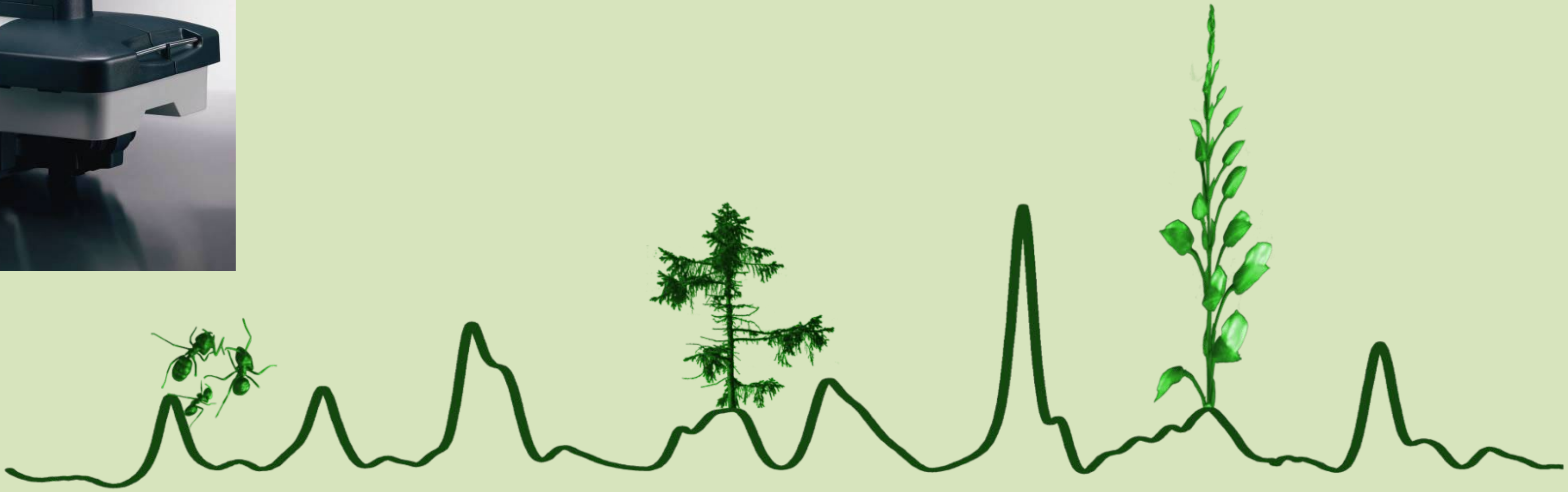
NICOLET CZ
MOLECULAR SPECTROSCOPY

# Raman spectroscopy / microscopy of bone fragments



# Samples

- Sample 1: 1400
- Sample 2: CCB01

Powdered and sieved bone fragments (> 25 microns) / Untreated samples.

**Measurement technique**
**DXR3 Raman microscope, 2023 (3th gen.)**

# Sir Chandrasekhara Venkata Raman
## 1888 – 1970

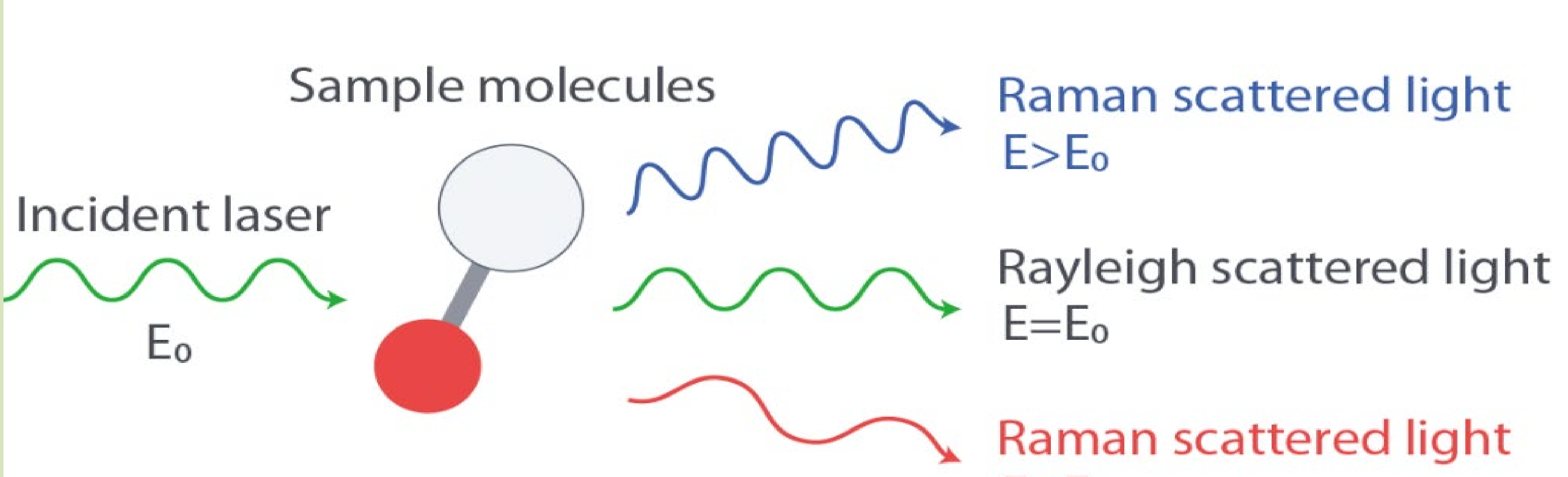
## Nobel Price in Physic 1930

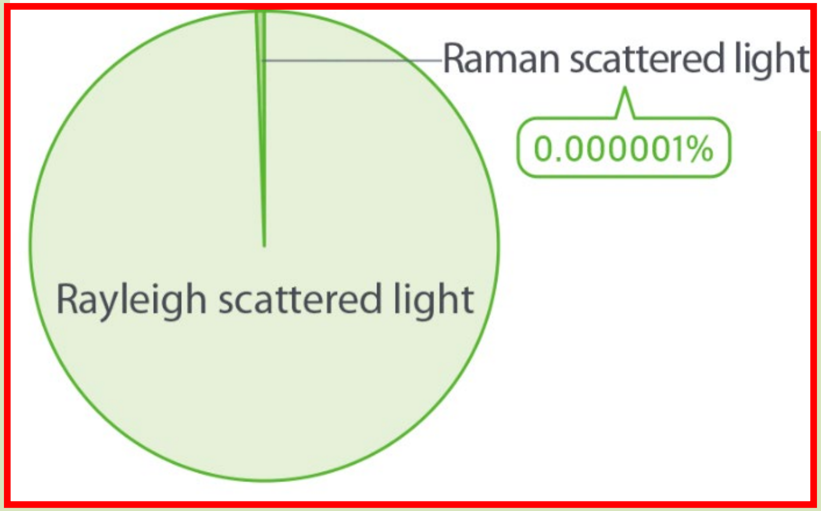
## A New Type of Secondary Radiation

**C. V. Raman and K. S. Krishnan, Nature,** ***121(3048)*****, 501, March 31, 1928**

The experiments we have made have confirmed this anticipation, and shown that in every case in which light is scattered by the molecules in dust-free liquids or gases, the diffuse radiation of the ordinary kind, having the same wavelength as the incident beam, is accompanied **<u>by a modified scattered radiation of degraded frequency</u>**.

# Raman microscopy: theory

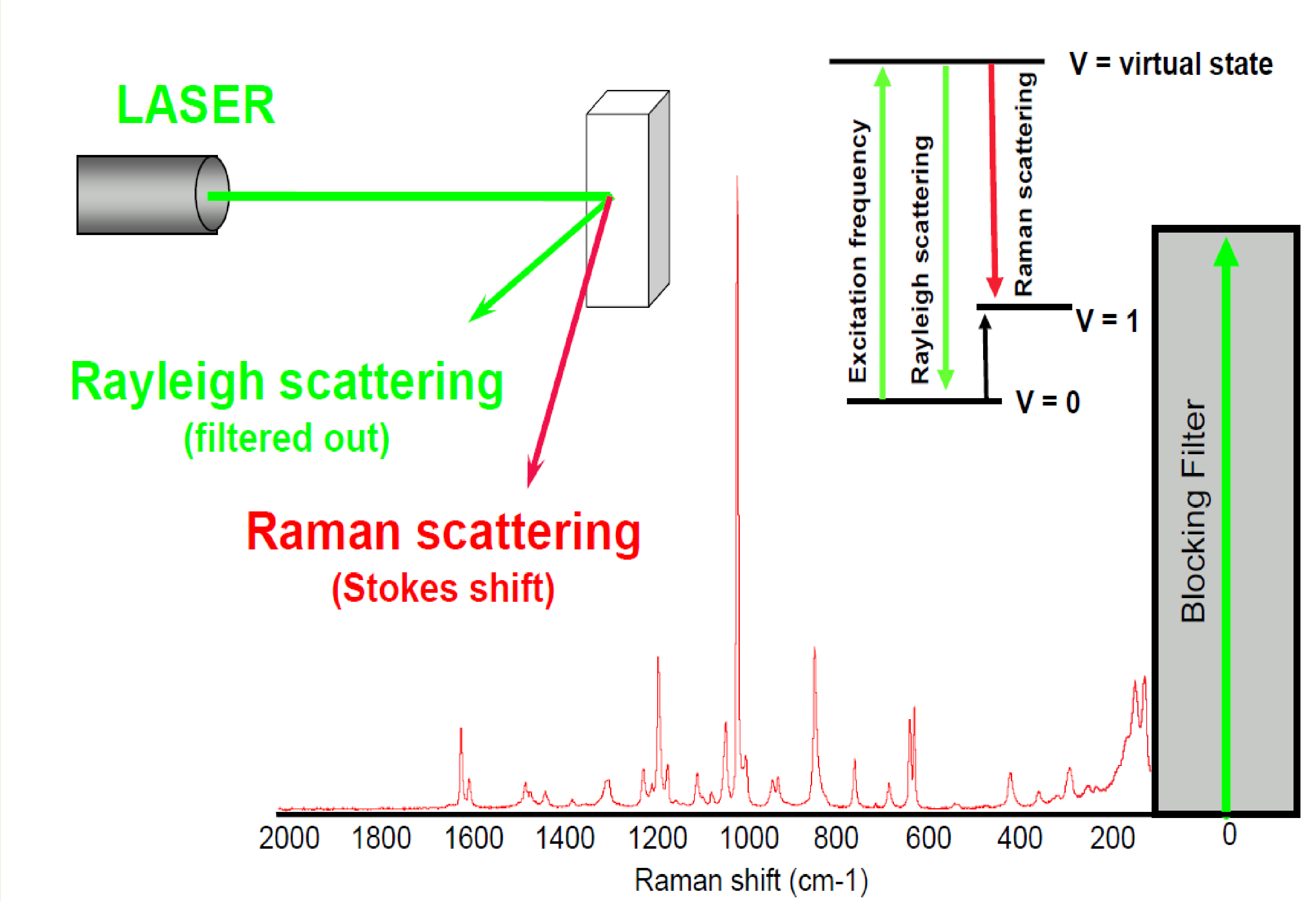




**Optical inelastic scattering**

Dominant process is Rayleigh scattered light, Ramanův is extremely weak
One photon from $10^6$ – $10^8$ photons is Raman scattering!

# Raman microscopy: theory



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# Raman versus infrared spectroscopy

| Raman | IR |
|---|---|
| It is due to the **scattering** of light by the vibrating molecules. | It is the result of **absorption** of light by vibrating molecules. |
| The vibration is Raman active if it causes a change in **polarisability.** | The vibration is IR active if there is a change in **dipole moment** during the vibration. |
| The molecule need not possess a permanent dipole moment. | The vibration concerned should have a change in dipole moment |
| Water can be used as a solvent | Water cannot usually be used due to its intense absorption IR spectrum |
| Sample preparation is not very elaborate sample can be almost in any state | Sample preparation is elaborate for some techniques |
| Gives an indication of covalent character in the molecule. | Gives an indication of ionic character in the molecule. |
| More suitable for anorganic features<br>Cutoff usually 50 $cm^{-1}$ | Cutoff usually 400 $cm^{-1}$ |
| **Microscopy: 0,4 micron resolution** | **Microscopy: 10 x 10 micron resolution** |

# DXR3 Raman microscope

**Several possibilities of Raman spectra measurement modes:**

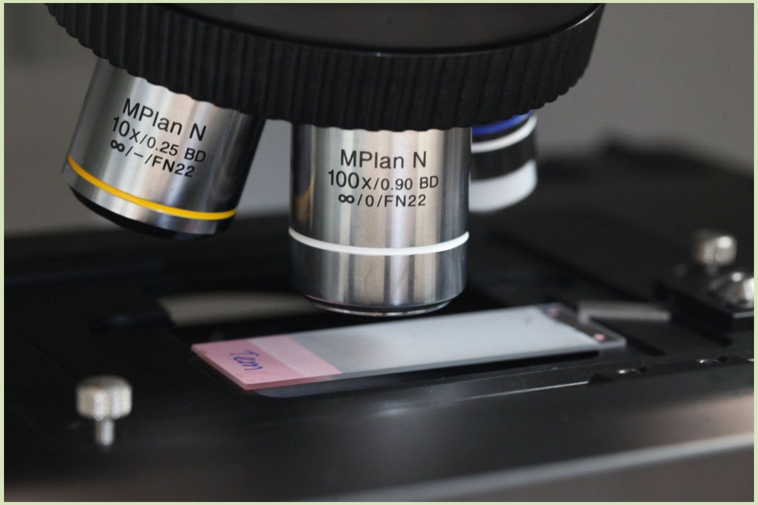
- „Point and Shoot“: manual selection of measurement spot on the samples (software, or hardware joystick)
- Point map: selection of measurements points on visual picture or overall sample image mosaic – number of points is not limited
- Linear map: line of measurement points – microlayers, cuts, linear section of sample
- Area map 2D: area grid of measurement points – 2D imaging of samples
- Depth profiling: microscope confocality allows depth profiling without any sample preparations. Depth profile resolution usually lower than 2 micrometers
- Combination of depth profiling and area/linear mapping: 3D imaging

**Possibility of great selection of excitation lasers wavelengths, gratings with different range and spectral resolution. Software tracks all parts and optimizing measurement parameters.**

**Choice of excitation in nm, maximum laser power on sample in mW. Polarizer/analyser experiments option etc.**

# Raman spectroscopy of bones

- Usually in literature FT-IR spectroscopy/microscopy option preffered
- Raman spectroscopy / microscopy as complementary method
- Raman has better spatial resolution = better understanding to material homogeneity + small particle detection
- „Inorganic“ peaks in Raman spectra (vibrational modes) of bone materials mostly present with minimum fluorescence
- Measurement range 6 700 – 50 $cm^{-1}$ (FT-IR cut-off usually around 400 $cm^{-1}$)
- Raman easier for fiber optics coupling, remote/on-site analysis etc.



| Vibration description | Frequency (cm$^{-1}$) |
|---|---|
| $CaCO_3$ / hydroxypatite lattice vibrations | 50 – 280 cm$^{-1}$ |
| $\nu_2(PO_4^{3-})$ | 423 - 433 cm$^{-1}$ (P-O stretching/ O-P-O bending) |
| $\nu_2(PO_4^{3-})$ | 446 - 449 cm$^{-1}$ (O-P-O bending) |
| $\nu_4(PO_4^{3-})$ | 561 – 617 cm$^{-1}$ (O-P-O bending) |
| Proline | 851 – 855 cm$^{-1}$ (benzene ring) |
| Hydroxyproline | 873 - 875 cm$^{-1}$ (benzene ring) |
| $\nu_2(CO_3^{2-})$ | 880 cm$^{-1}$ (C-O stretching) |
| Proline | 917 cm$^{-1}$ |
| $\nu_1(PO_4^{3-})$ | 955 - 962 cm$^{-1}$ (dominant P-O symmetric stretch) |
| $\nu_3(PO_4^{3-})$ | 1023 – 1054 cm$^{-1}$ (P-O asymmetric stretch) |
| $\nu_1(CO_3^{2-})$ | 1065 - 1077 cm$^{-1}$ (B-type substitution / C-O stretching in plane) |
| $\nu_1(CO_3^{2-})$ | 1079 – 1080 cm$^{-1}$ (amorphous $K_2CO_3$) |
| $\nu_1(CO_3^{2-})$ | 1095 - 1105 cm$^{-1}$ (A-type substitution / C-O stretching in plane |
| Amide III | 1230 - 1289 cm$^{-1}$ (C-N-H stretch, bending N-H) |
|  | **Continued on next slide …..** |

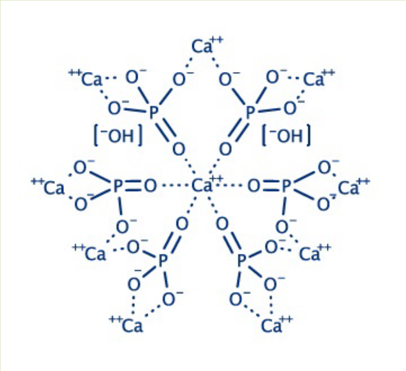
Bone is a material comprising usually a protein component (mostly collagen I) with inorganic matrix of hydroxyapatite ($Ca_{10}(PO_4)_6OH_x$), HA, where hydroxyl (OH) and phosphate ($PO_4$) groups being partly substituted by carbonate – $CO_3$

**Raman vibrations in table are prepared mostly according to literature, not all these vibrations are present in analyzed samples**

**(main factor will be sample preparation and origin)**

| Vibration description | Frequency (cm$^{-1}$) |
|---|---|
| $CH_2$ wag | 1455 – 1465 cm$^{-1}$ |
| Amide II | 1455 – 1465 cm$^{-1}$ (C-N-H stretch) (bending N–H) |
| Tyrosine Y8a | 1610 – 1620 cm$^{-1}$ (resonance-enhanced side-chain vibrations) |
| Amide I | 1655 – 1675 cm$^{-1}$ (C=O stretch) |
| ν(CH) | 2820 – 3035 cm$^{-1}$ (hydrocarbons?) |
| ν (OH) | 3570 – 3576 cm$^{-1}$ |
| **unknown** | 4367, 4397, 4500 cm$^{-1}$ |

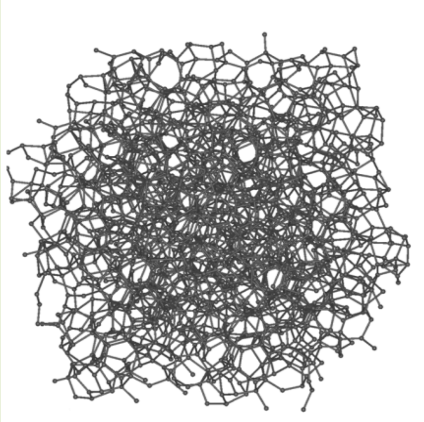
Hydroxyapatite HA:
$(Ca_{10}(PO_4)_6OH_x)$



**Both samples are not perfectly pure, there are black particles visible with microscope, ID is carbon (form of „ash“), with amorphous carbon Raman peaks:**

| Vibration description | Frequency (cm$^{-1}$) |
|---|---|
| D-band | 1320 – 1360 cm$^{-1}$ |
| G-band | 1500 – 1600 cm$^{-1}$ |

Carbon

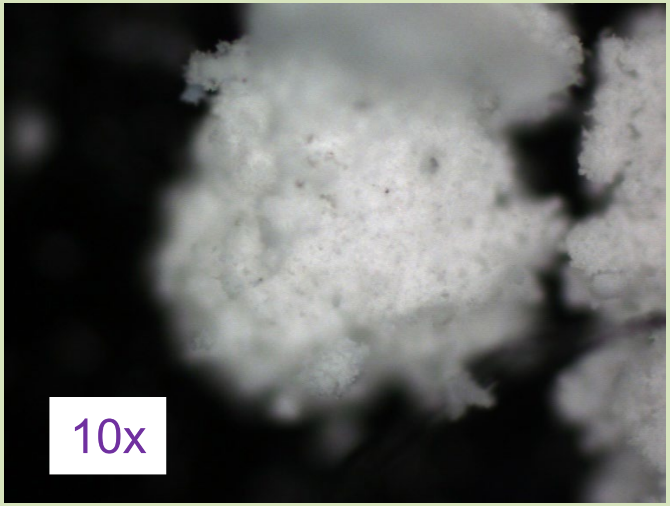


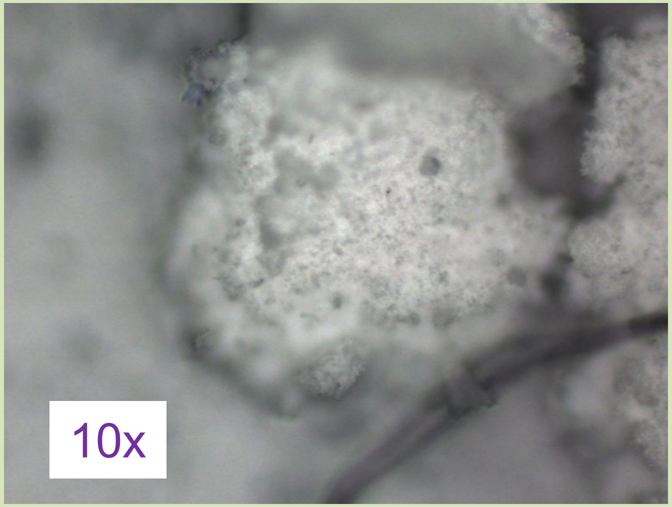
# Raman spectroscopy of bones: theory

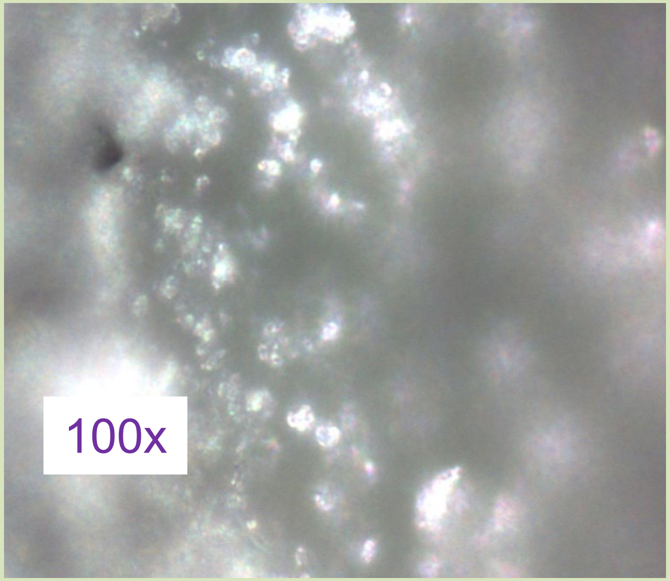
## Notes from literature

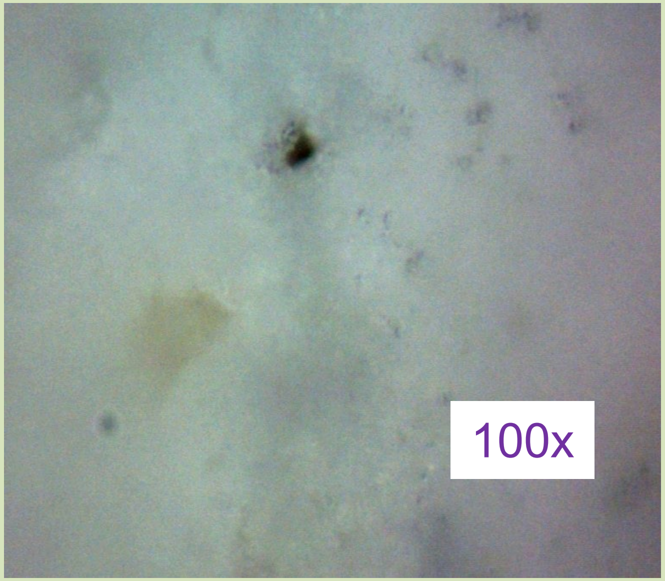
- Two main types of carbonate ($CO_3$) substitution that occur in hydroxyapatite:
  - A-type: when $CO_3$ replaces OH groups
  - B-type. when $CO_3$ replaces $PO_4$ in the apatite structure
  - If these substitutions take place concurrently, an AB-type substitution occurs.
- Broadening of the phosphate symmetric stretching ($v_1$ $PO_4^{3-}$ 955 - 962 $cm^{-1}$) for decreasing temperatures reflects a higher disorder of the bioapatite lattice, associated with a larger amount of $CO_3^{2-}$ ions (substituting either $OH^-$ or $PO_4^{3-}$) in hydroxyapatite
- Symmetric stretching vibration $v_1(PO_4^{3-})$ has a high sensitivity to the mineral encirclement: the frequency and form of this line depends on the local surroundings and change as a result of substitution of anionic groups and changes in the degree of crystallinity.
- Substituted carbonate-apatite B-type phosphate $v_1$ line appears in the range of 955–959 $cm^{-1}$. In a crystalline unsubstituted hydroxyapatite line $v_1$ shifted to 962–965 $cm^{-1}$
  Frequency line 945–950 $cm^{-1}$ indicates the presence of a disordered lattice phosphate apatite.
- Phosphate band $v_1$ is a superposition of three components; it usually has an asymmetric shape due to the disordered vibrations contribution of phosphate and unsubstituted hydroxyapatite.
- Variation in the bone’s carbonate content is closely related to alterations in the growth morphology and crystallite size, that are known to take place upon temperature changes – a higher atomic disorder corresponding to smaller crystal dimensions, lattice becoming progressively more ordered with increasing temperatures (upon $CO_3^{2-}$ loss)
- Etc….

# Samples - description







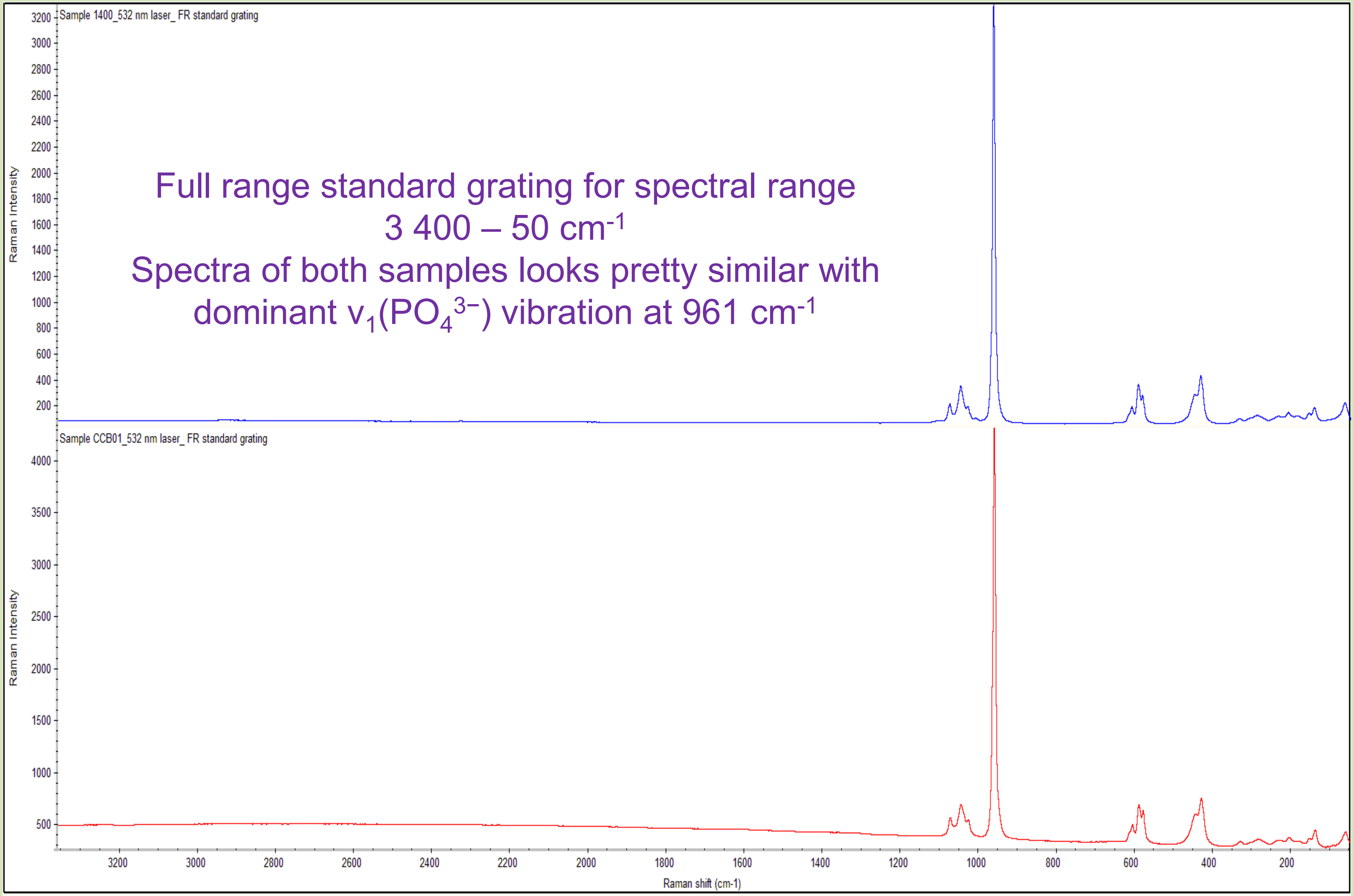


Both samples are homogenic white powders with randomly scattered black dots

Raman microscope allows classic microscopy with glass objectives, polarization and mapping options

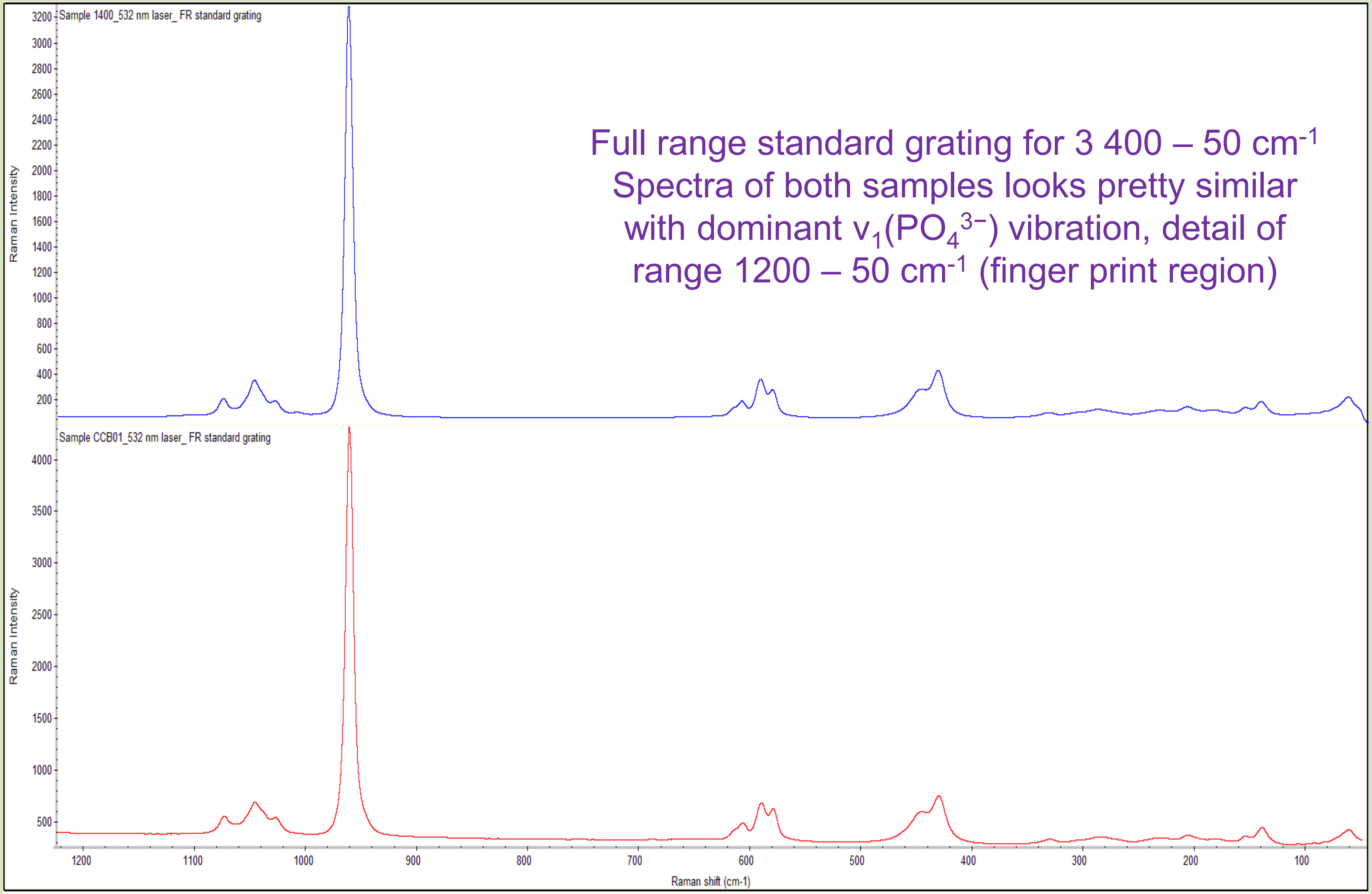
Sample volume needed for Raman analysis is very low, just couple of 25 micron particles (even one particle is enough)

# Raman spectra of samples



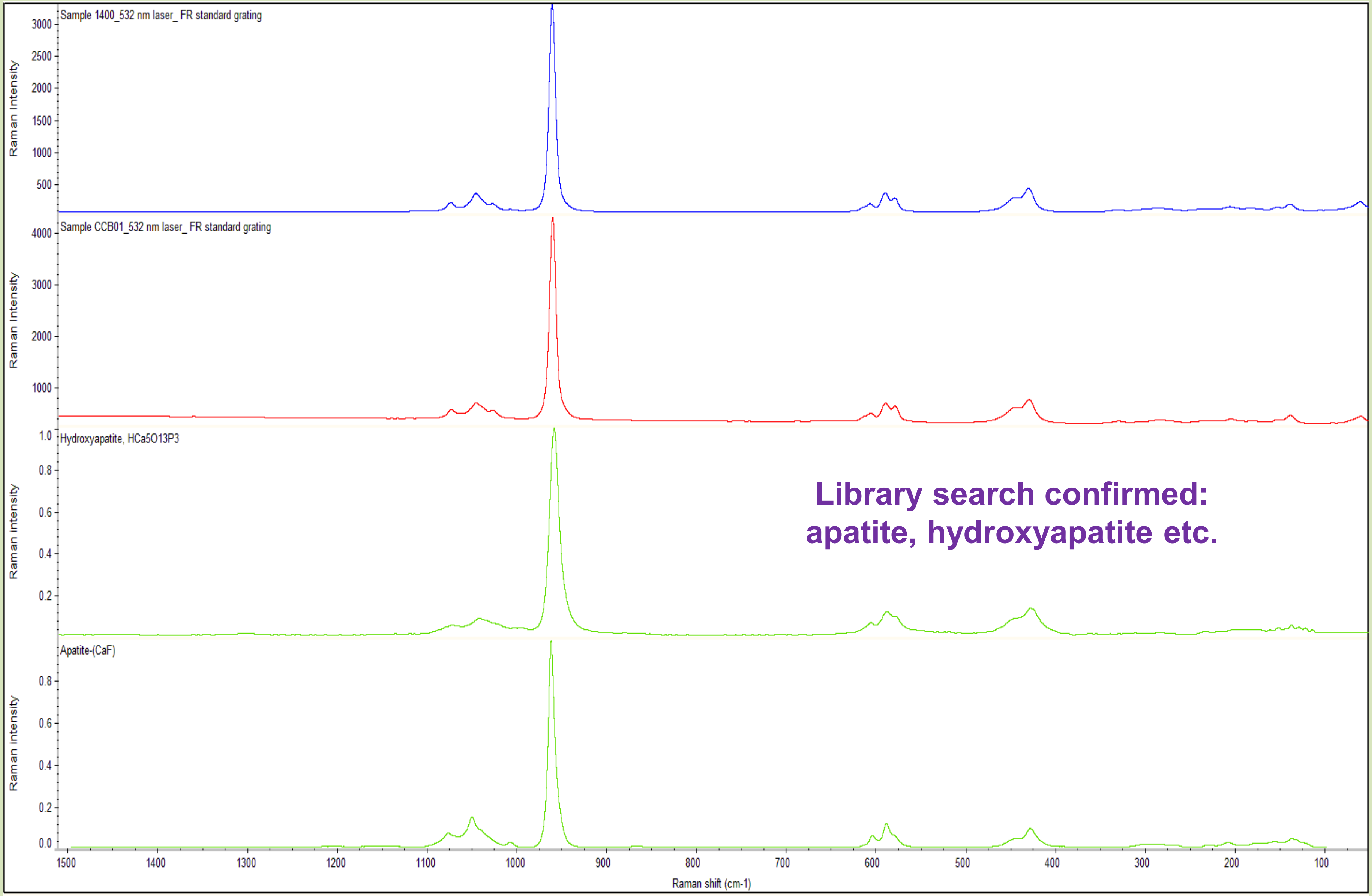
Full range standard grating for spectral range
3 400 – 50 cm$^{-1}$
Spectra of both samples looks pretty similar with
dominant $v_1(PO_4^{3-})$ vibration at 961 cm$^{-1}$

# Raman spectra of samples



Full range standard grating for 3 400 – 50 cm$^{-1}$
Spectra of both samples looks pretty similar with dominant $v_1(PO_4^{3-})$ vibration, detail of range 1200 – 50 cm$^{-1}$ (finger print region)

# Raman spectra of samples



**Library search confirmed: apatite, hydroxyapatite etc.**

# Raman spectra of samples

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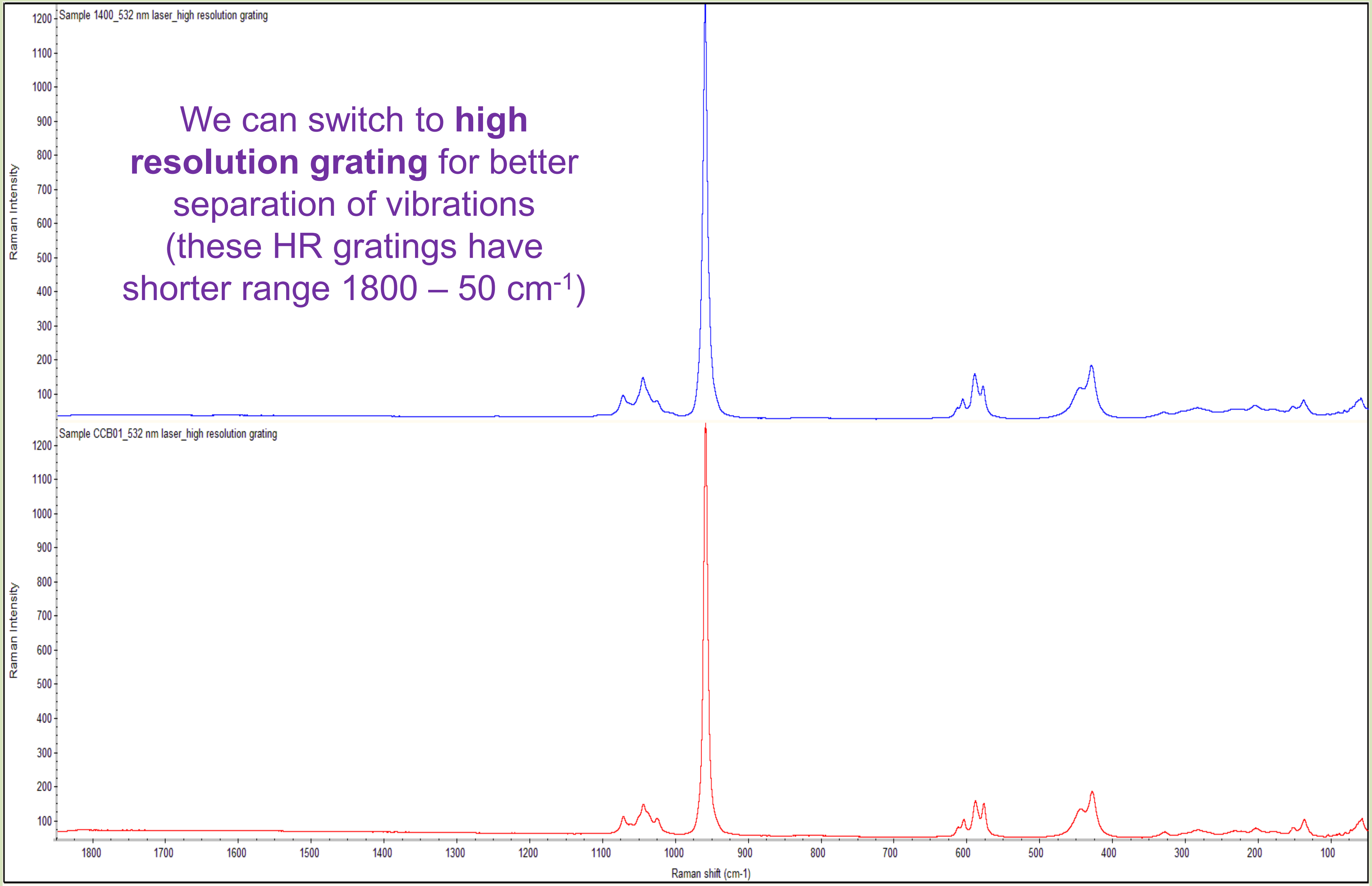
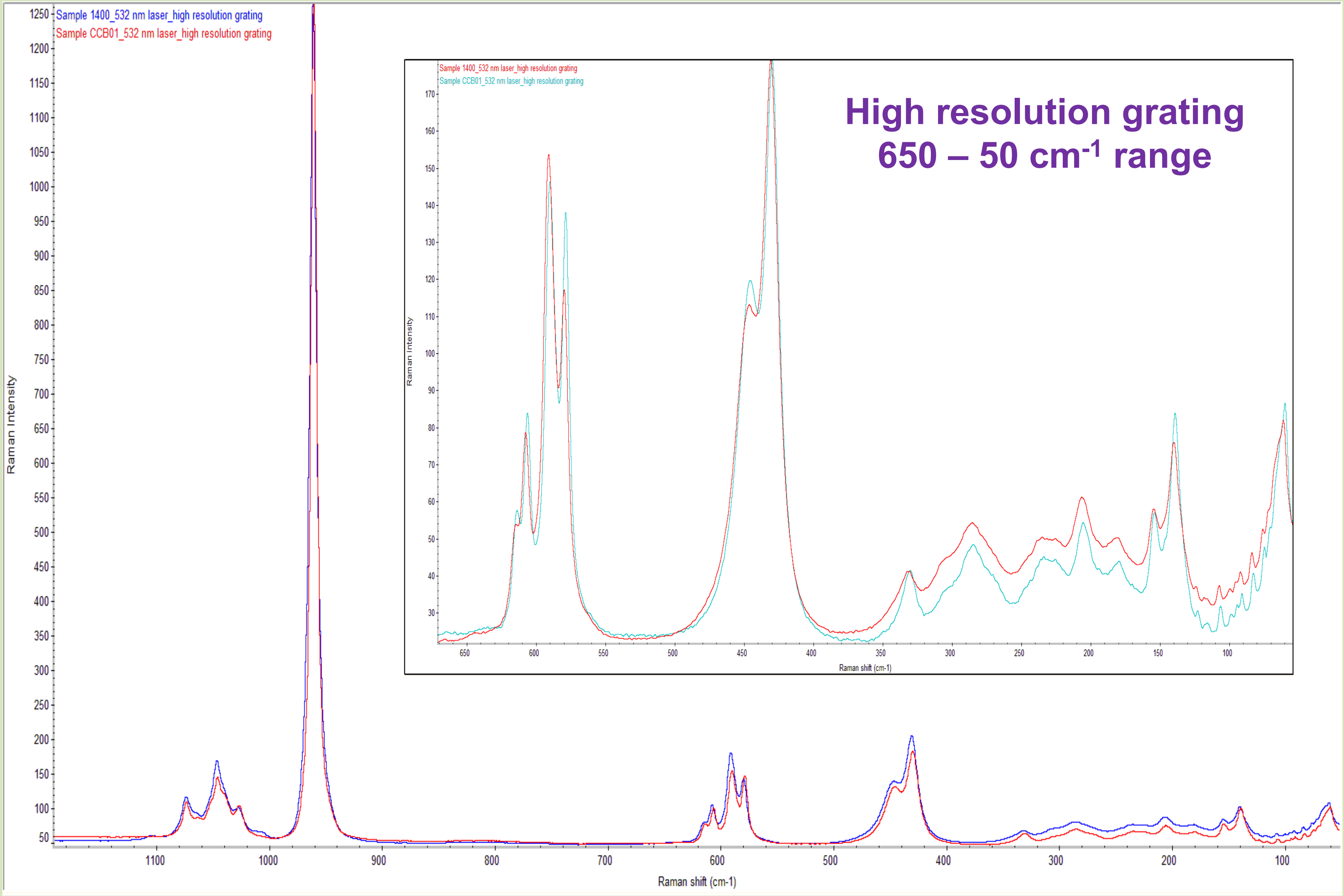


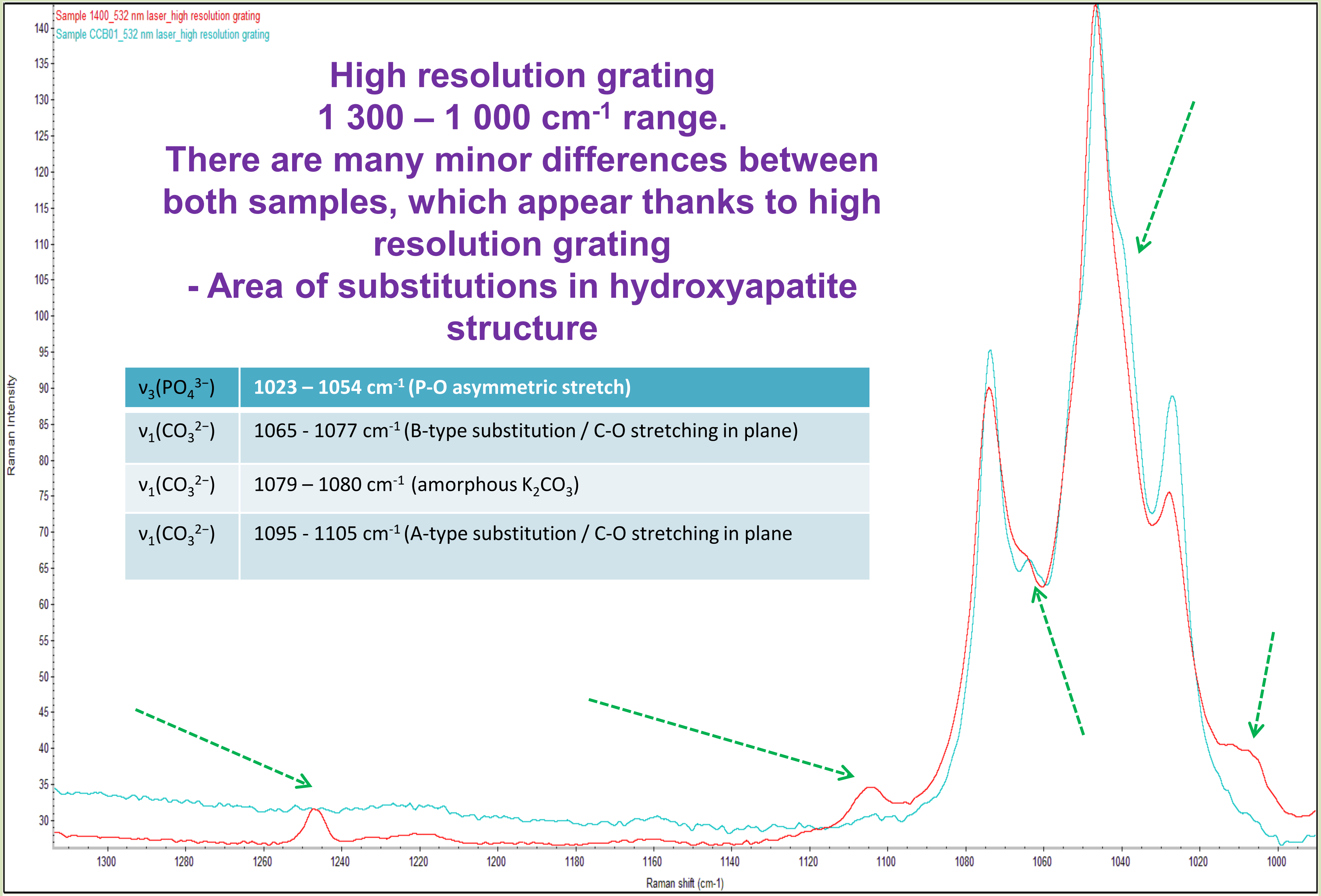
We can switch to **high resolution grating** for better separation of vibrations (these HR gratings have shorter range 1800 – 50 $cm^{-1}$)

# Raman spectra of samples



High resolution grating
650 – 50 cm$^{-1}$ range

# Raman spectra of samples



**High resolution grating**
**1 300 – 1 000 cm$^{-1}$ range.**
**There are many minor differences between both samples, which appear thanks to high resolution grating**
**- Area of substitutions in hydroxyapatite structure**

| $v_3(\mathrm{PO_4}^{3-})$ | **1023 – 1054 cm$^{-1}$ (P-O asymmetric stretch)** |
|---|---|
| $v_1(\mathrm{CO_3}^{2-})$ | 1065 - 1077 cm$^{-1}$ (B-type substitution / C-O stretching in plane) |
| $v_1(\mathrm{CO_3}^{2-})$ | 1079 – 1080 cm$^{-1}$ (amorphous $K_2CO_3$) |
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# Raman spectra of samples

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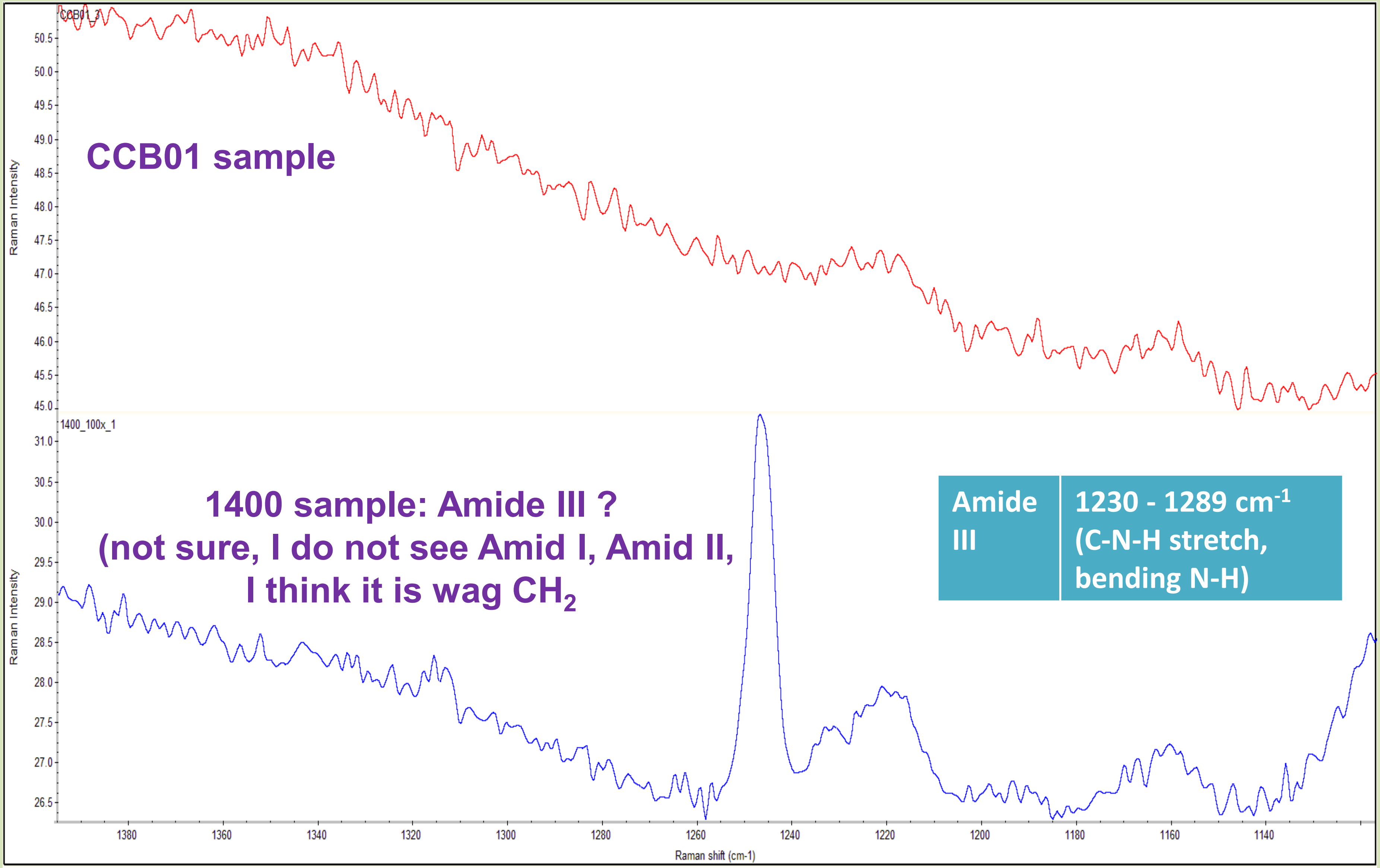
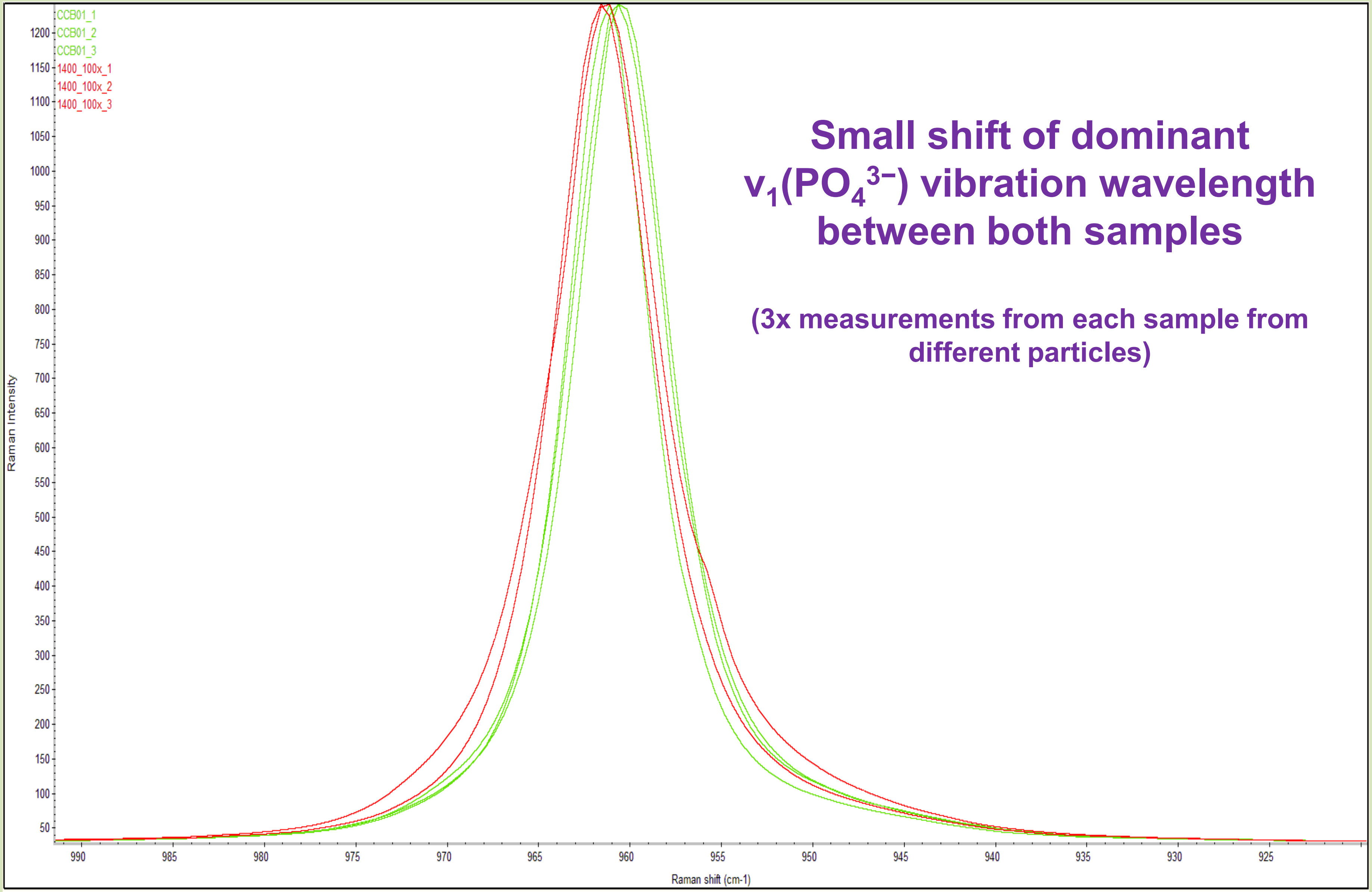


**CCB01 sample**

**1400 sample: Amide III ?**
**(not sure, I do not see Amid I, Amid II,**
**I think it is wag $CH_2$**

| | |
|---|---|
| Amide III | 1230 - 1289 $cm^{-1}$ (C-N-H stretch, bending N-H) |

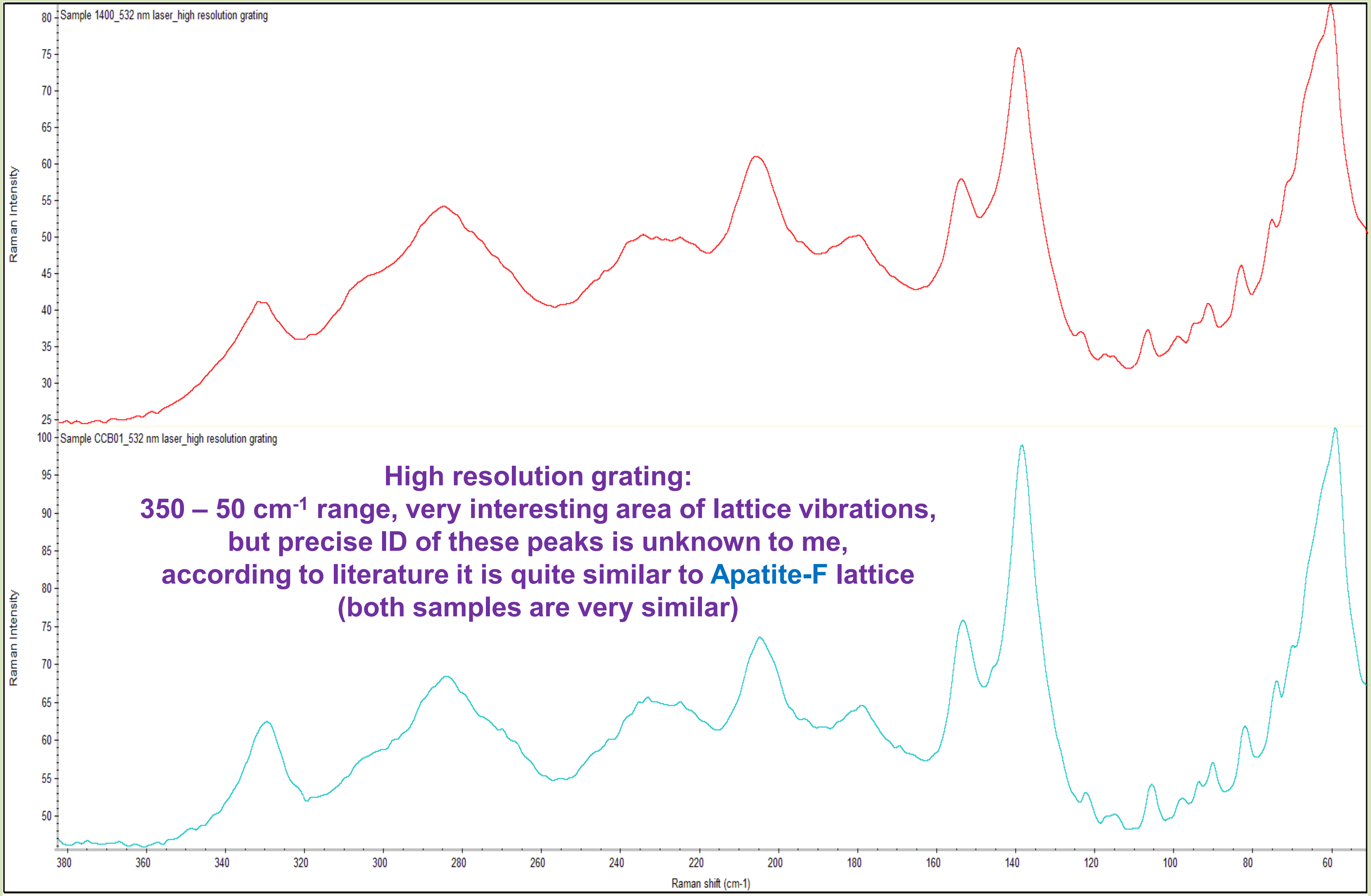
# Raman spectra of samples



**Small shift of dominant $v_1(PO_4^{3-})$ vibration wavelength between both samples**

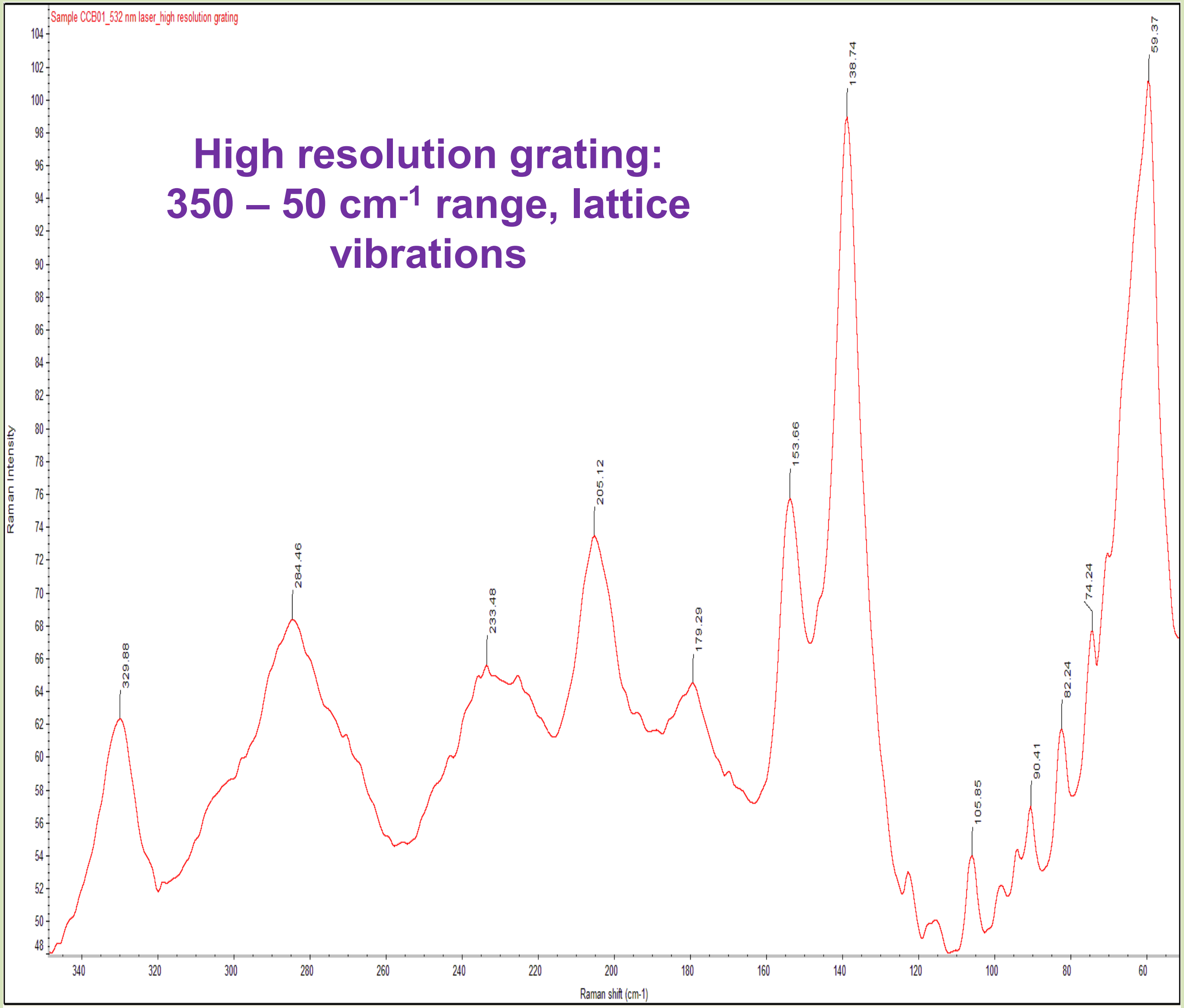
**(3x measurements from each sample from different particles)**

# Raman spectra of samples



**High resolution grating:**
**350 – 50 $\text{cm}^{-1}$ range, very interesting area of lattice vibrations,**
**but precise ID of these peaks is unknown to me,**
**according to literature it is quite similar to Apatite-F lattice**
**(both samples are very similar)**

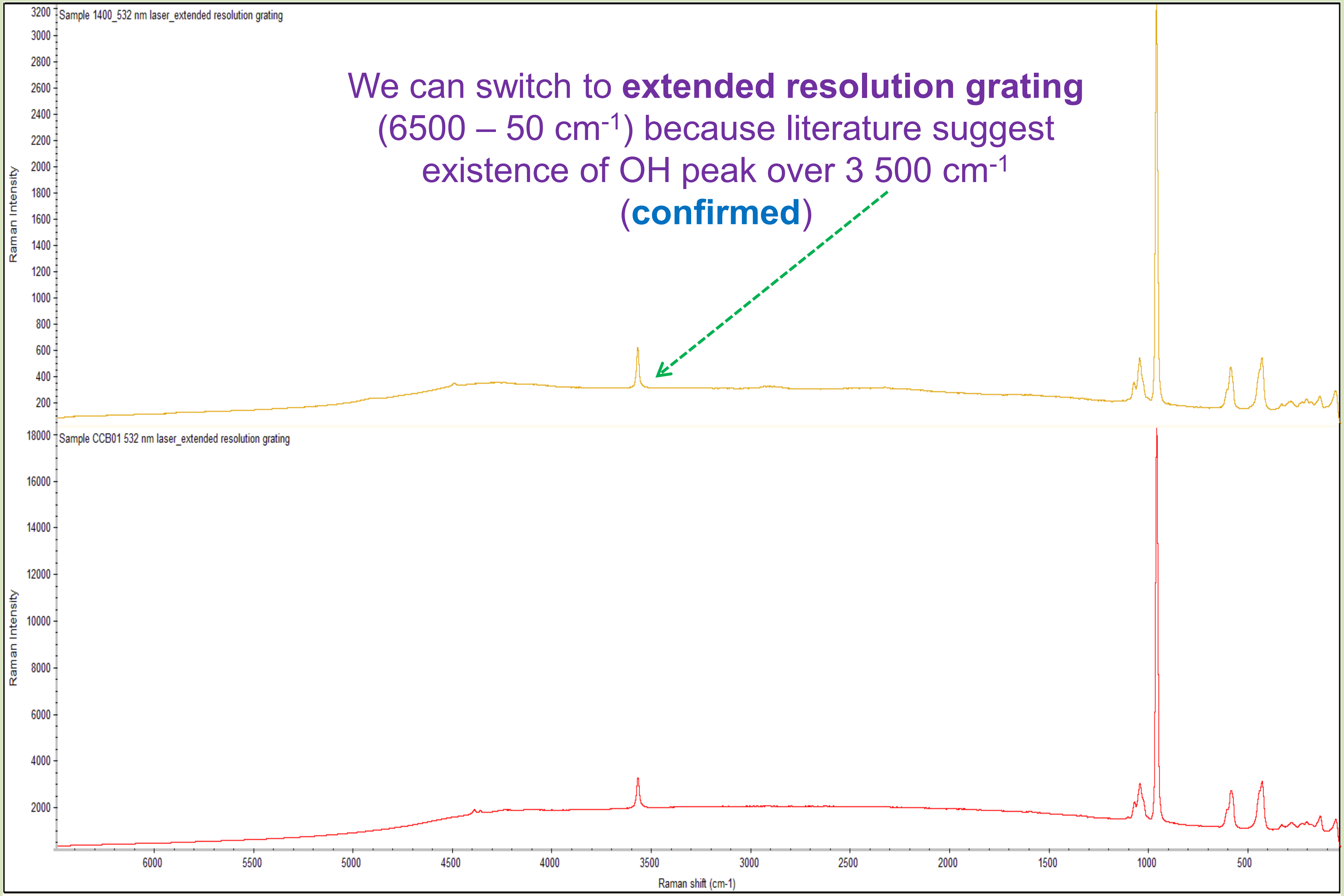
# Raman spectra of samples



**High resolution grating: 350 – 50 cm$^{-1}$ range, lattice vibrations**

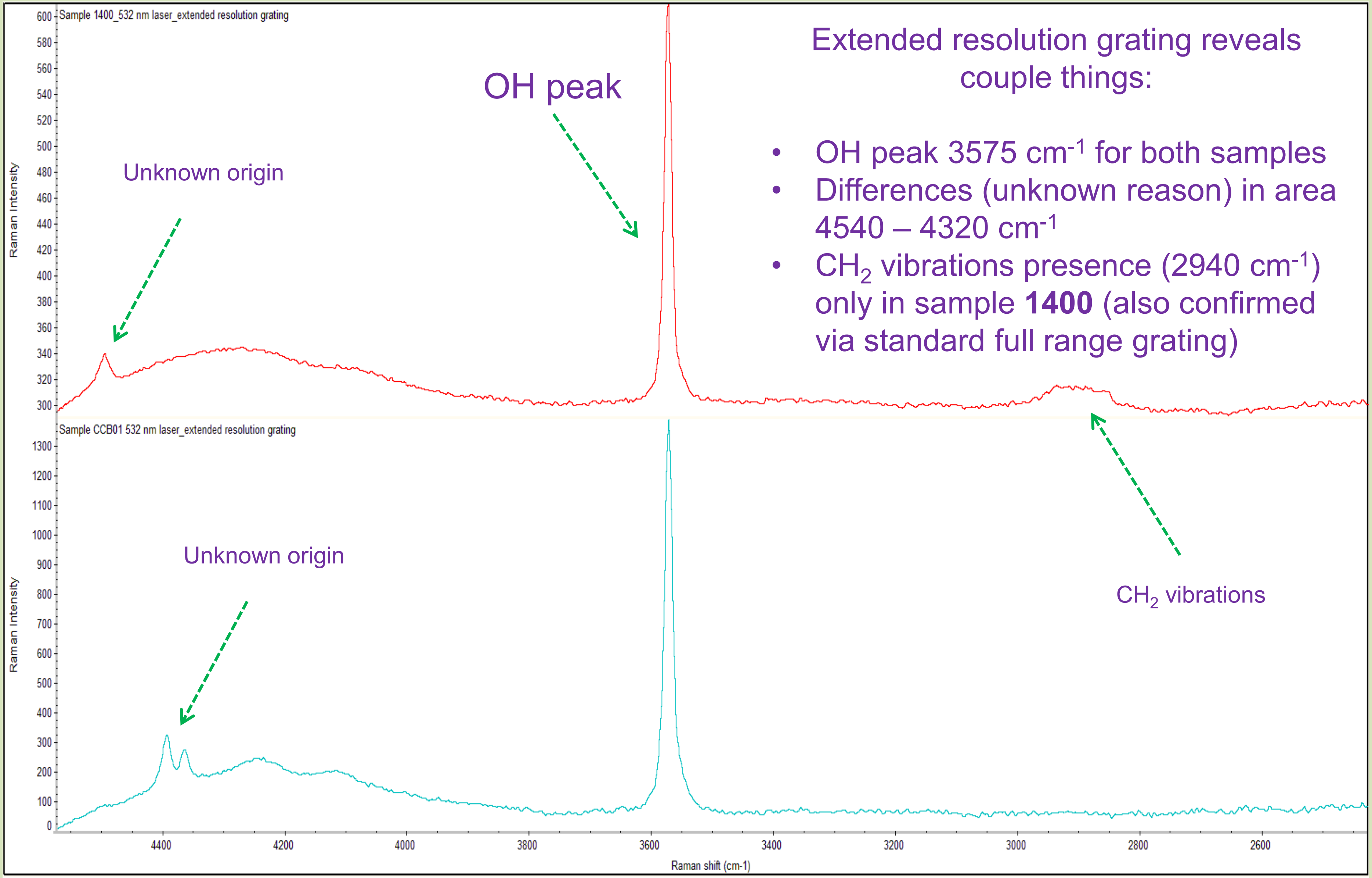
| X Position (lattice) |
|---|
| 59.4 |
| 74.2 |
| 82.2 |
| 90.4 |
| 105.9 |
| 138.7 |
| 153.7 |
| 179.3 |
| 205.1 |
| 233.5 |
| 284.5 |
| 329.9 |

# Raman spectra of samples



We can switch to **extended resolution grating** (6500 – 50 $cm^{-1}$) because literature suggest existence of OH peak over 3 500 $cm^{-1}$ (**confirmed**)

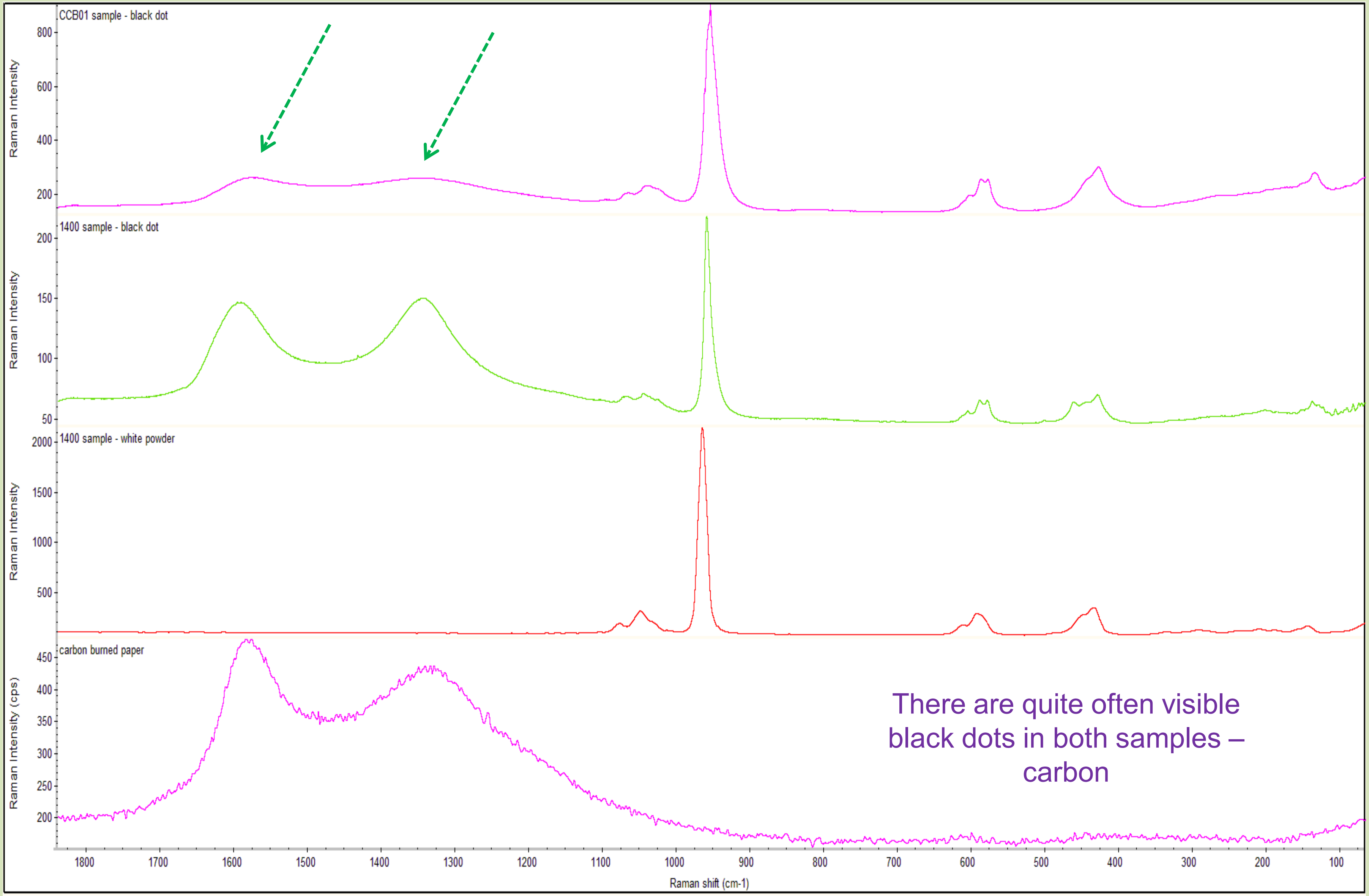
# Raman spectra of samples



Extended resolution grating reveals couple things:

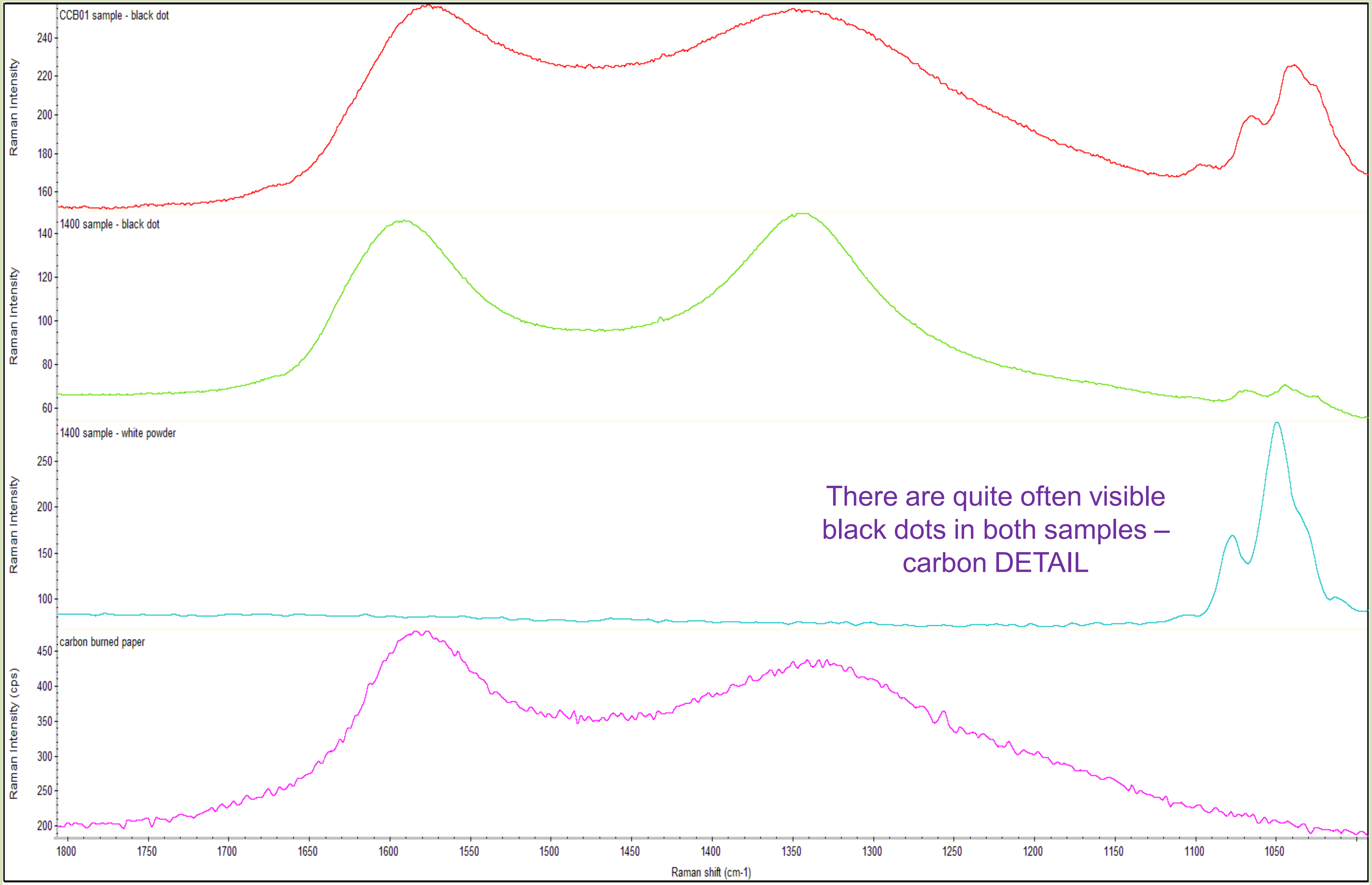
- OH peak 3575 $cm^{-1}$ for both samples
- Differences (unknown reason) in area 4540 – 4320 $cm^{-1}$
- $CH_2$ vibrations presence (2940 $cm^{-1}$) only in sample **1400** (also confirmed via standard full range grating)

# Raman spectra of samples



There are quite often visible black dots in both samples – carbon

# Raman spectra of samples



There are quite often visible black dots in both samples – carbon DETAIL

# Raman spectra of samples: Summary and future research ideas

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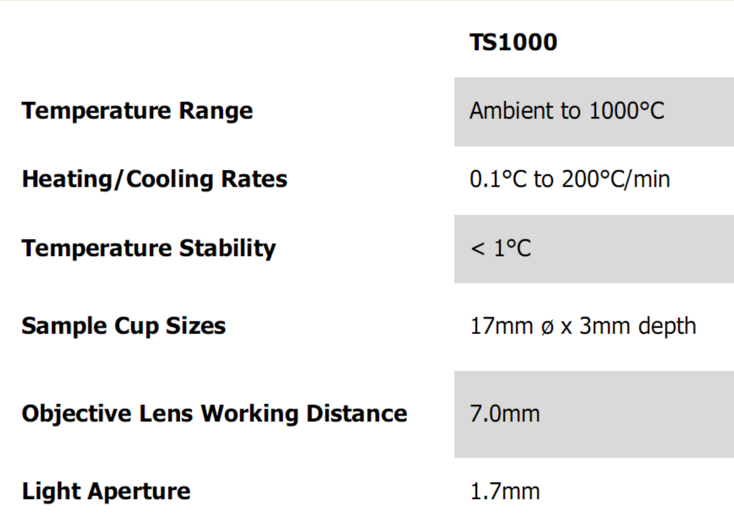
- Raman features of both samples are very similar, nevertheless there are differences, f.e. sample 1400 includes hydrocarbons $CH_2$ vibrations and unknown features over 4320 $cm^{-1}$ range
- More samples Raman measurements required for better understanding of spectral features, maybe collect Raman spectra during whole sample preparation process / processes.
- Measurement of hydroxyapatite and apatite standards with high resolution gratings, again for better understanding of spectra and their differences
- More research according to notes on slide 11 (from literature)
- Temperature dependency experiments with high resolution grating: Linkam environmental chamber(s):



| | **TS1000** |
|---|---|
| **Temperature Range** | Ambient to 1000°C |
| **Heating/Cooling Rates** | 0.1°C to 200°C/min |
| **Temperature Stability** | < 1°C |
| **Sample Cup Sizes** | 17mm ø x 3mm depth |
| **Objective Lens Working Distance** | 7.0mm |
| **Light Aperture** | 1.7mm |

https://www.linkam.co.uk/ts1000