg)	2.0
Minimum peak intensity (counts)	2000

Figures 2a and b are microphotographic views of two laying hen eggshells under cross-polarised light, while Fig. 2c shows the microstructure of a partridge eggshell. Eggshells are composed of columnar calcite crystal units (palisades). These units arise from spherulitic aggregates of smaller calcite crystals (mamillary cores) anchored onto the eggshell membranes. Average crystal unit sizes in eggshells vary from one avian species to another, as well as among samples of the same species (Fig. 2a and b). Columnar unit sizes in these samples decrease in the following order: hen (a), hen (b) and partridge (c). The 2D X-ray diffraction patterns produced by these eggshells can be seen in Figs. 2d, e, and f. It should be noted that both the number of spots increases and spot intensity decreases in the same order as crystal sizes decreases.

Figure 3 describes two cases of eggshells showing peculiar microstructure characteristics and illustrates how these characteristics are manifested in the diffraction pattern. Figure 3a shows the microstucture of an ostrich eggshell as seen under cross-polarized light. It is constituted by slender columnar calcite crystals oriented with their ***c***-axes aligned ((001) fiber texture) and perpendicular to the eggshell outer surface. Crystals reflect in the same section of the Debye ring because they share almost the same crystallographic orientation (Fig. 3c). Continuous arcs are produced rather than separated reflection spots. The effect of the strong texture can also be observed in the calculated powder diffractogram for ostrich eggshell (Fig. 3e). In the transmission experiments, crystals are preferentially oriented with their ***c***-axes aligned nearly parallel to the X-ray beam so that reflection from crystallographic planes belonging to the [001] zone are reinforced. The intensity of the 110 reflection is therefore reinforced and is now the strongest one instead of the 104 which is almost absent. The second case is that of samples in which submicrostructure is similar to that of muscovy duck eggshells constituted by microcrystals arranged in greater columnar units. One would expect that such a microcrystalline material would yield a diffraction pattern constituted by constinuous rings like that produced by a fine powder sample. Instead a spotty pattern, typical of a coarsely grained sample, is formed. This is due to that crystals within each columnar unit share an almost identical crystallographic orientation and all contribute to the same reflection spots acting like a single or mosaic crystal.

It has been shown in the above mentioned 2D patterns, though just qualitatively, that as crystal size increases the number of reflection spots in the pattern decreases and their intensity increases. Following, it will be described how microstrutural parameters and XRD parameters are related. In particular, correlations among parameters determined by OM (length and width of crystal units) and different parameters determined by XRD (np_{hkl}, A_{hkl}) have been studied in order to compare the microstructure information obtained by both techniques. Results are summarized in Tables 3 and 4. It is important to say that eggshells from ostrich, muscovy duck and pekins falcon because of their peculiar microstructural characteristics were excluded from these analyses. Highly significant co-variation can be seen between XRD-determined parameters and OM-determined average crystal dimensions. The number of peaks (np_{hkl}) in the Debye-Scherrer rings associated to Bragg reflections 104, 110, 202, 113 and 108 are negatively correlated to average crystal dimensions. On the contrary, peak intensity (A_{hkl}) in Debye-Scherrer rings are positively, and more significantly, correlated with crystal dimensions. The sum of average peak intensity (TA) for this group of reflections is even more highly correlated to crystal dimensions. Interestingly, peak intensity (A_{104}) in the 104 ring is highly correlated to the length of columnar units (CUL). On the other hand, Tnp is positively correlated to the aspect ratio of crystal units (defined as the length to width ratio of columnar crystals).

These results make it possible to calculate a calibration curve for the crystal sizes from parameters determined by XRD. Figure 4 shows a set of cross-plots showing the relationship among crystal dimensions and parameters determined by XRD. Figure 4a shows that, as the width of crystal units (CUW) increases, total peak intensities (TA) increases linearly in accordance with the regression line model (TA = b* CUW + m; b = 15037 and m = 401350; N = 11; R^2 = 0.913; $p < 0.0001$). Interestingly, peak intensities in 104 Debye ring, A_{104}, strongly correlate with crystal unit lengths (CUL; Fig. 4b). The volume of crystals is also positively correlated to the sum of peak intensities in all five rings (TA). Figure 4d illustrates the positive correlation between crystal aspect ratio (defined as the length to width ratio of columnar crystals) and the total number of peaks (Tnp) in the five rings. Any of these curves can be used to determine crystal sizes in unknown samples with a high degree of confidence, and also to obtain information regarding the shape of crystals. This is especially valid when using intregrated peak intensities and, in particular, the sum of averaged peak intensities for several rings (*i.e.*, TA). For instance, if we back-calculate the width of columnar units making the eggshell using the calibration curve of Fig. 4a, there is an average error of 2.9 μm corresponding to an error of 5 % in the estimation.

Discussion

Microstructural information of eggshell obtained by OM is highly detailed regarding size, shape and orientation of crystals. However, this data is limited to a 2-D radial section of a very small area (at ×10, field of view is approximately 400×400 μm). Crystal size measurements are taken from a small number of crystals (typically 20–30), Thus, it

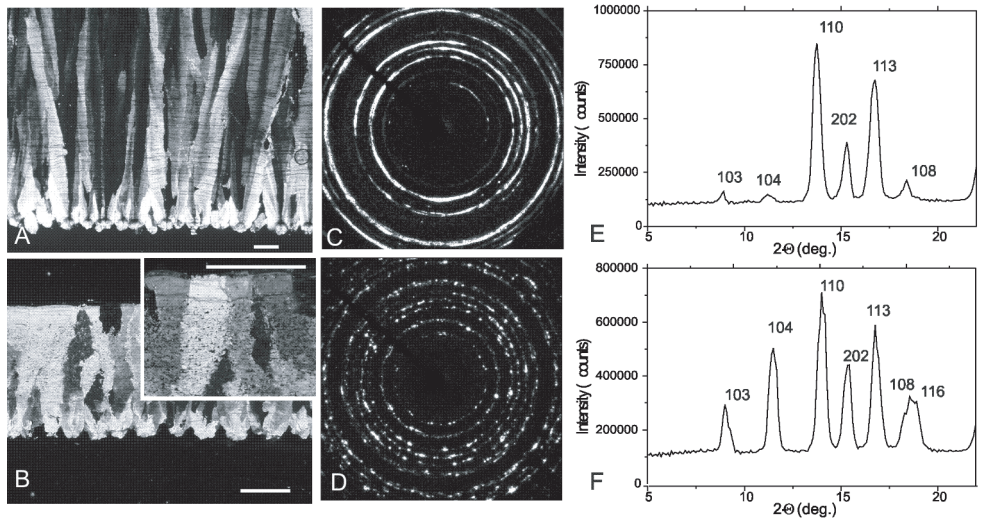

Fig. 3. Effects of specific microstructural characteristics on diffraction patterns. (**A**) Microphotographic view of an ostrich eggshell section under cross-polarised light. Calcite crystals are long columns which are aligned with ***c***-axes ((001) fiber texture) perpendicular to shell surfaces. (**B**) microphotographs of muscovy duck eggshell microstructure. Note that calcite columnar units display diffuse boundaries and are composed of equiaxed calcite microcrystals (about 5 micron in size). Scale bar is 100 μm. (**C**) Diffraction pattern of ostrich eggshell (from the top 400 μm) is formed by continuous arcs as a consequence of the high degree of crystal preferential orientation. (**D**) Diffraction pattern of muscovy duck eggshell. This pattern reveals the sizes of columnar units and not those of microcrystalline subunits. (**E**) and (**F**) Calculated powder diffractograms from ostrich and muscovy duck eggshells. Note that in the ostrich pattern (**E**) the strongest calcite 104 reflection is almost absent, while the lower intensity 110 reflection is now the strongest. Reflections associated to (110) calcite planes are reinforced due to preferential orientation of crystals.

Table 3. Eggshell properties determined by optical microscopy and parameters determined from X-ray diffraction patterns. Average peak intensities (A_{hkl}). Values for np_{hkl} and Tnp correspond, respectively, to the number of peaks in a single *hkl* Debye ring and to the total number of peaks in the five analysed rings. The number of samples (N) is 11. CUW and CUL stand for crystal unit width and crystal unit length, respectively.

	Properties	Value	N
Optical M	CUW (μm)	55 ± 12	11
	CUL (μm)	270 ± 40	11
	Thickness (μm)	351 ± 46	11
	Aspect Ratio	5.0 ± 0.8	11
	Volume (μm^3)	882925 ± 442711	11
X-ray diffraction	np_{104}	39 ± 11	11
	A_{104}	147622 ± 73832	11
	np_{110}	19 ± 7	11
	A_{110}	68023 ± 34188	11
	np_{202}	31 ± 7	11
	A_{202}	57164 ± 20513	11
	np_{113}	26 ± 8	11
	A_{113}	82716 ± 46069	11
	np_{108}	54 ± 10	11
	A_{108}	71368 ± 41378	11
	Tnp	170 ± 39	11
	TA	426894 ± 195023	11

is necessary to repeat the procedure in different locations along the eggshell thin section. However, the differences in optical extinction, as well as the presence of a colour gradient, does not enable an unambiguous differentiation among crystals. This makes it difficult to measure crystal dimensions. In addition, it makes it even more difficult to automatize this process by means of image analysis. The methodology is further complicated by the fact that thin-sections preparation is so tedious and impractical, except within the context of studies using small numbers of samples. Regarding X-ray diffraction analyses and considering that the diameter of columnar units in eggshell is about 50 microns and the X-ray beam diameter is 500 microns, we are illuminating about 100 crystals in every location analyzed using a transmission setting. Thus, the number of crystals contributing to the diffraction pattern is larger than the number of crystals analyzed by OM allowing for more accurate statistics.On the other hand, the present study reveals that crystal sizes measured by OM were highly correlated to parameters determined by XRD technique and that a calibration curve can be calculated relating average crystal sizes and peak intensities. Information regarding the shape of crystals was also determined from XRD data. This proves that the analyses of 2D diffraction patterns could be a powerful tool for studying the microstructure of polycrystalline materials such as ceramics and metals, composed of crystal with sizes within the microscopic range, as it has been previously demonstrated using powder samples of SiC and α-Al_2O_3 abrasives (Rodriguez-Navarro *et al.*, 2006). It should also be kept in mind that the material used in this study, the avian eggshell, is highly complex. It is composed of calcite crystals that display an anisotropic shape and varying degrees of preferential orientation (Nys *et al.*, 1999; Rodriguez-Navarro *et al.*, 2002). Eggshell thicknesses also change from one sample to another. In any case, the XRD technique has been proved to

be very sensitive allowing the detection of small changes in eggshell microstructure even between samples belonging to the same avian species. For instance, in a previous study this technique allowed the detection of subtle but still significant differences in hen eggshell microstructure associated to moulting. XRD data indicated that the size of eggshell constituting crystals decreased after birds were forced to moult their feathers (Ahmed *et al.*, 2005). However, in that previous study, no calibration curve was established so that only qualitative information could be inferred.

From the calibration curves determined here, calcite crystal sizes can be calculated at an estimated error of within 5%, similar to that obtained with abrasive powder of graded sizes (Rodriguez-Navarro *et al.*, 2006). This error may be smaller for other materials which are not as heterogeneous and complex as avian eggshells. Furthermore, since data collection is fast, it is possible to analyze a large number of samples for studies requiring statistical analyses. For instance, to correlate material properties (*i.e.*, mechanical, electrical) to their microstructure characteristics.

The following subsections discuss in detail the influence of various factors which need to be considered for the correct application of the methodology:

a) Crystal size and sample thickness

The intensity of an *hkl* reflection depend on several factors including crystal structure, crystallinity of the material, absorption, temperature, geometry of the diffractometer (Azaroff, 1969; Cullity, 1977; Rodriguez Gallego, 1982). Nevertheless, other factors being equal, the intensity of reflections increases and is proportional to the volume of the crystal, provided that the (*hkl*) planes satisfy the Bragg condition. As crystal sizes increase, so also does the intensity of the reflected X-ray though intrinsic properties of crystals (crystallinity, mosaicity, and other imperfections) could modify this relationship. Also, a slight deviation in crystal orientation from Bragg condition would decrease the intensity reflected by the crystal, since a tail of the reflection peak will be measured. Therefore, it is not possible, in principle, using this technique to determine a size distribution but an average size (Rodriguez-Navarro *et al.*, 2006). In any case, for the different materials studied, we found a very good linear relationship between average crystal size and integrated peak intensities. Also keep in mind that this technique requires samples of known sizes for calibration. In case these samples are not readily available, still we can get valuable qualitative data.

On the other hand, as the X-ray beam passes through the sample thickness, its intensity is attenuated by absorption. In the case of eggshell, the dependence of reflection intensity on crystal size and sample thickness is as follows:

$$I = I_o\, a\, b^2 \exp(-\mu t) \quad (1)$$

where a is the crystal units length, b is the crystal units width, t is shell thickness and μ, the linear absorption coefficient. There is, thus, an optimum sample thickness ($1/\mu$) which produces the maximum intensity of diffraction (Buerger, 1942; Cullity, 1977; Rodriguez Gallego, 1982). For our measurements, it is about 450 μm (similar to the thickness of the eggshell samples studied here). Above this thickness value, the absorption effect will be dominant and the diffracting beam intensity will decrease. Thus, the optimum thickness value is an upper limit for the crystal sizes that can be determined using this technique. This is so because absorption effect became too strong and intensity decreases with crystal size. Another difficulty regarding the measurement of such coarse crystal sizes is that only a few crystals are illuminated by the beam (0.5 mm in diameter in this case) and, thus, the resulting data are not sufficiently reliable. This upper limit can be extended using larger beam diameters. Minimum crystal size limit is attained when crystal reflections begin to overlap so that individual peak intensities can no longer be measured. This lower limit is also determined by area detector size and by the number of pixels of the area detector circumscribing a Debye ring. In order to be resolved, peak width must be at least 3 pixels. In our study, for instance, a 104 Debye ring is composed of approximately 750 pixels. Therefore, a maximum of around 250 peaks can be measured without overlapping. Thirty peaks appear using a collimator of 0.5 mm in diameter for a sample whose crystal is 50 microns. A minimum crystal size of about 5 μm can be estimated which is similar to that determined experimentally for abrasive powders (Rodriguez-Navarro *et al.*, 2006). However, the limit can be lowered by decreasing the size of the beam using a smaller collimator. This makes it possible to illuminate a smaller number of crystals so that their reflections can be measured (Hirsch & Kellar, 1952). Commercially available collimators of 0.05 mm are able to generate spotty rings even with samples whose crystal sizes are 1 μm or below. Thus, by selecting an adequate X-ray beam size, different crystal sizes ranges can be analyzed.

b) Sample aggregation state

One of the main advantages of the technique based on X-ray diffraction is that it does not depend on the aggregation state of the material. The reflection intensity of an individual crystal is always proportional to its volume (size), regardless of whether crystals are part of a fully dense ceramic as in this case study or in powder form. However, the number of spots is proportional to the number of crystals illuminated by the beam and, thus, depends on the degree of crystal dispersion in a matrix (Rodriguez-Navarro *et al.*, 2006). That is why the latter parameter (used in earlier studies; Hirsch and Kellar, 1952; Andrews & Johnson, 1959; Cain & Heyn, 1964; Ichikawa *et al.*, 1996) is a poorer estimate of crystal sizes.

c) The influence of the preferential orientation and submicrostructure

Figure 3 illustrates two cases in which this technique is not applicable. The first is that of strongly textured samples formed by highly oriented crystals. Figure 3a shows the microstucture of an ostrich eggshell. Columnar calcite crystals display aligned ***c***-axes ((001) fiber texture)

Table 4. Correlation among eggshell properties determined by optical microscopy and parameters determined from X-ray diffraction patterns. R is the Pearson correlation coefficient and p is the significance (for $p < 0.05$ correlation are significative). The number of samples (N) is 11.

	CUW		CUL		Aspect Ratio		Volume	
	R	p	R	p	R	p	R	p
CUW	1.000		0.587	0.058	–0.806	0.003	0.967	< 0.001
CUL	0.587	0.058	1.000		–0.005	0.989	0.719	0.013
Aspect Ratio	–0.806	0.003	–0.005	0.989	1.000		–0.666	0.025
Volume	0.967	< 0.001	0.719	0.013	–0.666	0.025	1.000	
np_{104}	–0.680	0.021	–0.064	0.852	0.789	0.004	–0.643	0.033
A_{104}	0.764	0.006	0.762	0.006	–0.426	0.192	0.813	0.002
np_{110}	–0.909	< 0.001	–0.471	0.144	0.748	0.008	–0.887	< 0.001
A_{110}	0.909	< 0.001	0.345	0.299	–0.844	0.001	0.836	0.001
np_{202}	–0.899	< 0.001	–0.403	0.218	0.770	0.006	–0.862	0.001
A_{202}	0.951	< 0.001	0.557	0.075	–0.765	0.006	0.914	< 0.001
np_{113}	–0.859	0.001	–0.397	0.227	0.783	0.001	–0.850	0.001
A_{113}	0.887	< 0.001	0.330	0.321	–0.843	0.001	0.862	0.001
np_{108}	–0.719	0.013	–0.018	0.957	0.868	0.001	–0.640	0.034
A_{108}	0.931	< 0.001	0.408	0.212	–0.821	0.002	0.883	< 0.001
Tnp	–0.872	< 0.001	–0.256	0.447	0.876	< 0.001	–0.828	0.002
TA	0.956	< 0.001	0.572	0.066	–0.763	0.006	0.942	< 0.001

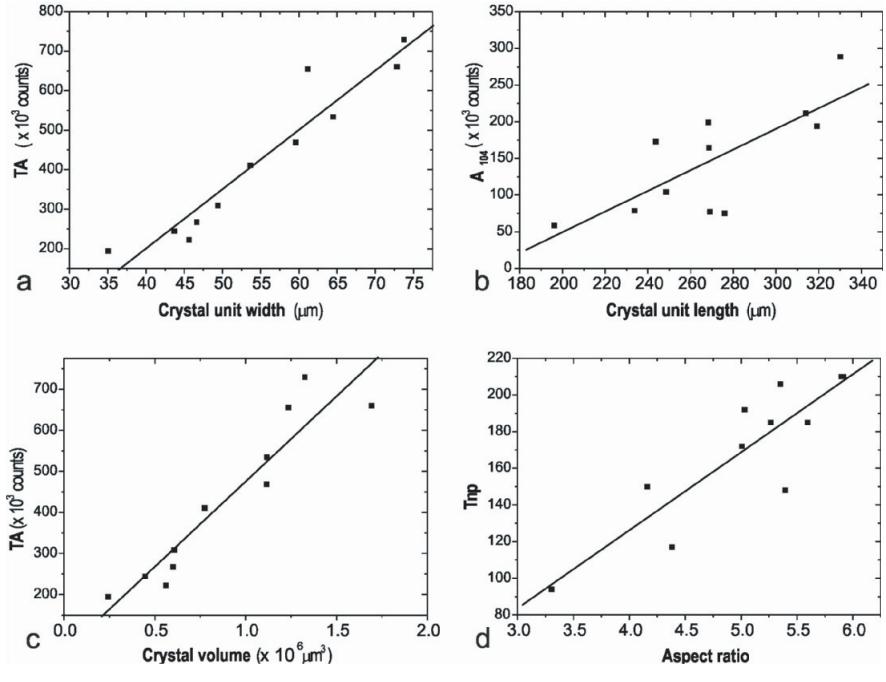

Fig. 4. Calibration curves relating calcite crystal dimensions to XRD-parameters. (**a**) Relationship between total peak intensity (TA) and columnar unit width (CUW). (**b**) Relationship between peak intensity in 104 ring (A_{104}) and columnar unit length (CUL). (**c**) Relationship between total peak intensity (TA) and crystal volume. (**d**) Relationship between total number of peaks (*Tnp*) and crystal aspect ratio (defined as the length to width ratio).

which are oriented perpendicular to the eggshell outer surface. Crystals reflect in the same section of the Debye ring because they share almost the same crystallographic orientation (Fig. 3c). Continuous arcs are produced rather than separated reflection spots. Therefore, the abovementioned technique can not be successfully used with strongly textured materials, since diffraction spots from individual crystals are not resolvable (Ichikawa *et al.*, 1996). The effect of the strong texture can also be observed in the calculated powder diffractogram for ostrich eggshell (Fig. 3e). Crystals are preferentially oriented with their ***c***-axes aligned nearly parallel to the X-ray beam so that reflection from crystallographic planes belonging to the [001] zone are reinforced. The intensity of the 110 reflection is therefore reinforced and is now the strongest one. The second case is that of samples in which submicrostructure is similar to that of muscovy duck eggshells and the columnar crystals units are formed by microcrystals that share an almost identical crystallographic orientation. Here, whereas the technique measures the size of larger

columnar units, it can not determine their subunit size. Thus, a certain amount of information regarding the type of sample microstructure must be known if crystal sizes are to be correctly determined by X-ray diffraction. For instance, it is necessary to know to some degree the size range of crystal units making the samples and their shape as well as their organization and degree of orientation. Note also that, each crystal, according to their orientation relative to the X-ray beam, will have different sets of {*hkl*} planes reflecting, on an associated Debye-Scherrer ring. Thus, measuring peak reflections from several rings will improve sample statistics and minimise the effects of preferential orientation of crystals.

d) Comparison with Scherrer methodology

It is highly important that the technique described in this paper not be confounded with the widely used Scherrer method. The latter is based on the fact that when crystal sizes are smaller than about 0.1 μm, there is an important broadening of diffraction peaks in powder diffractograms (within the 2-θ angle) (Azaroff, 1968; Cullity, 1977; Rodriguez Gallego, 1982). The Scherrer methodology provides information regarding sample crystallinity and particle size (or more exactly the size of domains of coherent diffraction) when this is in the submicrometer range. However, our technique is much more quantitative and is able to determine the physical size of crystals. It can be used for a larger size range. In any case, both techniques are complementary within the size range to which each is applicable and with regard to the type of information they provide.

Conclusions

The X-ray diffraction methodology, based on the detailed analyses of 2D diffraction patterns, displays several advantages in the quantification of polycrystalline sample microstructure in comparison to optical microscopy. No sample preparation is required and the data regarding all of the crystals illuminated by the beam in a volume of the sample is integrated into one single pattern. Measurements are independent of the aggregation state and this technique can be applied to any polycrystalline material whose crystal size is within the micrometer range. Results obtained using X-ray diffraction are in good agreement with those generated by optical microscopy. However, the X-ray based technique is much faster and convenient for studying a large number of samples. Also, the number of crystals contributing to the diffraction pattern is greater and allows for more accurate statistics than techniques based on OM. Additionally, data analyses are fully automatised by applying XRD2DScan software. Although this kind of analyses must be done using an X-ray diffractometer equipped with an area detector, such equipment is now available in many laboratories and does not therefore represent a significant limitation.

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