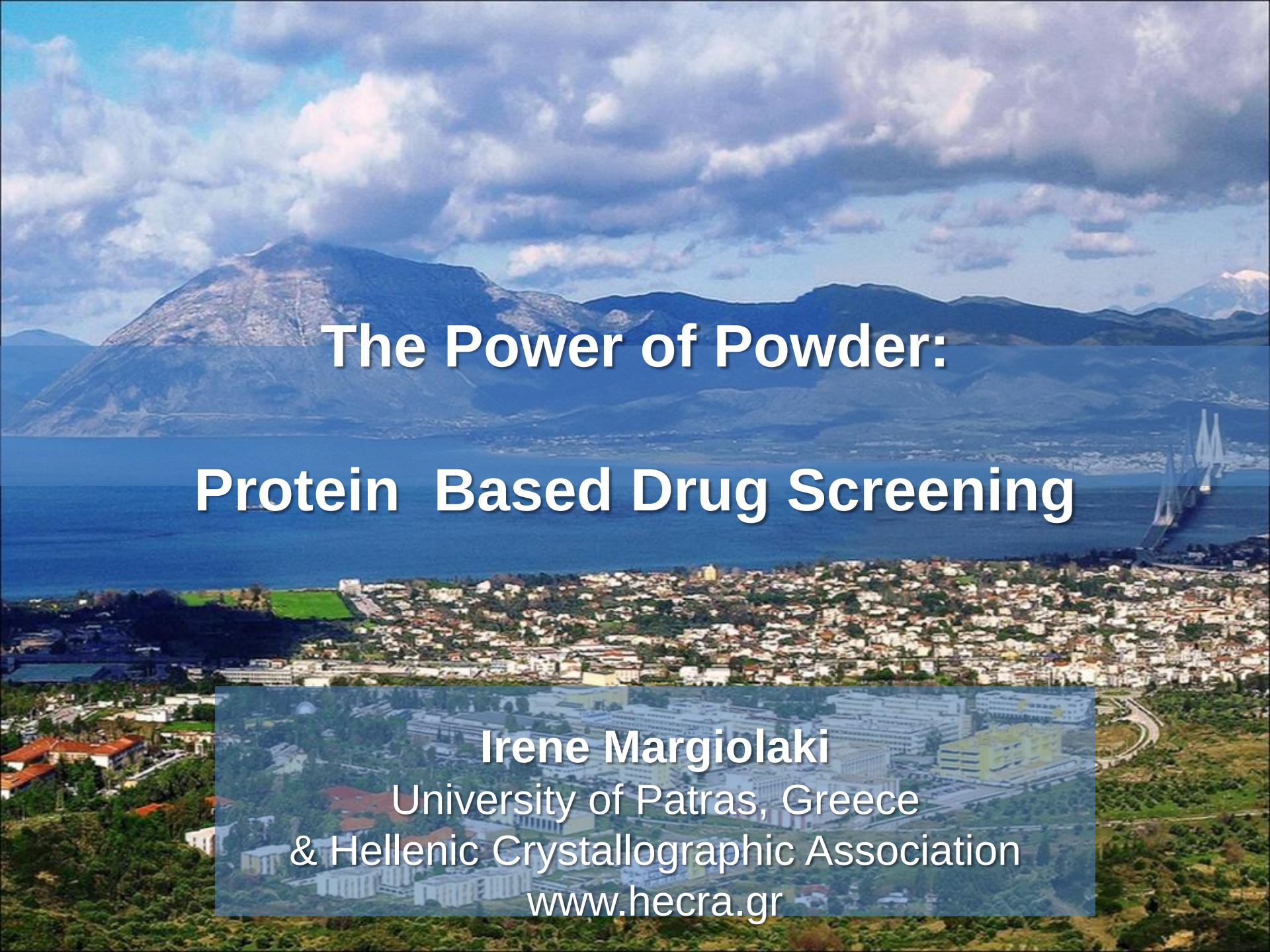


The Power of Powder:

Protein Based Drug Screening

Irene Margiolaki
University of Patras, Greece
& Hellenic Crystallographic Association
www.hecra.gr

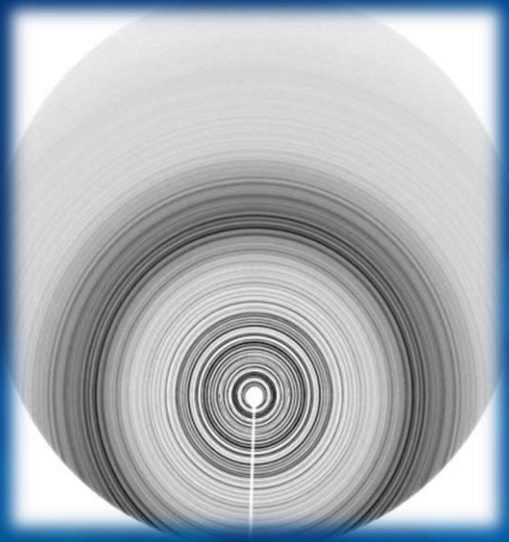
This document was presented at PPXRD - Pharmaceutical Powder X-ray Diffraction Symposium

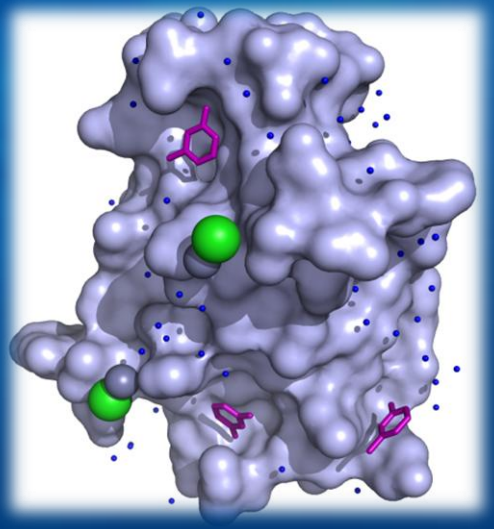
Sponsored by The International Centre for Diffraction Data

PPXRD Website – www.icdd.com/ppxrd

ICDD Website - www.icdd.com

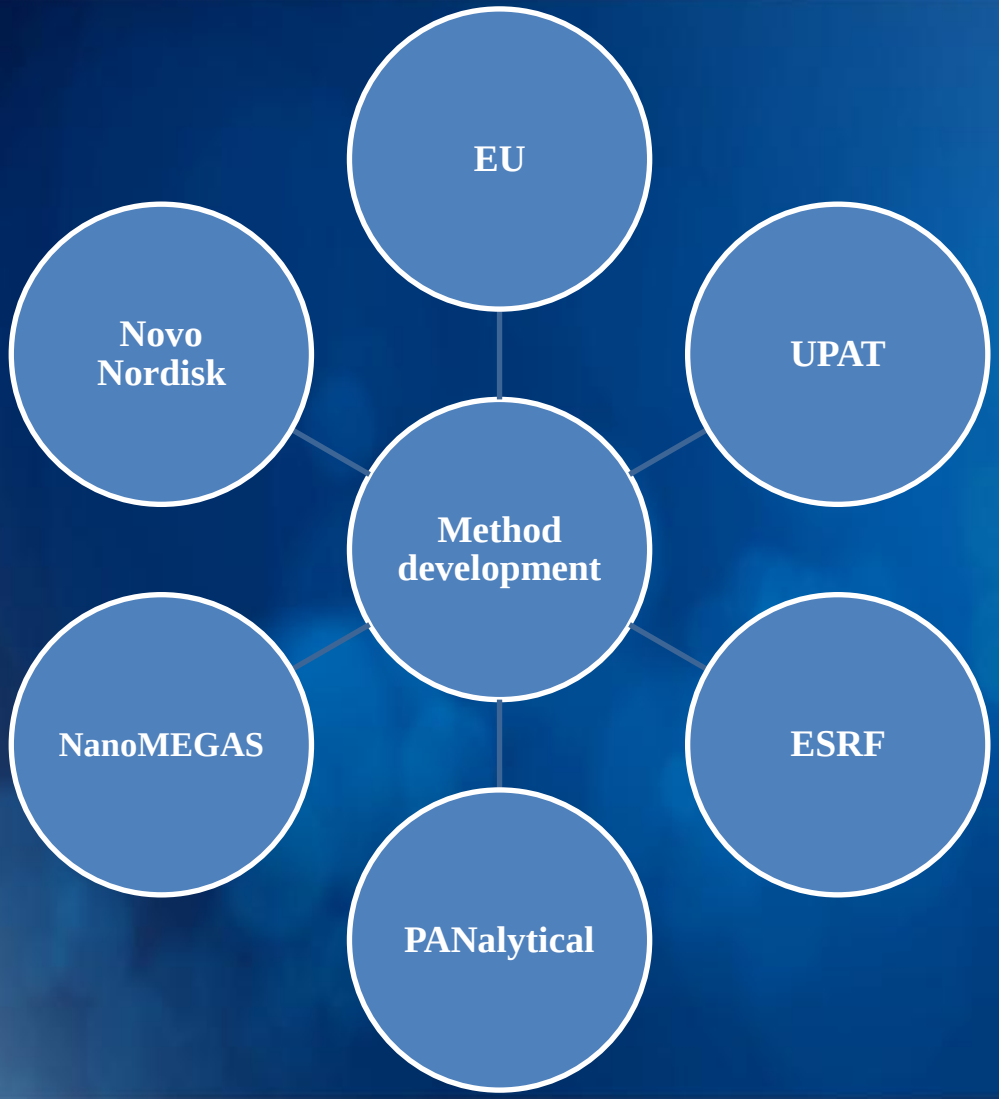
STRATEGY





WHO?

NETWORK


EU
Novo Nordisk
UPAT
Method development
NanoMEGAS
ESRF
PANalytical

X-RAY CRYSTALLOGRAPHY / STRUCTURAL BIOLOGY GROUP, DEPARTMENT OF BIOLOGY, UPAT

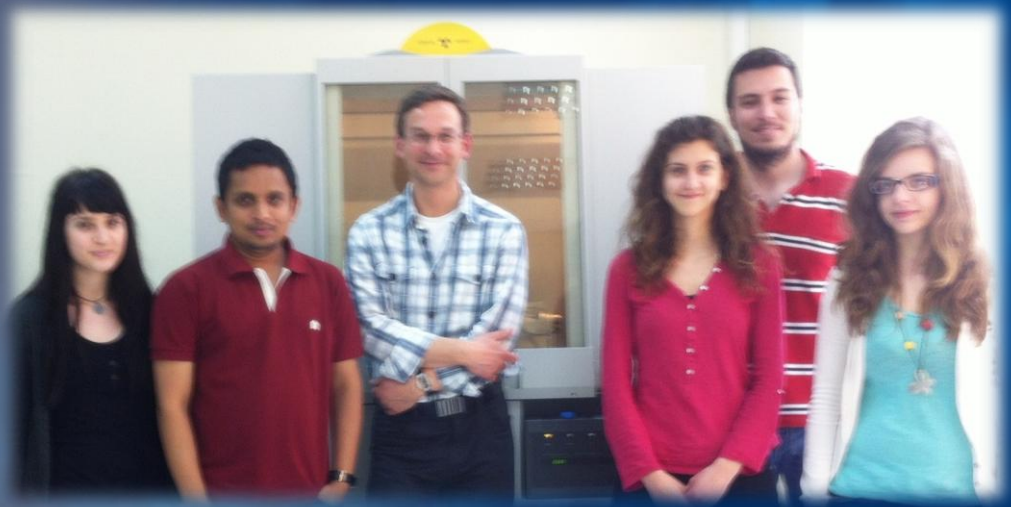
Our Team:

Fotini Karavassili (PhD)
Alexandros Valmas (PhD)
Stavroula Fili (M.Sc.)
Konstantina Magiouf (BSc.)
Danae Lachana (BSc.)
Suzanna Logotheti (BSc.)
Stefanos Saslis (B.Sc.)
Nikos Nikolopoulos (B.Sc.)

European Synchrotron Radiation Facility
ESRF, Grenoble, France

PANalytical

PANalytical, Almelo
Detlef Beckers
Thomas Degen
Celeste Reiss
Stjepan Prugovecki
Martijn Fransen
Gwilerm Nenert



UPAT, PATRAS
Stavroula Fili,
Partha Das (Former Scientist)
Fotini Karavassili
Alexandros Valmas
Konstantina Magiouf

I. Margiolaki & J. P. Wright, Acta Cryst. A - Invited Review Article for Special Issue of IUCR[1]

Reviews

• *"Powder Crystallography on Macromolecules",*
Acta Cryst. A64, 169-180 (2008)
I. Margiolaki & J. P. Wright

• "Macromolecular Powder Diffraction",
Book Chapter for the International Tables of Crystallography-Volume H: Powder Diffraction, In Press
I. Margiolaki

• "Macromolecular Powder Diffraction: Ready for genuine biological problems"
Protein & Peptide Letters, In Press
F. Karavassili & I. Margiolaki

What are the options?

NMR Spectroscopy

No need of crystals

Maximum MW limit

Bieri M, Kwan AH, Mobli M, King GF, Mackay JP, Gooley PR. *Macromolecular NMR spectroscopy for the non-spectroscopist: beyond macromolecular solution structure determination.* **FEBS J. 2011 Mar;278(5):704-15. 2011**

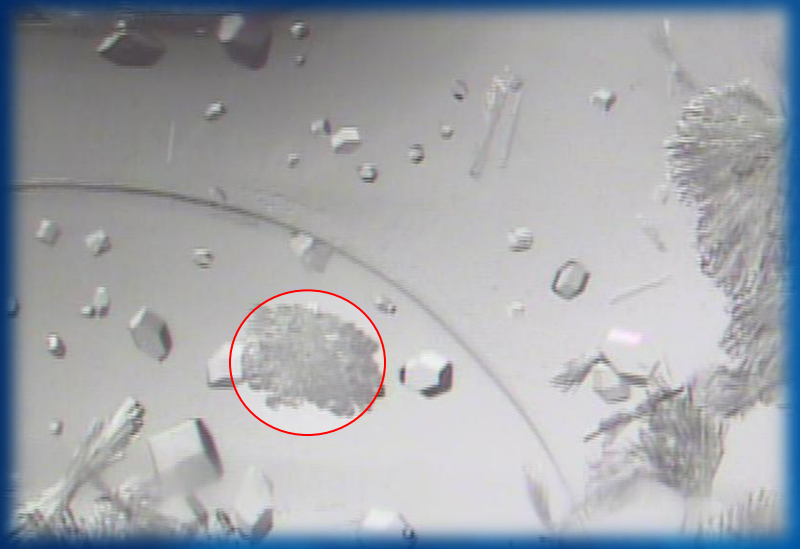
X-ray Single Crystal Diffraction

Crystals of ~ 50 - 100 µm

Crystallization may be problematic

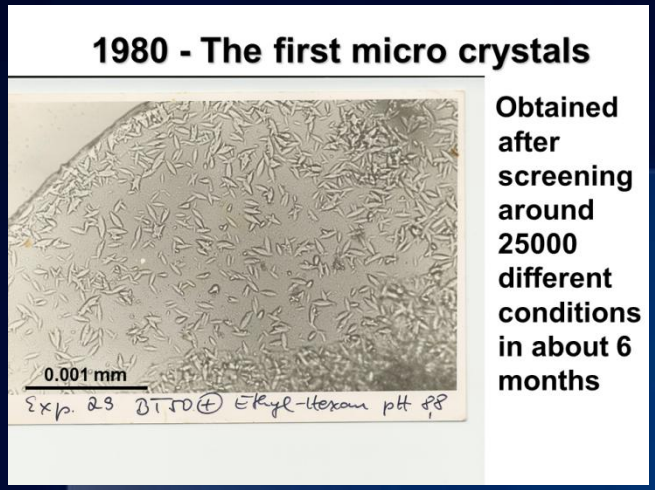
Evans, G., Axford, D., Waterman, D., Owen, R.L. *Macromolecular microcrystallography*. **Crystallography Reviews. 17, 105-142. 2011**

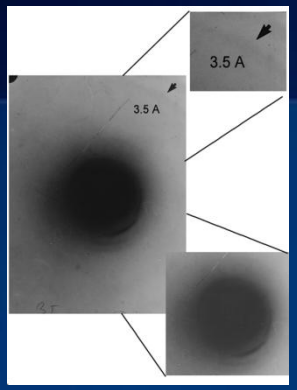
Protein Crystalline Precipitates



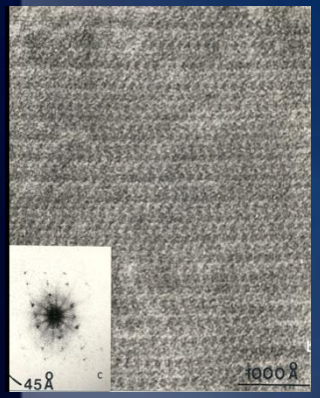
Can we extract any structural information from a protein polycrystalline sample?

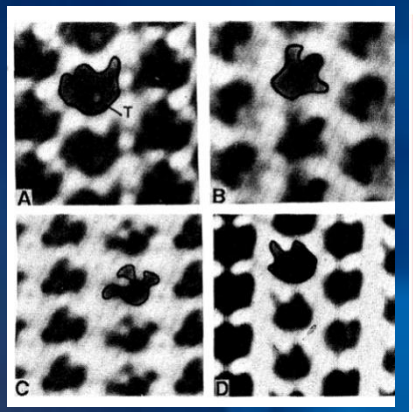
Preliminary powder data in the 80s

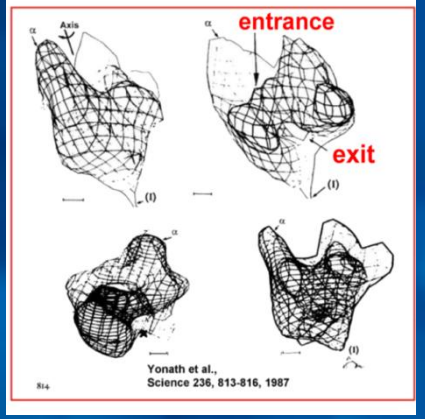




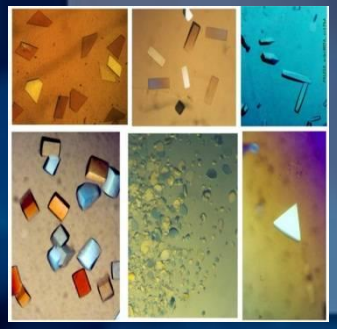
1980-1984: Micro-crystals diffracting to 30-40 Å (powder patterns to 3.5 Å)

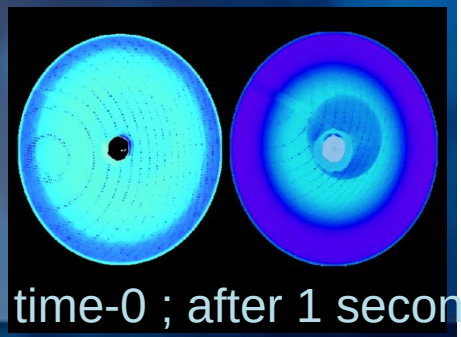


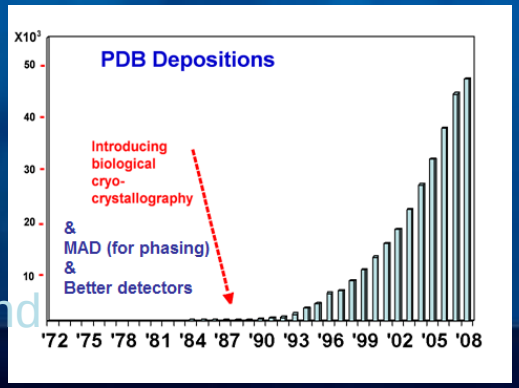




1985-1987
3 dimensional image reconstruction by EM at 25 Å showing the internal ribosomal tunnel



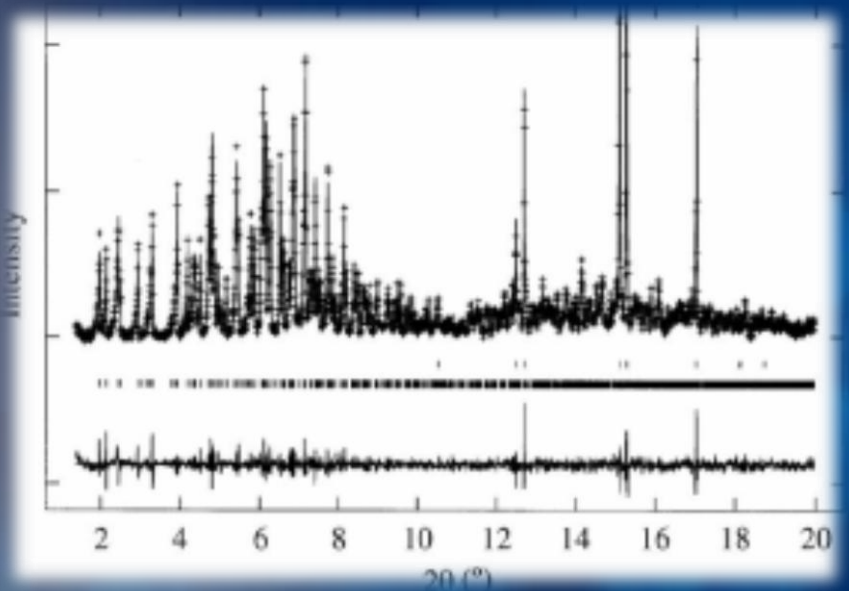


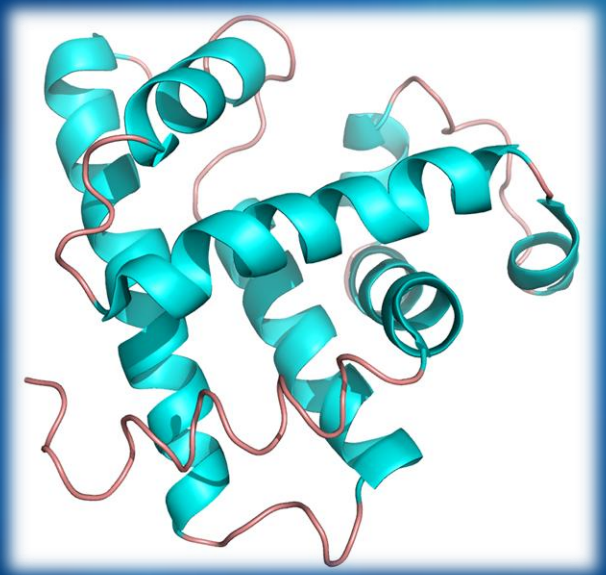


1986: excellent diffraction, but sever X-ray damage: Introducing cryo bio- crystallography

Courtesy: Ada Yonath

The first protein structure refinement using powder diffraction data: Whale Metmyoglobin





R. B. Von Dreele
J. Appl. Cryst. (1999). 32, 1084-1089

Protein Crystalline Precipitates

GOOD VS BAD PRECIPITATES

UPPSALA UNIVERSITET

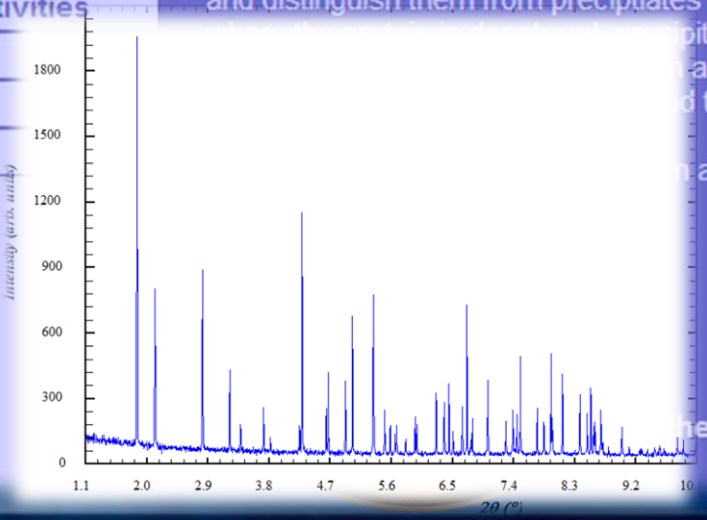
Protein Crystallization

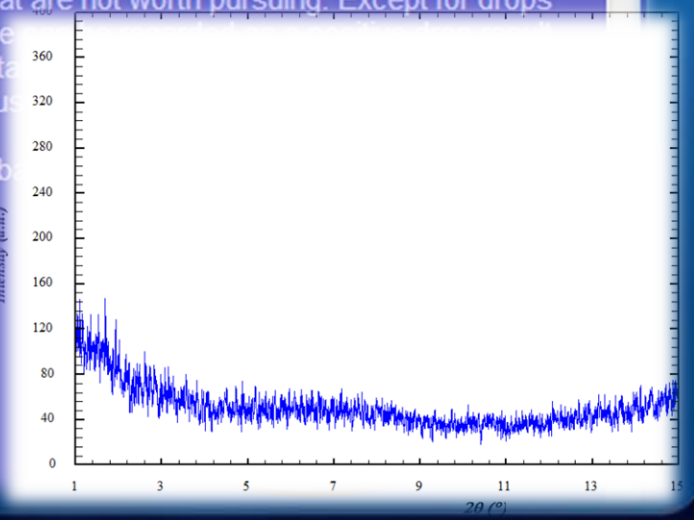
- Home
- Calendar
- Publications
- Presentations
- Teaching
- Crystallization Tutorials
- Professional Activities
- Links
- Me
- Contact

Tutorial 2.

Precipitates

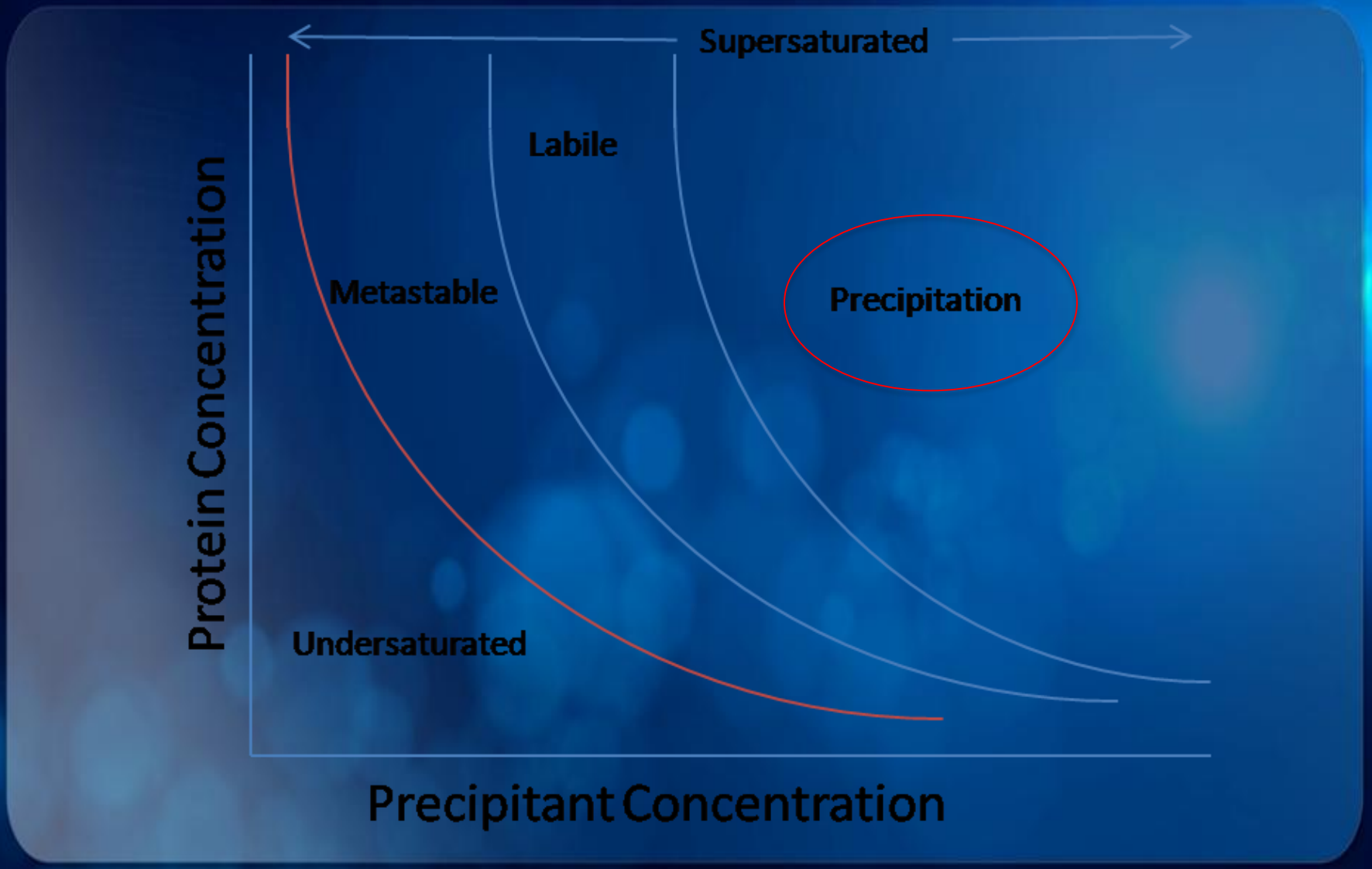
One of the most difficult things for beginners is to recognize promising precipitates and distinguish them from precipitates that are not worth pursuing. Except for drops





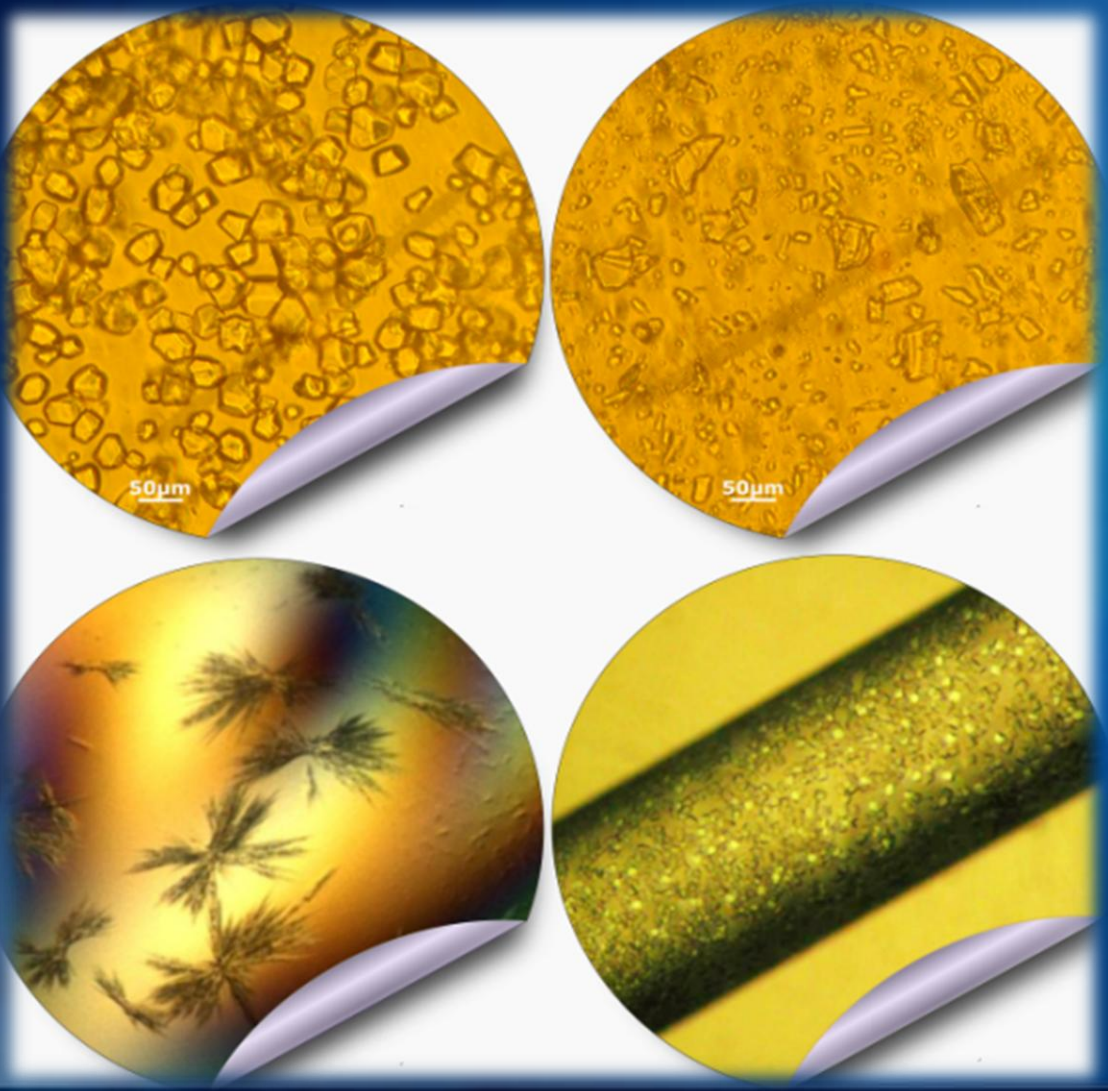
Courtesy: Prof. Terese Bergfors, http://xray.bmc.uu.se/~terese/tutorial2.html

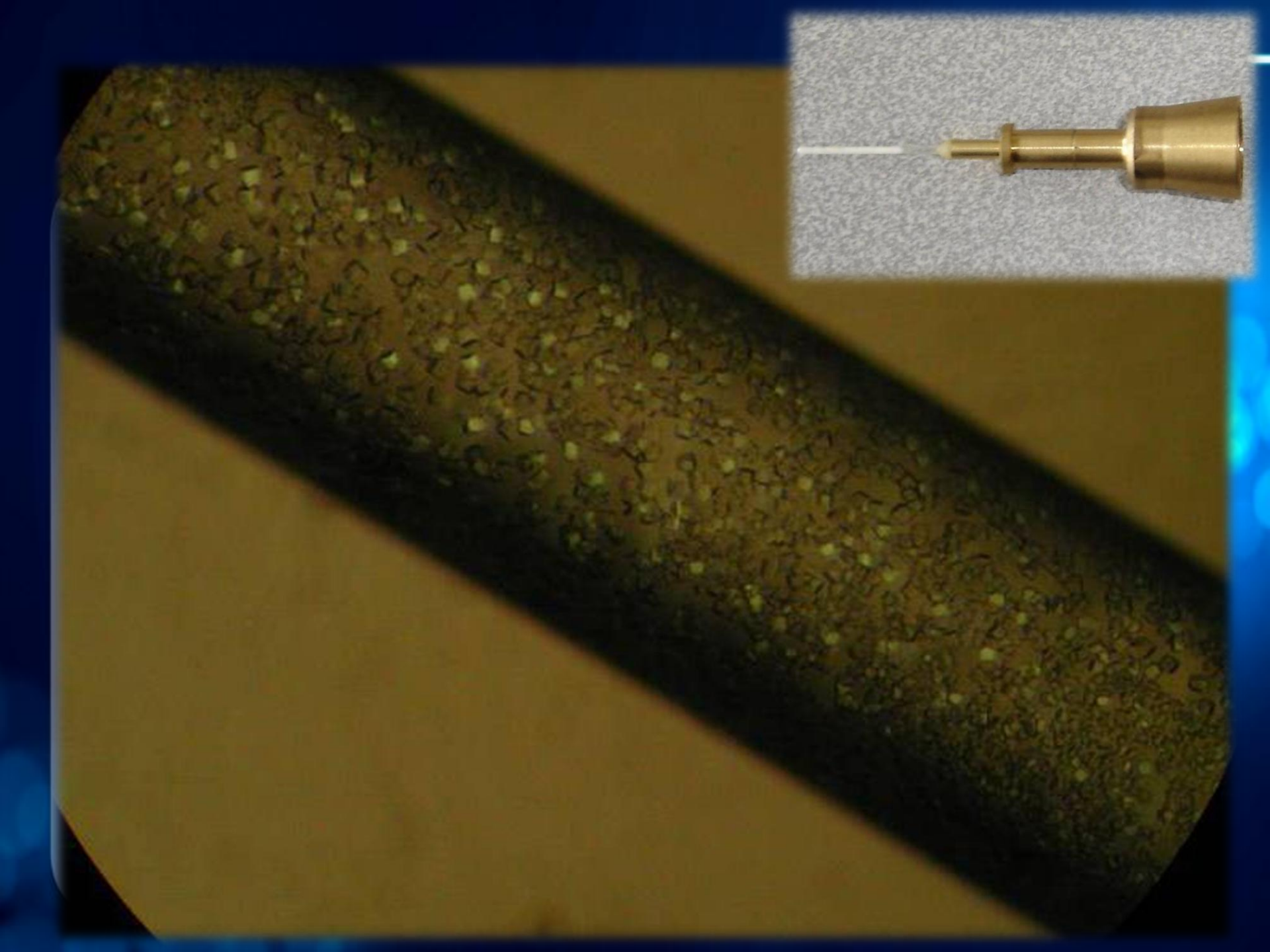
Protein Crystallization & Phase Diagrams



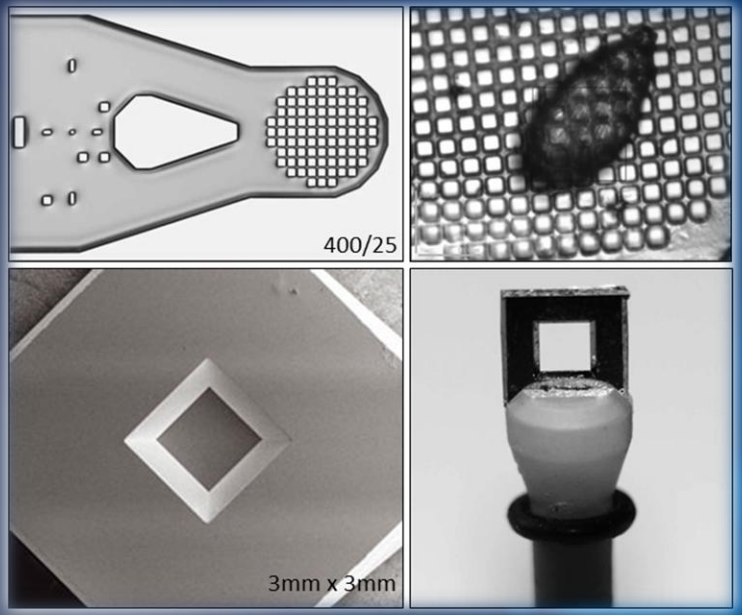
Protein crystallization and phase diagrams: Neer Asherie, Methods 34 (2004) 266–272

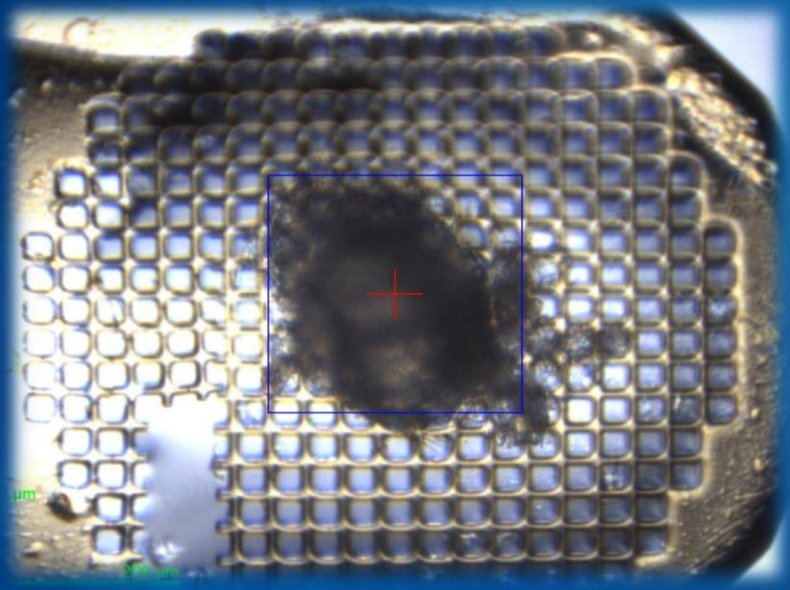
PROTEIN POWDERS



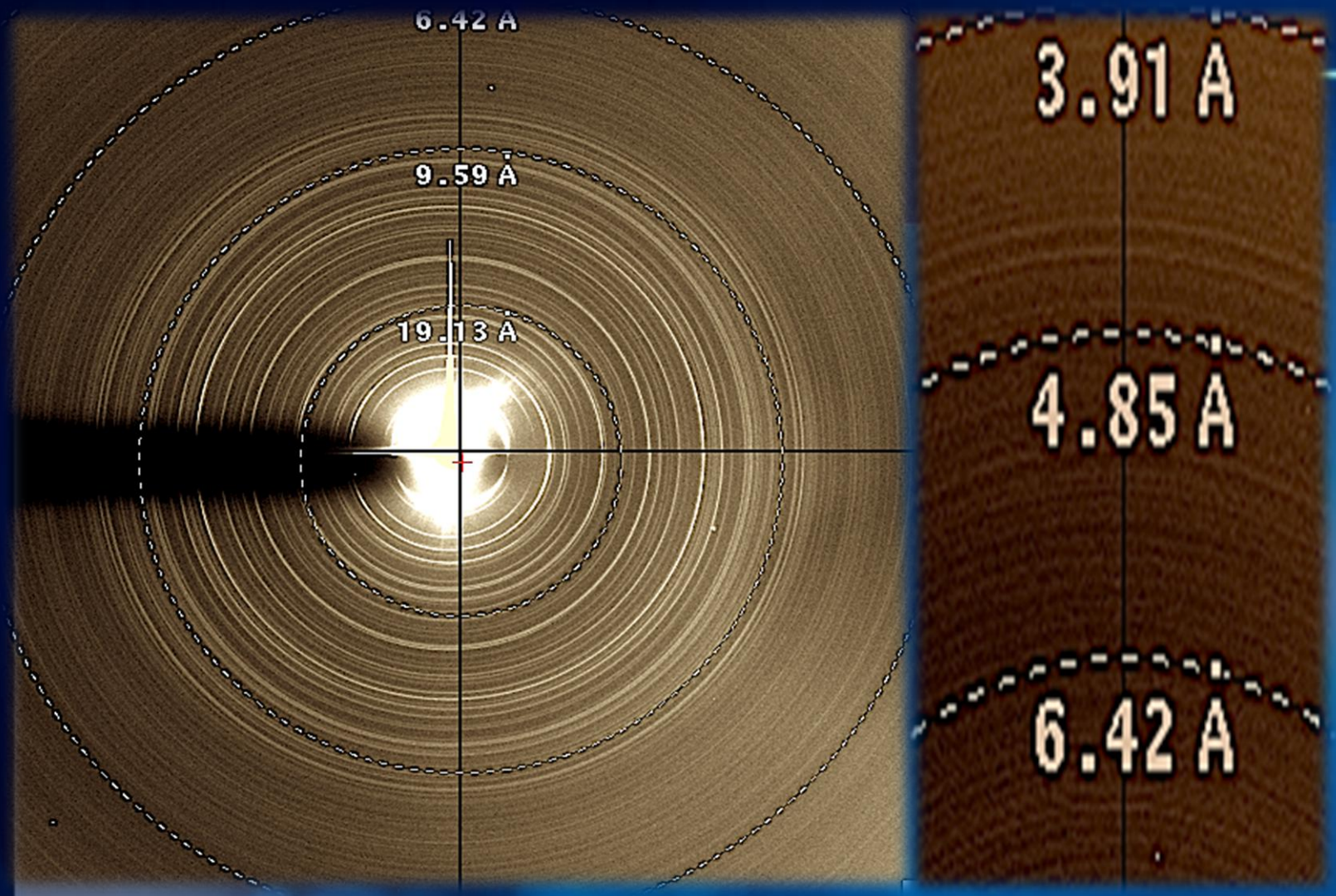


A VIRUS PROTEIN DOMAIN “SINGLE URCHIN” IN A GRID





http://www.mitegen.com/



ID14- 2, PILATUS area detector
λ=0.9934 Å

Matthew Bowler, Yves Watier,
Nicolas Papageorgiou

EMBL HC1b device

Papageorgiou et al., Z. Kristallogr. 225, 576–580 (2010)

- Data Collection

SR-XRPD + LAB XRPD

1999 - present
ESRF- Grenoble, France

New collaborations with other synchrotrons

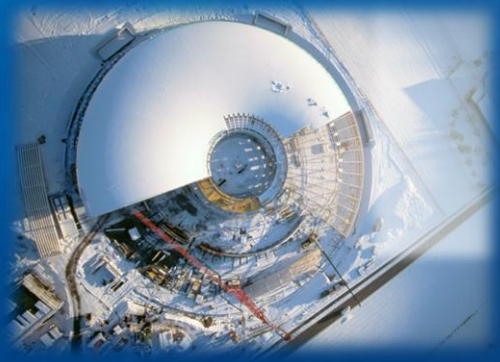
FRANCE

http://www.synchrotron-soleil.fr/

JAPAN

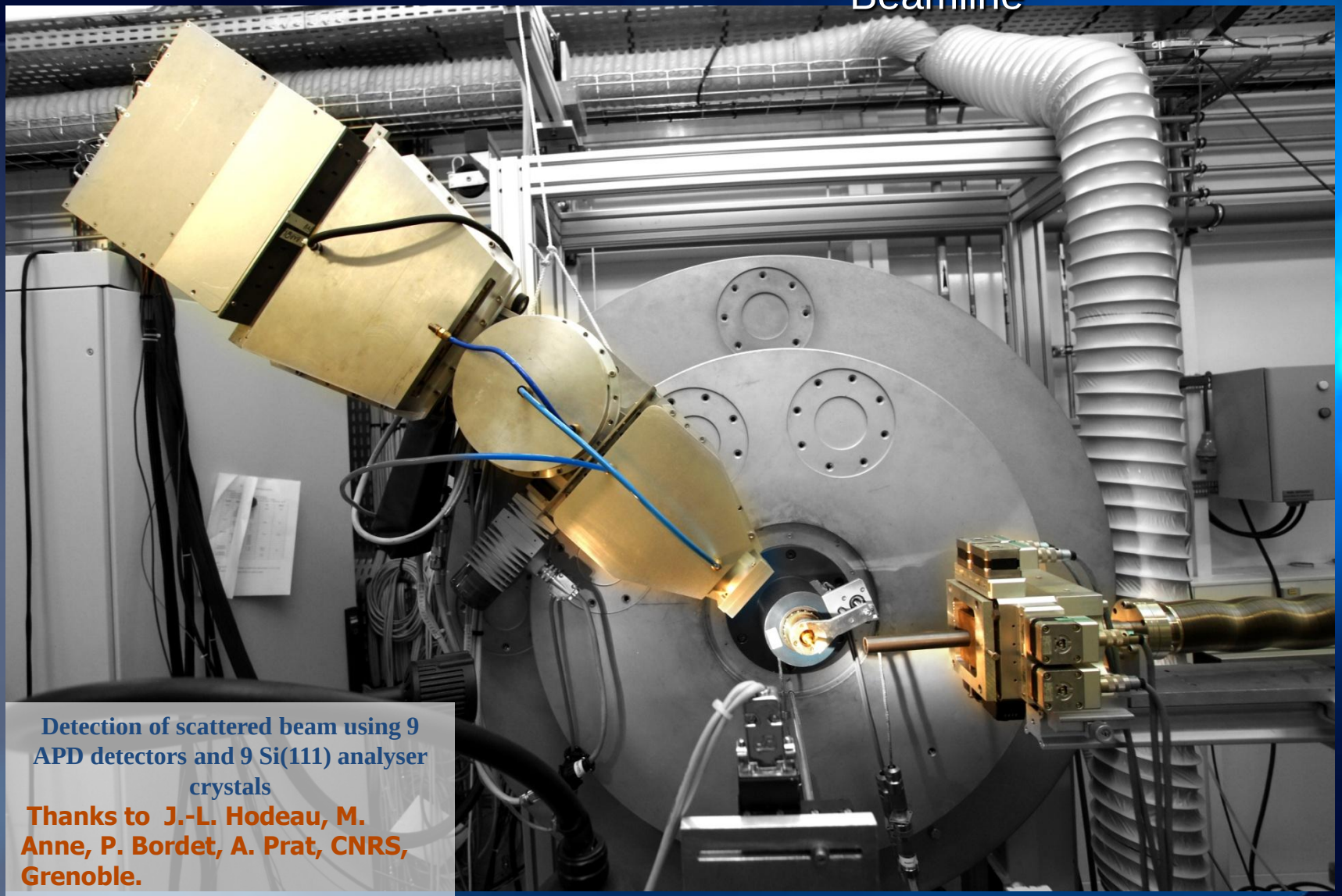
http://www.spring8.or.jp/en/

SWITZERLAND



http://www.psi.ch/sls/

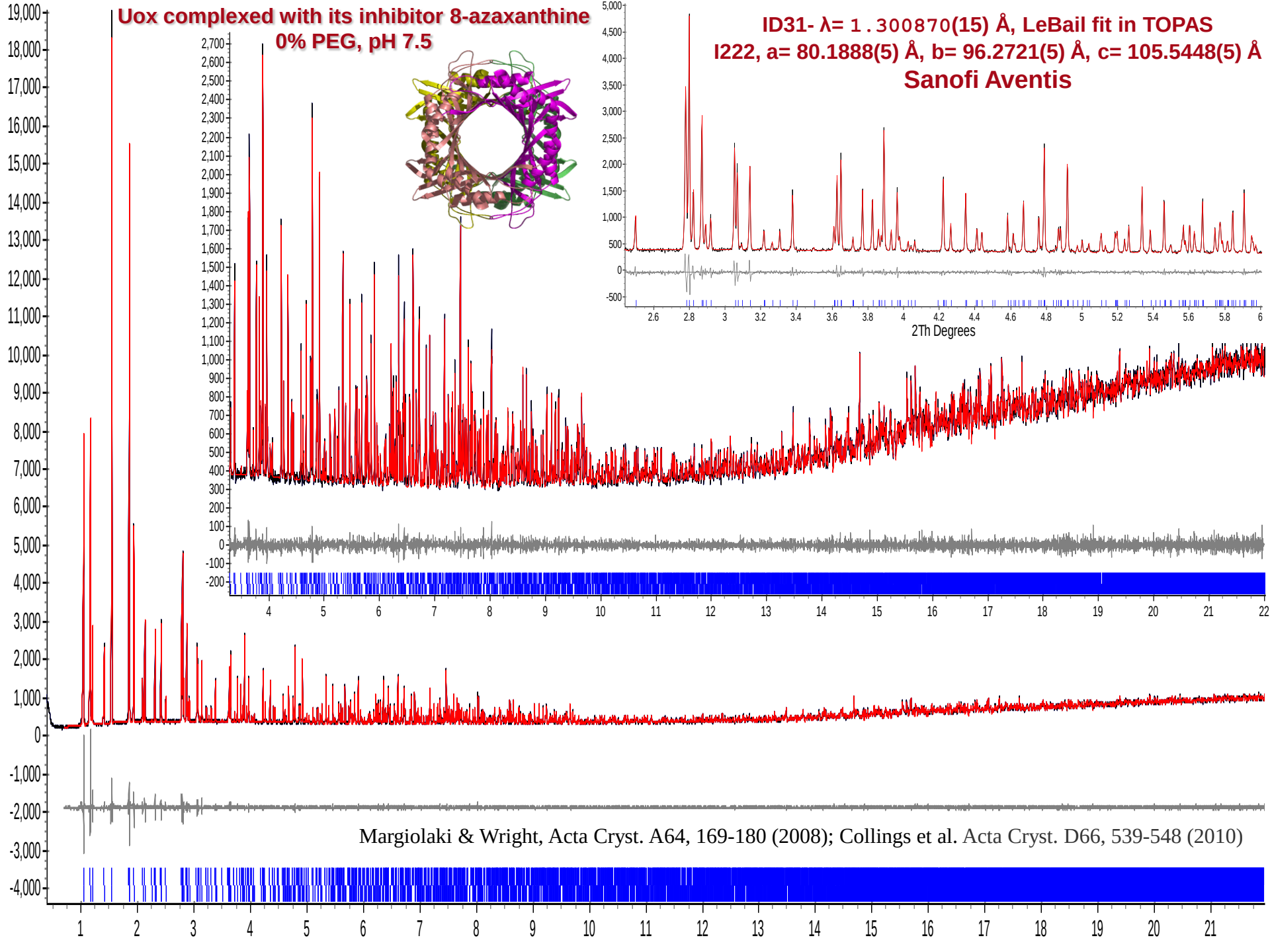
ID31: High Resolution Powder diffraction Beamline



Detection of scattered beam using 9 APD detectors and 9 Si(111) analyser crystals

Thanks to J.-L. Hodeau, M. Anne, P. Bordet, A. Prat, CNRS, Grenoble.

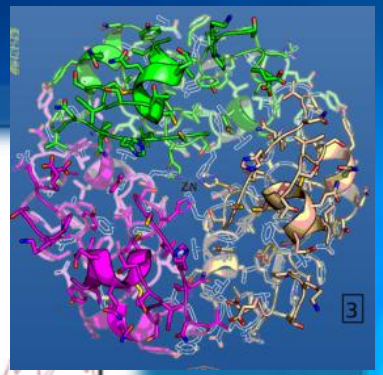
Fitch, J. Res. Natl. Inst. Stand. Technol. 109, 133-142 (2004)

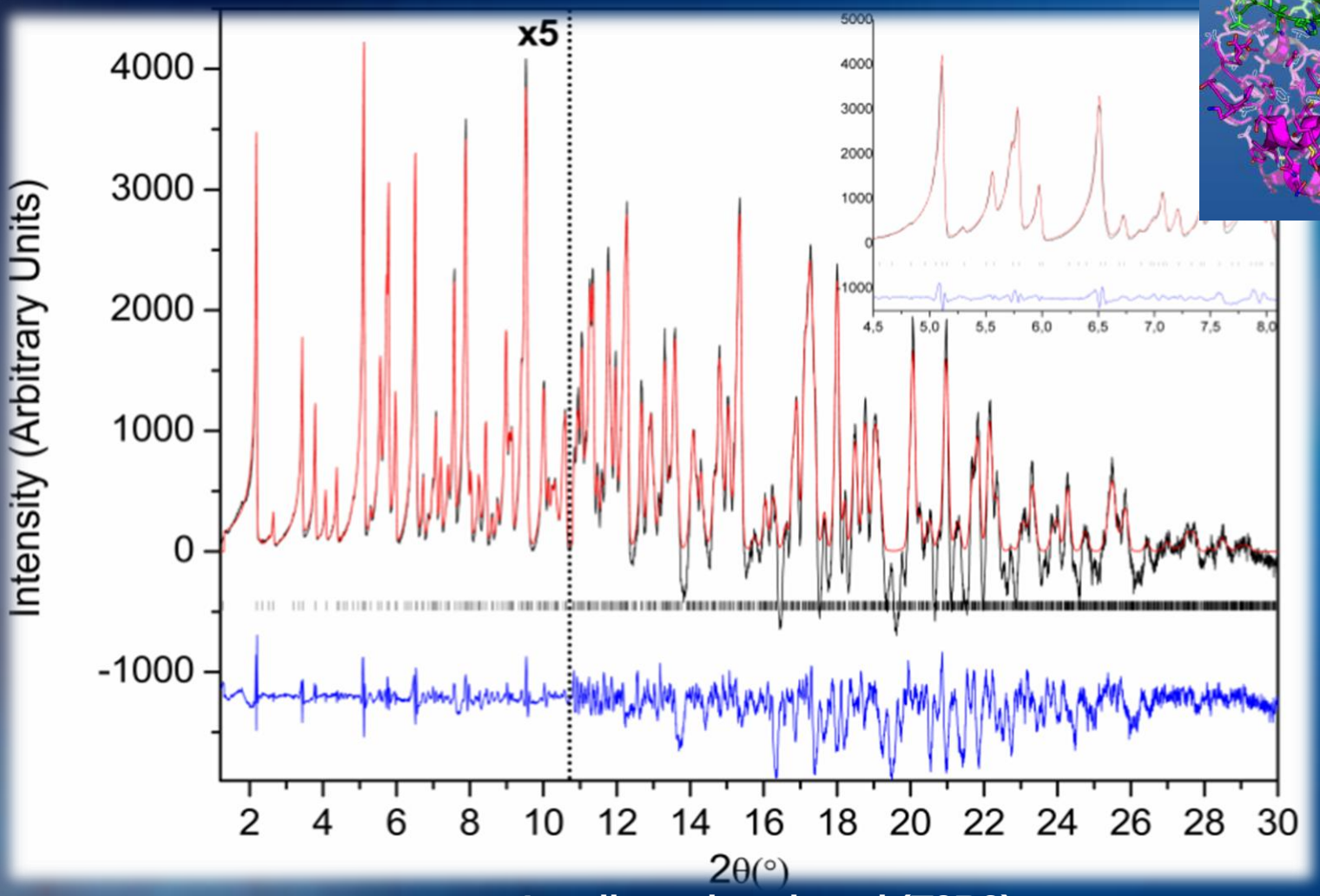

Uox complexed with its inhibitor 8-azaxanthine
0% PEG, pH 7.5
ID31- λ= 1.300870(15) Å, LeBail fit in TOPAS
I222, a= 80.1888(5) Å, b= 96.2721(5) Å, c= 105.5448(5) Å
Sanofi Aventis
2Th Degrees
Margiolaki & Wright, Acta Cryst. A64, 169-180 (2008); Collings et al. Acta Cryst. D66, 539-548 (2010)

LAB DIFFRACTOMETER
X’ PERT PRO BY PANALYTICAL

PAWLEY FIT OF LAB DATA

Novo Nordisk, Copenhagen





Insulin + nitrophenol (T3R3)

Valmas et al. Acta Cryst. (2015). D71, 819–828;

POWDER DIFFRACTION:

A POWERFUL QUALITY CONTROL TOOL FOR PROTEIN- BASED DRUG SCREENING

ASPECTS OF OUR STUDIES

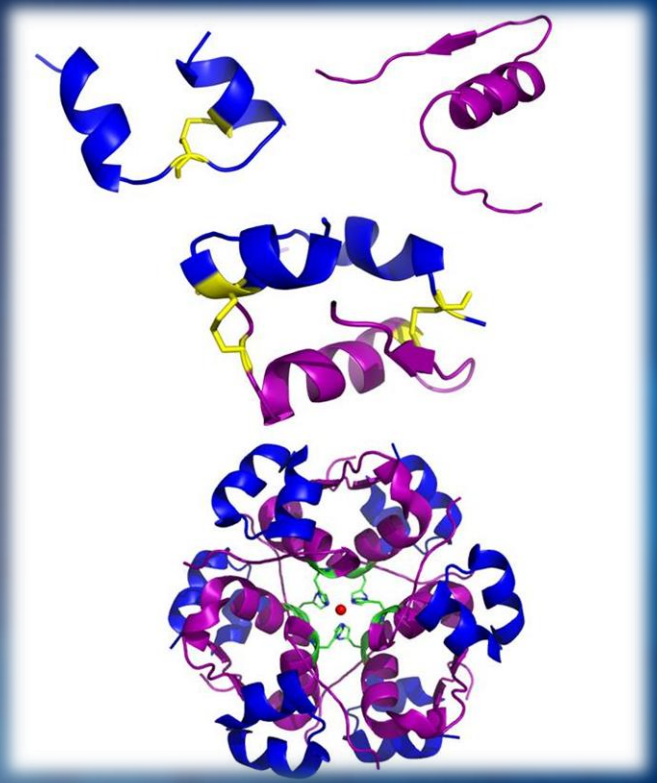
1. **Polymorph Identification**
2. **Structure solution of novel polymorphs**
3. Structure refinement
   Ligand identification using powder data

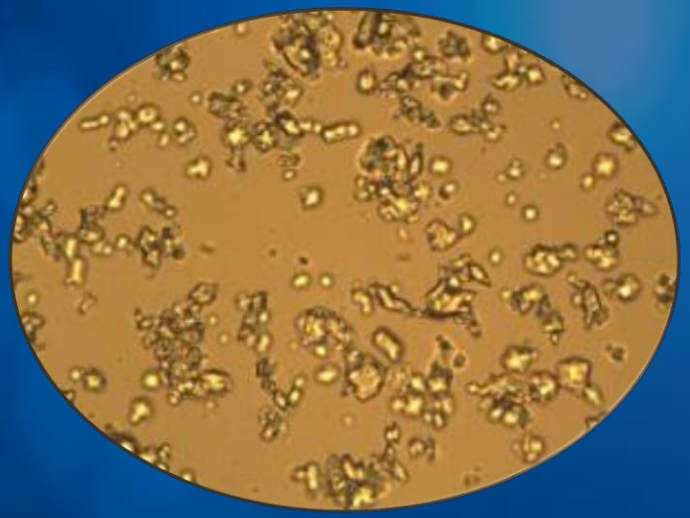
UNIVERSITY - INDUSTRY COLLABORATION

Novo Nordisk, Copenhagen
Diabetes Protein Engineering

Research Collaborators
Gerd Schluckebier & Mathias Norrman

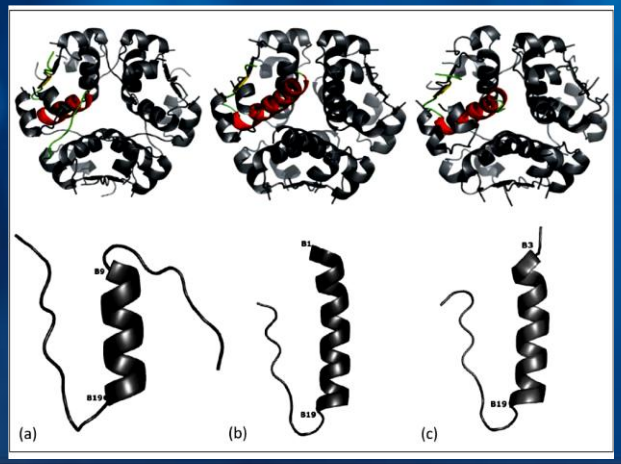
THE CASE OF HUMAN INSULIN





INSULIN HEXAMERIC CONFORMATIONS

- T_6 ⟶ B_1-B_8 EXTENDED CONFORMATION
- R_6 ⟶ B_1-B_8 HELICAL CONFORMATION
- T_3R_3 ⟶ 3 MONOMERS B_1-B_3 EXTENDED
  B_4-B_8 HELICAL
  3 MONOMERS : B_1-B_8 EXTENDED

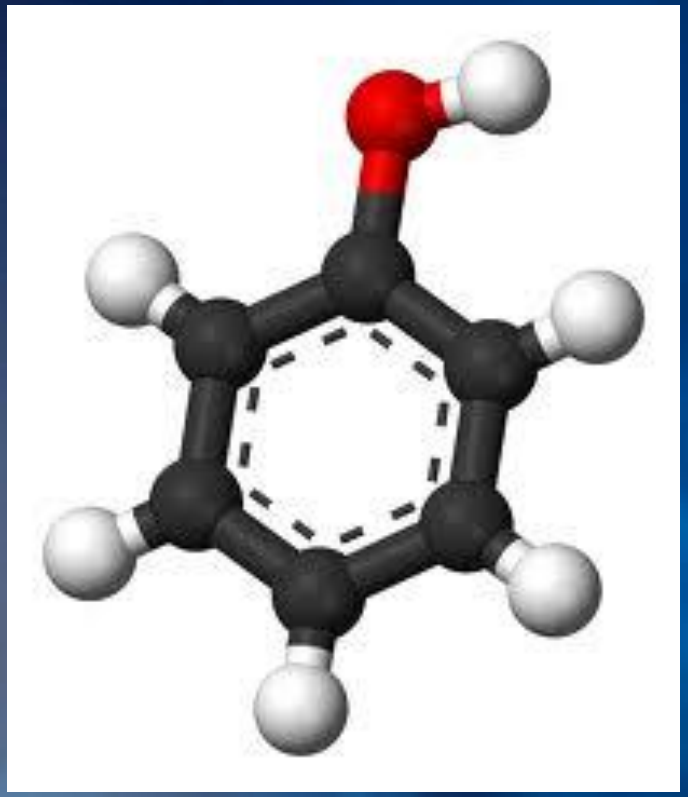


MICROCRYSTALLINE INSULIN

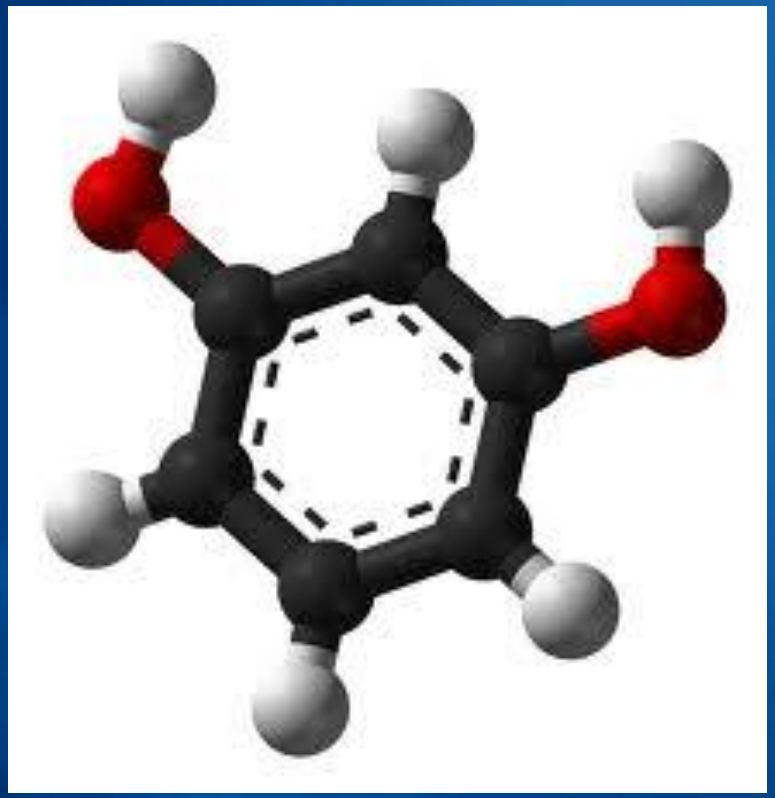
- Several polymorphs exist depending on pH of crystallization and concentration of additives.
- Great interest in finding new forms of potentially therapeutic applications.
- Study microcrystalline insulin crystallized as a function of pH (4 – 8.9) and with phenol – based additives.
- Extremelly rich phase diagram.

HUMAN INSULIN – LIGAND COMPLEXES

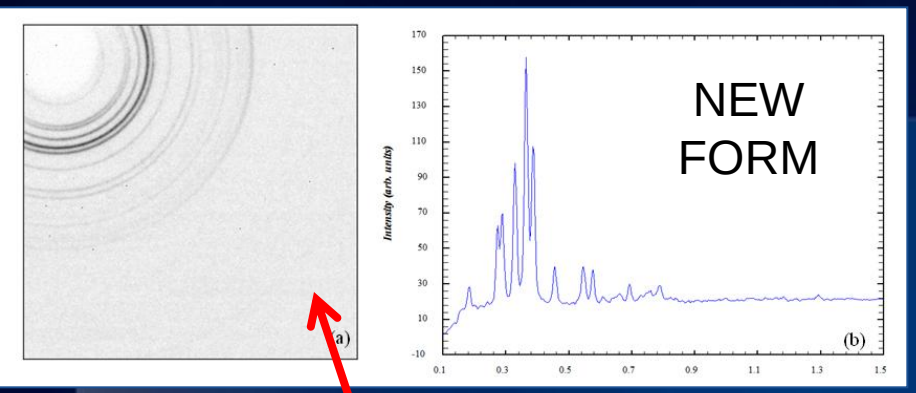
CRYSTALLINE PRECIPITATES

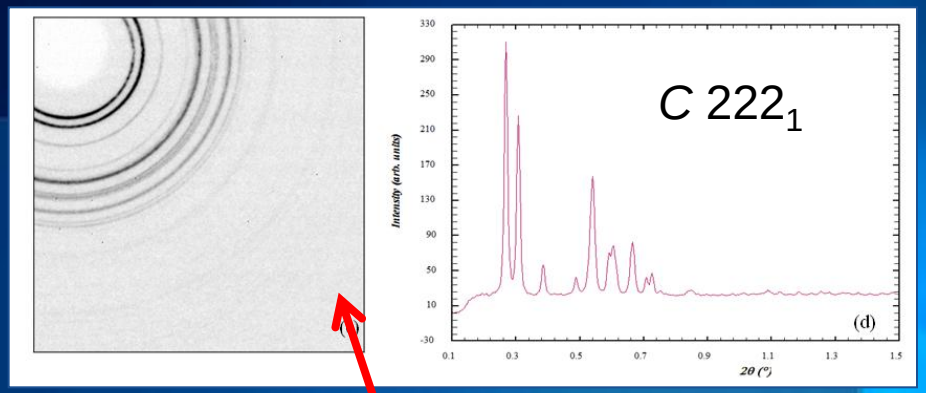


PHENOL

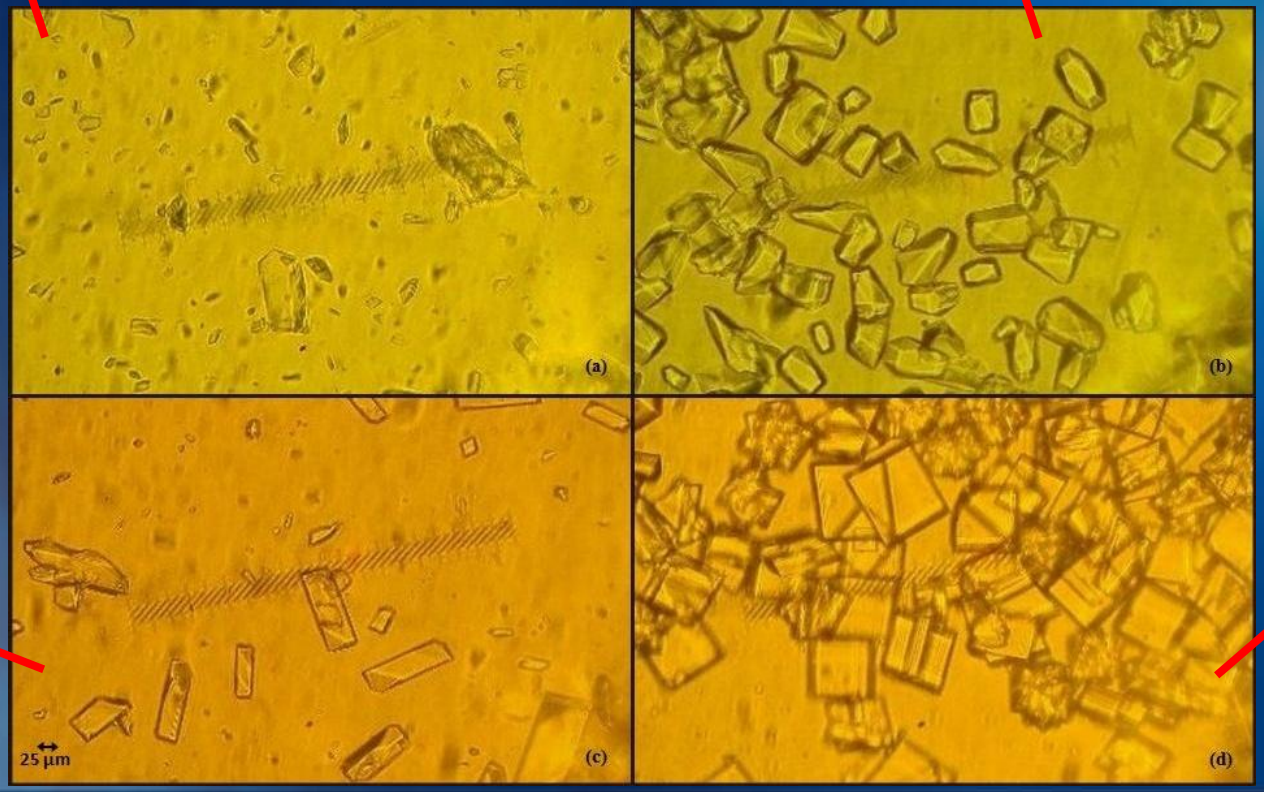


RESORCINOL


(a)
Intensity (arb. units)
(b)
NEW
FORM


(c)
Intensity (arb. units)
(d)
2θ (°)
C 222$_1$

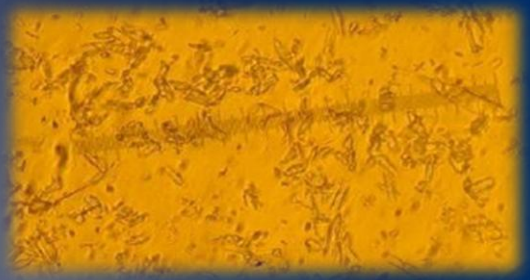
pH<5.6

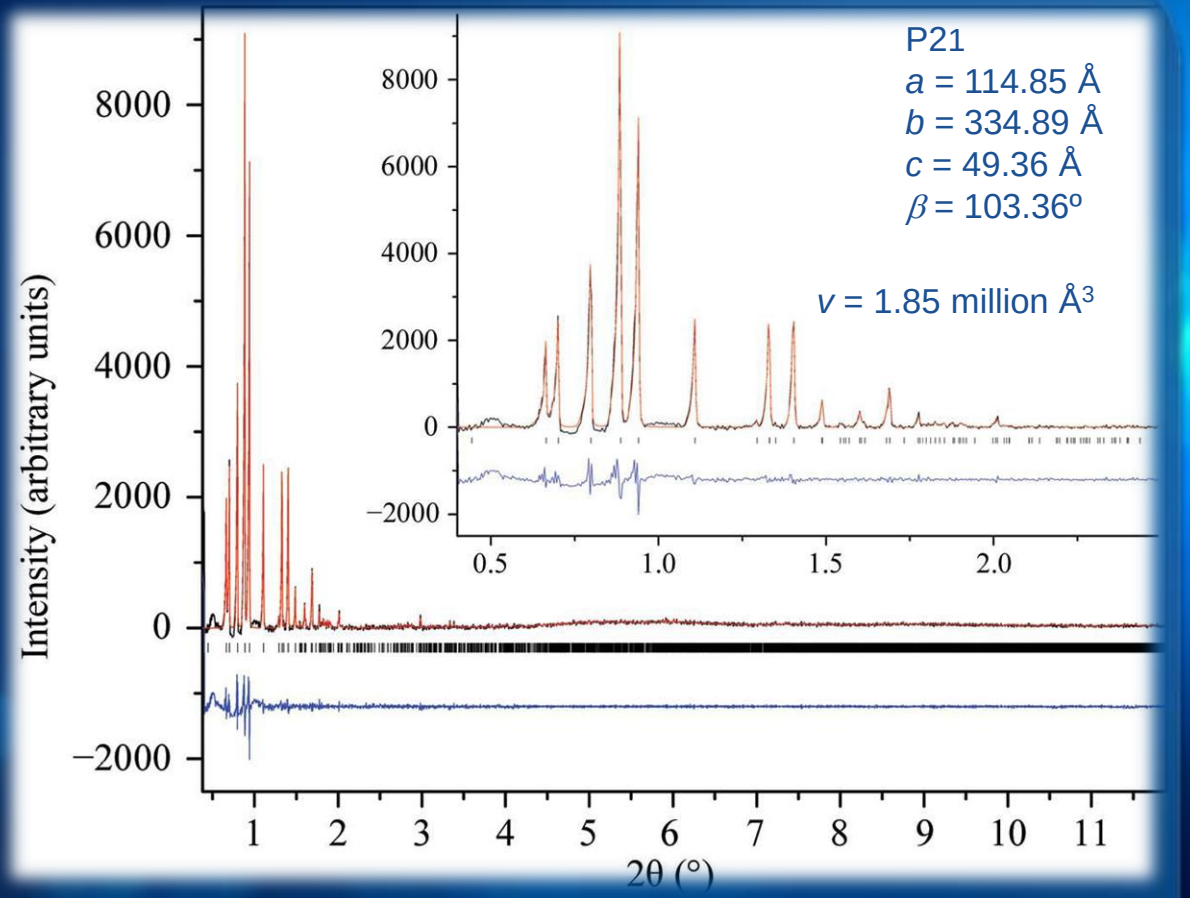

(a)
(b)
25 µm
(c)
(d)

C 2

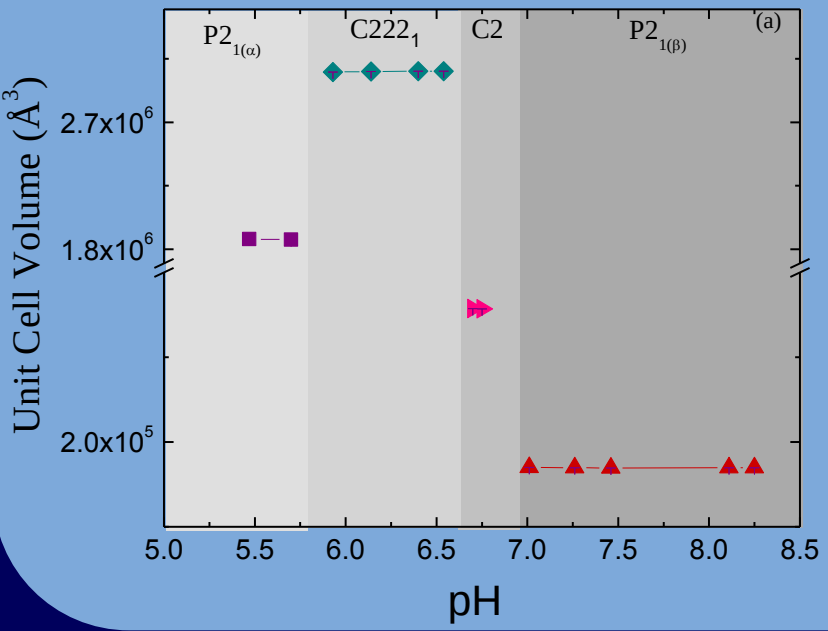
P 2$_1$

NEW POLYMORPH
pH 5.18 + resorcinol or phenol

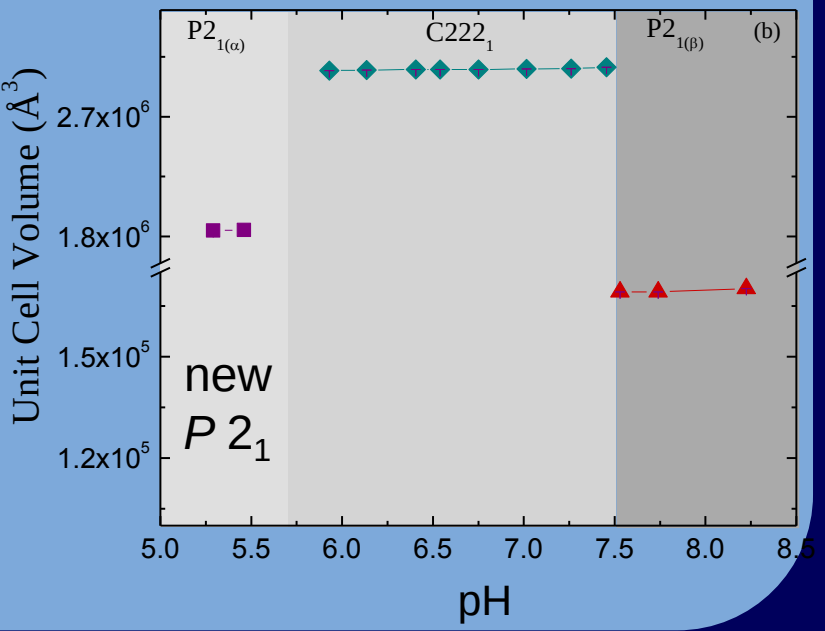


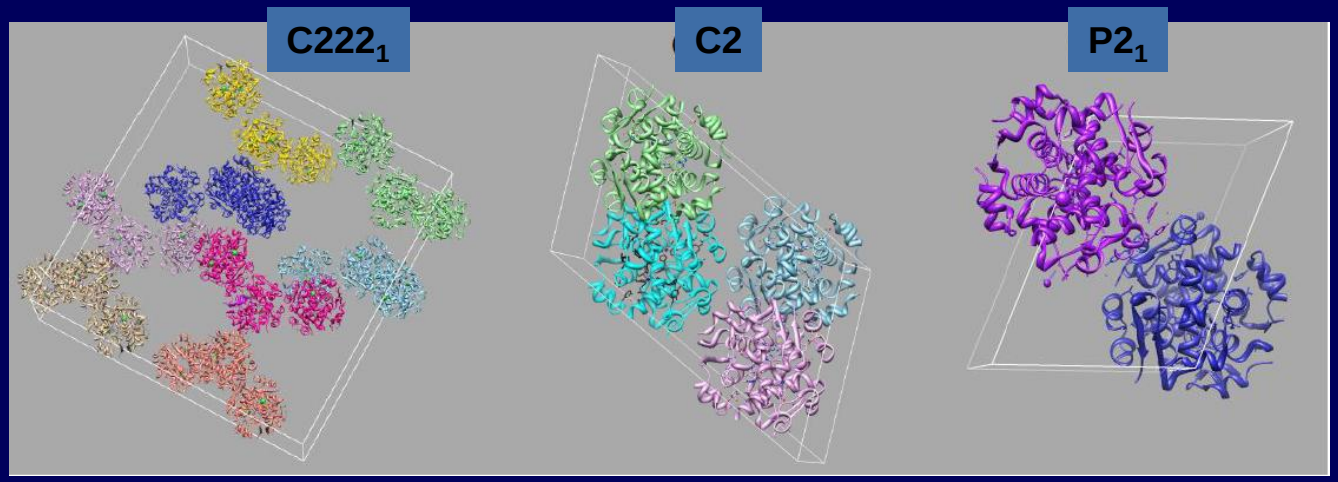


phenol



resorcinol

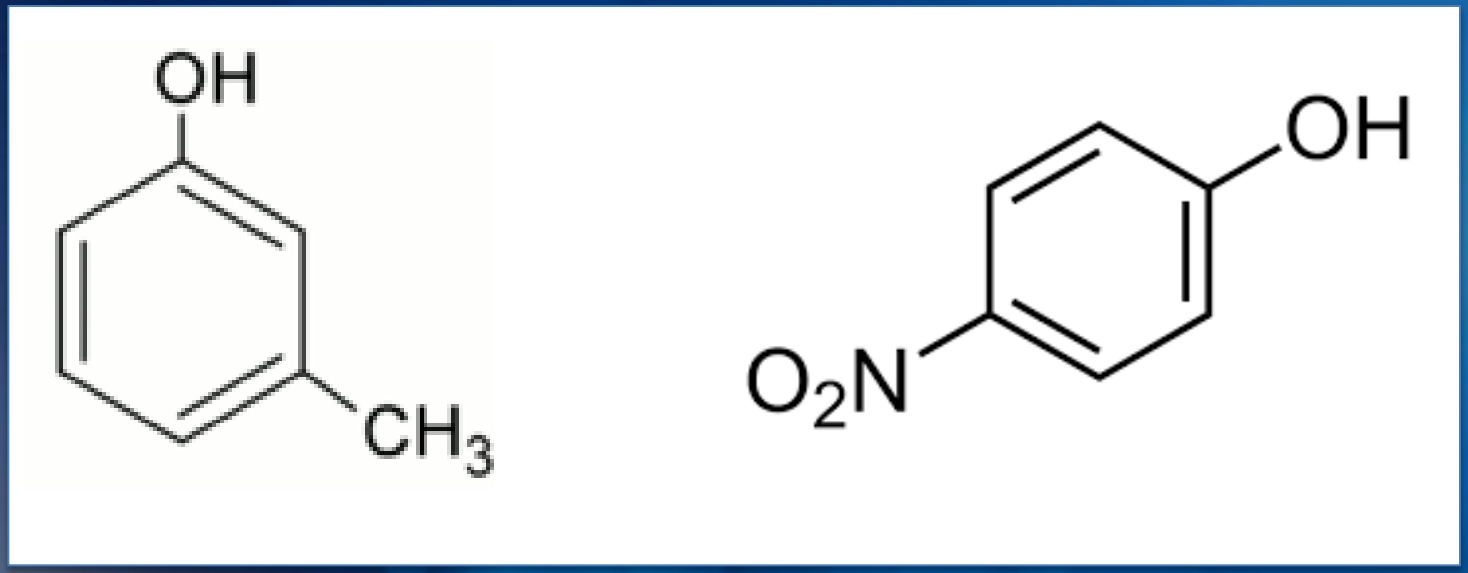




Karavassili et al., Acta Cryst. (2012). D68, 1632–1641

HUMAN INSULIN – LIGAND COMPLEXES

CRYSTALLINE PRECIPITATES

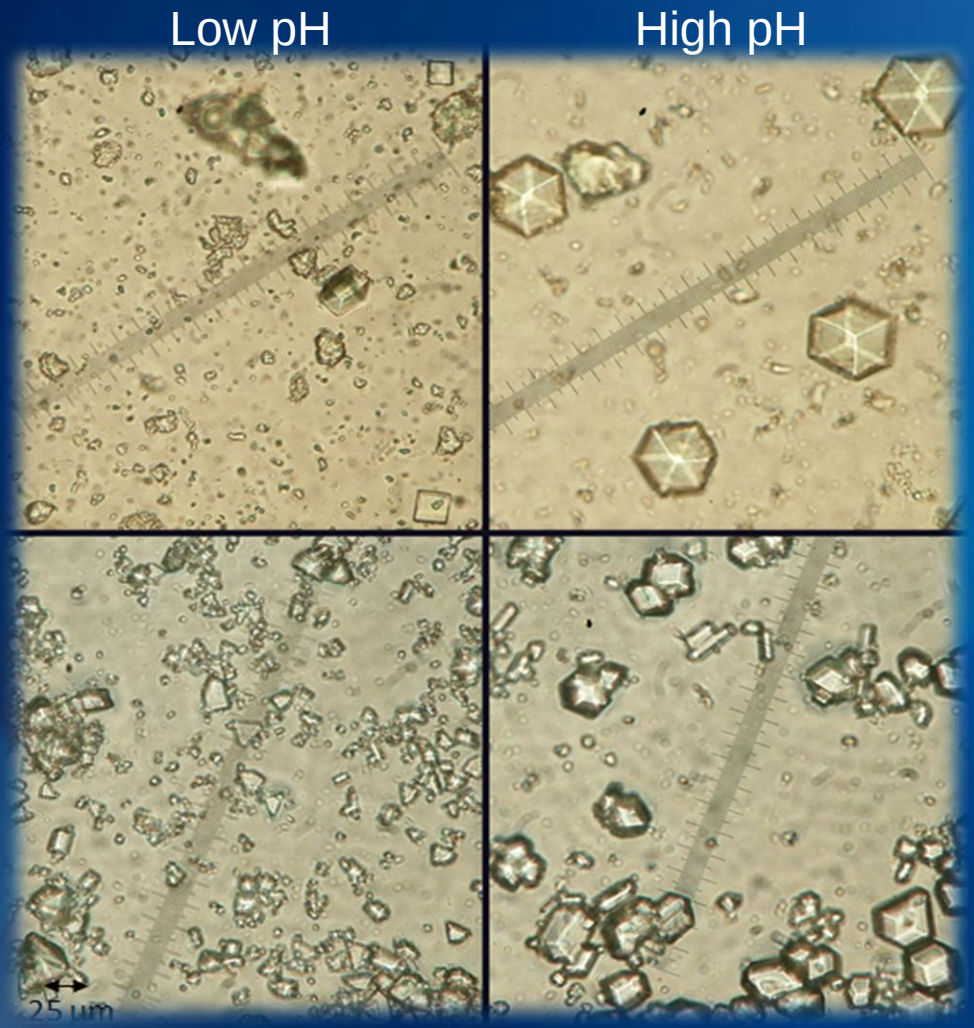


M-cresol

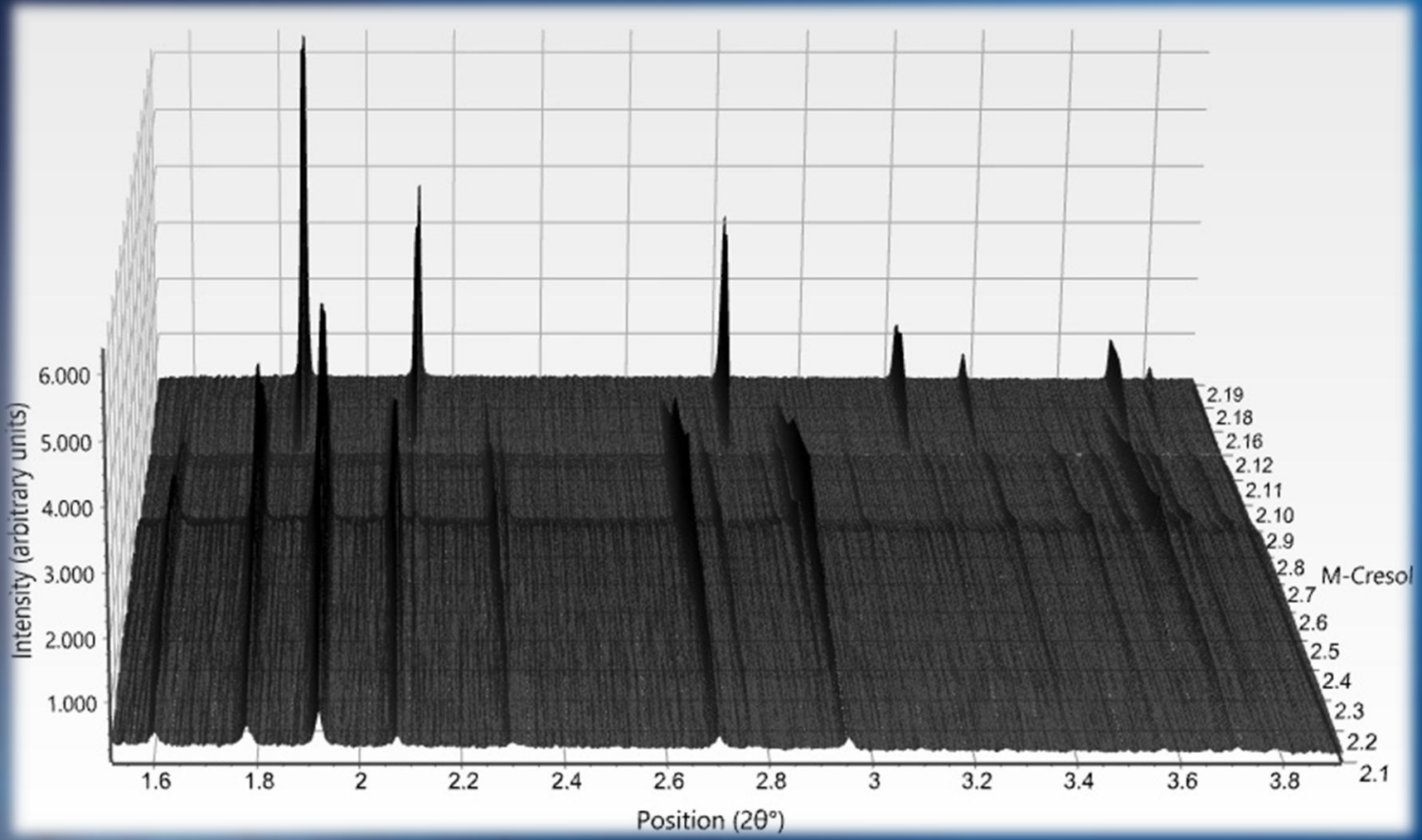
4-nitrophenol

HUMAN INSULIN – LIGAND COMPLEXES

CRYSTALLINE PRECIPITATES

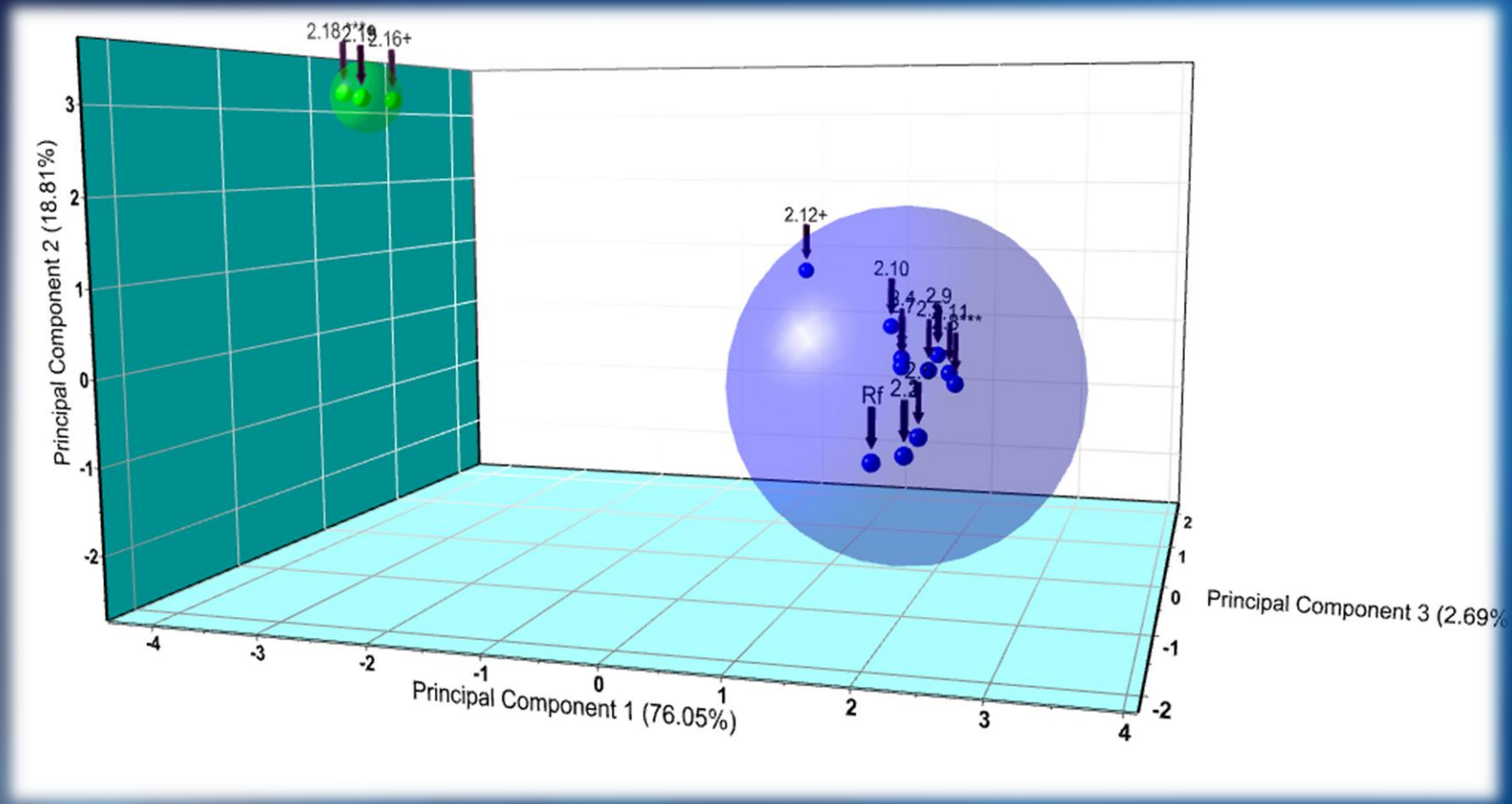


ESRF- ID31
[λ = 1.29989 (3) Å, RT]



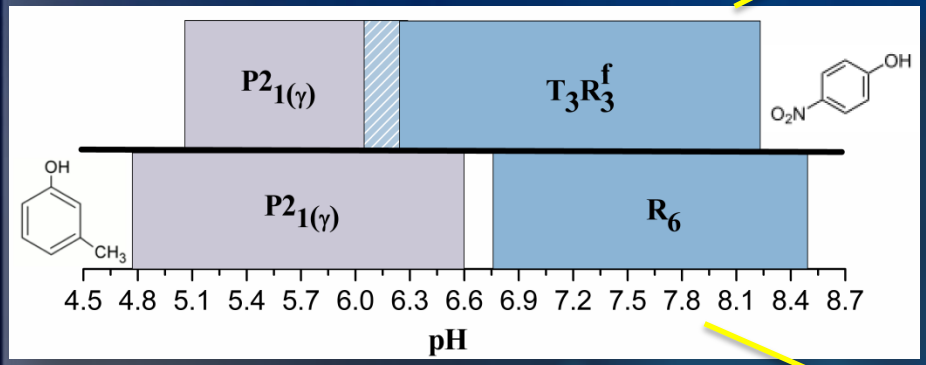
Valmas et al. Acta Cryst. (2015). D71, 819–828

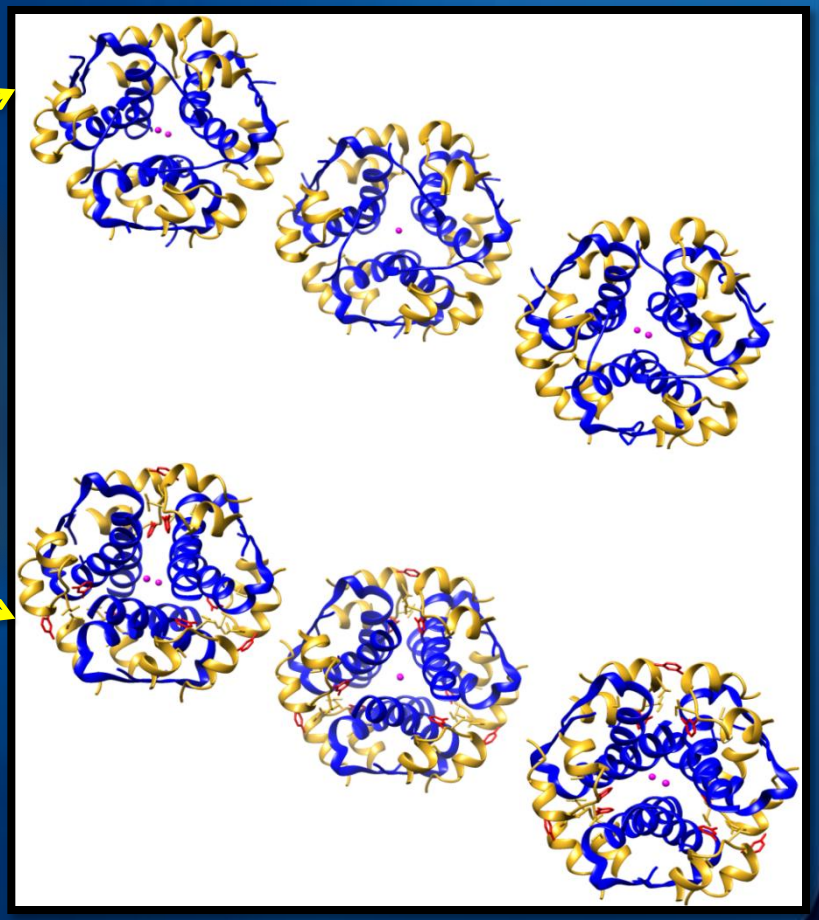
HighScore Plus software
(Degen et al., Powder Diffr. 29, S13–S18



Valmas et al. Acta Cryst. (2015). D71, 819–828

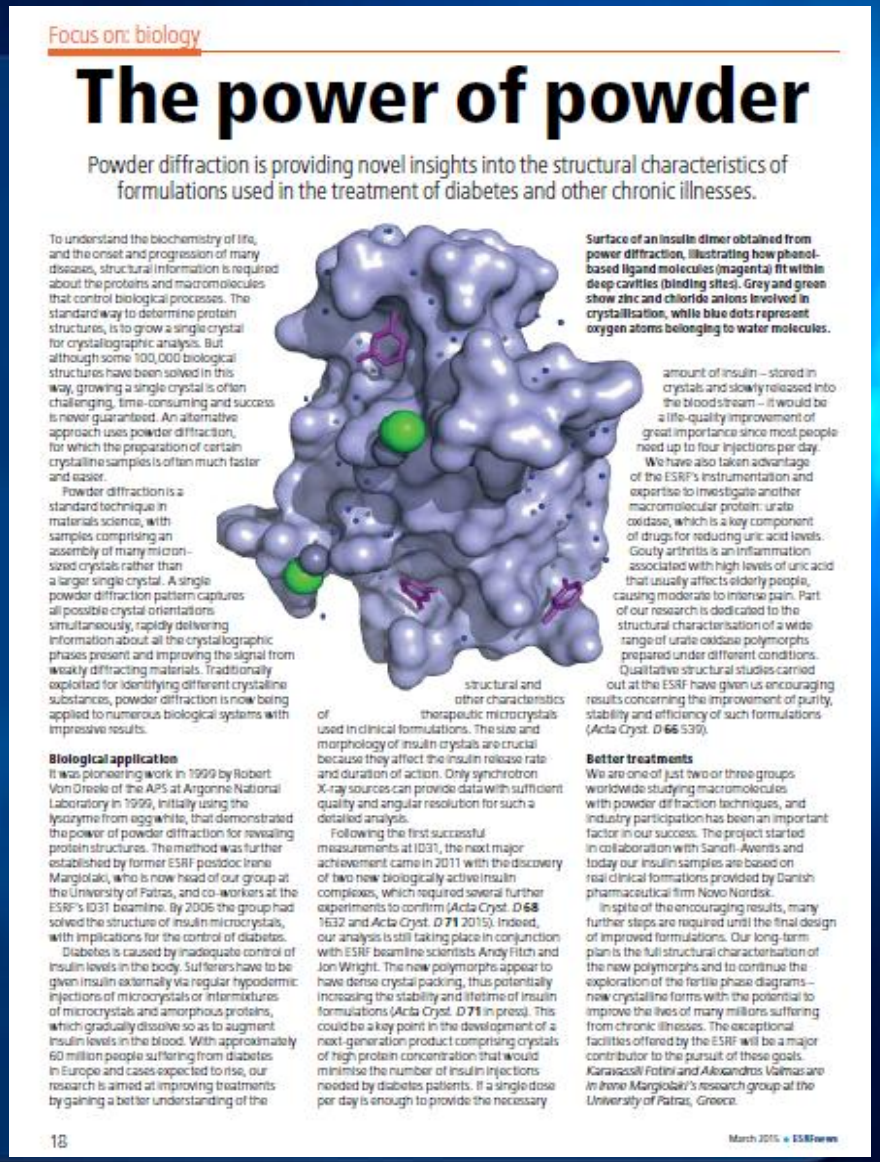
PHASE IDENTIFICATION





Valmas et al. Acta Cryst. (2015). D71, 819–828;

RESULTS ON THE NEWS



Focus on: biology

The power of powder

Powder diffraction is providing novel insights into the structural characteristics of formulations used in the treatment of diabetes and other chronic illnesses.

To understand the biochemistry of life, and the onset and progression of many diseases, structural information is required about the proteins and macromolecules that control biological processes. The standard way to determine protein structures, is to grow a single crystal for crystallographic analysis. But although some 100,000 biological structures have been solved in this way, growing a single crystal is often challenging, time-consuming and success is never guaranteed. An alternative approach uses powder diffraction, for which the preparation of certain crystalline samples is often much faster and easier.

Powder diffraction is a standard technique in materials science, with samples comprising an assembly of many micron-sized crystals rather than a larger single crystal. A single powder diffraction pattern captures all possible crystal orientations simultaneously, rapidly delivering information about all the crystallographic phases present and improving the signal from weakly diffracting materials. Traditionally exploited for identifying different crystalline substances, powder diffraction is now being applied to numerous biological systems with impressive results.

Biological application

It was pioneering work in 1999 by Robert Von Dreele of the APS at Argonne National Laboratory in 1999, initially using the lysozyme from egg white, that demonstrated the power of powder diffraction for revealing protein structures. The method was further established by former ESRF postdoc Irene Margiolaki, who is now head of our group at the University of Patras, and co-workers at the ESRF's ID31 beamline. By 2006 the group had solved the structure of insulin microcrystals, with implications for the control of diabetes.

Diabetes is caused by inadequate control of insulin levels in the body. Sufferers have to be given insulin externally via regular hypodermic injections of microcrystals or intermixtures of microcrystals and amorphous proteins, which gradually dissolve so as to augment insulin levels in the blood. With approximately 60 million people suffering from diabetes in Europe and cases expected to rise, our research is aimed at improving treatments by gaining a better understanding of the structural and other characteristics of therapeutic microcrystals used in clinical formulations. The size and morphology of insulin crystals are crucial because they affect the insulin release rate and duration of action. Only synchrotron X-ray sources can provide data with sufficient quality and angular resolution for such a detailed analysis.

Following the first successful measurements at ID31, the next major achievement came in 2011 with the discovery of two new biologically active insulin complexes, which required several further experiments to confirm (*Acta Cryst.* D**68** 1632 and *Acta Cryst.* D**71** 2015). Indeed, our analysis is still taking place in conjunction with ESRF beamline scientists Andy Fitch and Jon Wright. The new polymorphs appear to have dense crystal packing, thus potentially increasing the stability and lifetime of insulin formulations (*Acta Cryst.* D**71** in press). This could be a key point in the development of a next-generation product comprising crystals of high protein concentration that would minimise the number of insulin injections needed by diabetes patients. If a single dose per day is enough to provide the necessary amount of insulin – stored in crystals and slowly released into the bloodstream – it would be a life-quality improvement of great importance since most people need up to four injections per day.

Surface of an insulin dimer obtained from power diffraction, illustrating how phenol-based ligand molecules (magenta) fit within deep cavities (binding sites). Grey and green show zinc and chloride anions involved in crystallisation, while blue dots represent oxygen atoms belonging to water molecules.

We have also taken advantage of the ESRF's instrumentation and expertise to investigate another macromolecular protein: urate oxidase, which is a key component of drugs for reducing uric acid levels. Gouty arthritis is an inflammation associated with high levels of uric acid that usually affects elderly people, causing moderate to intense pain. Part of our research is dedicated to the structural characterisation of a wide range of urate oxidase polymorphs prepared under different conditions. Qualitative structural studies carried out at the ESRF have given us encouraging results concerning the improvement of purity, stability and efficiency of such formulations (*Acta Cryst.* D**66** 539).

Better treatments

We are one of just two or three groups worldwide studying macromolecules with powder diffraction techniques, and industry participation has been an important factor in our success. The project started in collaboration with Sanofi-Aventis and today our insulin samples are based on real clinical formulations provided by Danish pharmaceutical firm Novo Nordisk.

In spite of the encouraging results, many further steps are required until the final design of improved formulations. Our long-term plan is the full structural characterisation of the new polymorphs and to continue the exploration of the fertile phase diagrams – new crystalline forms with the potential to improve the lives of many millions suffering from chronic illnesses. The exceptional facilities offered by the ESRF will be a major contributor to the pursuit of these goals.

Karavassili Fotini and Alexandros Valmas are in Irene Margiolaki's research group at the University of Patras, Greece.

http://mag.digitalpc.co.uk/fvx/iop/esrf/1503/

ASPECTS OF OUR STUDIES

Polymorph Identification

- Crystal Screening

2

- **Structure solution of novel polymorphs**

- Structure refinement
- Ligand identification using powder data

- Structure Solution via MR

A NICE REVIEW ON MR

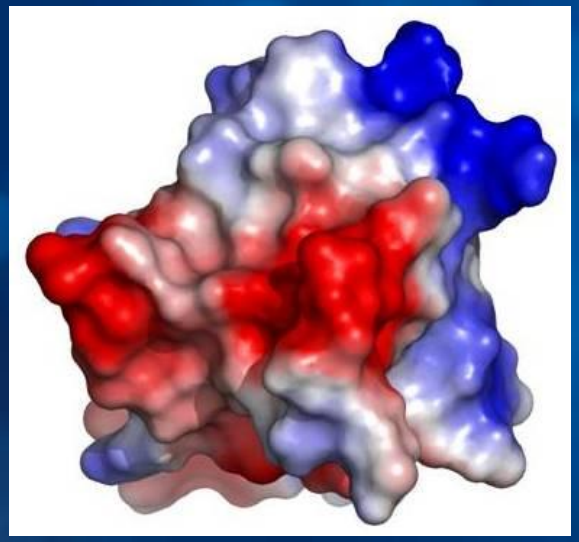
Doebbler, J. A. & Von Dreele, R. B.

Application of molecular replacement to protein powder data from image plates.

Acta Cryst. D65, 348–355 (2009)

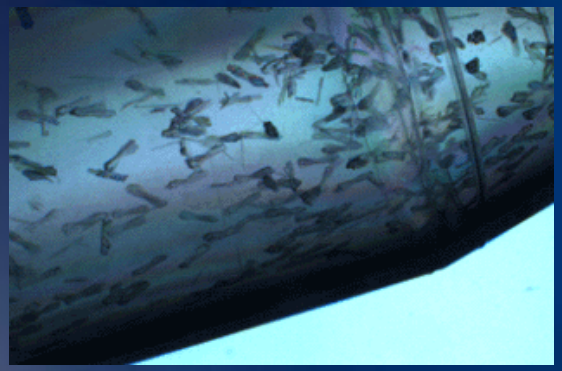
Collaboration with EMBL – Hamburg

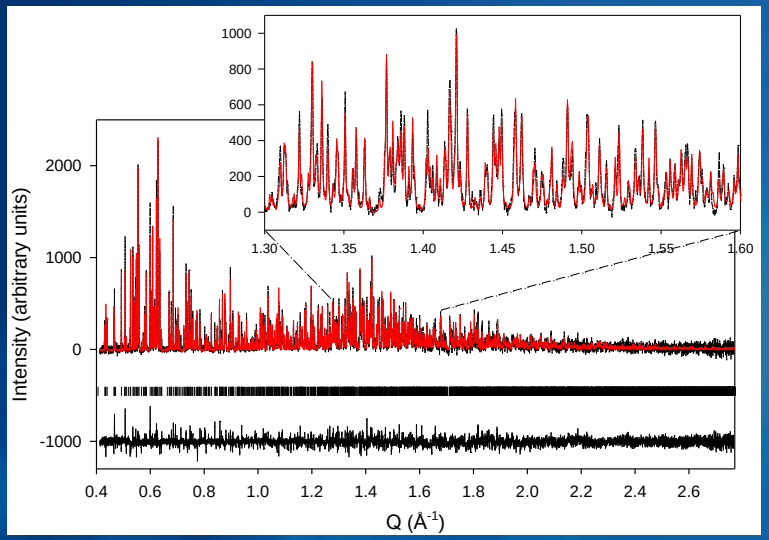
Mathias Wilmanns



The Second SH3 domain of Ponsin

AN UNKNOWN PROTEIN STRUCTURE SOLVED FROM POWDERS

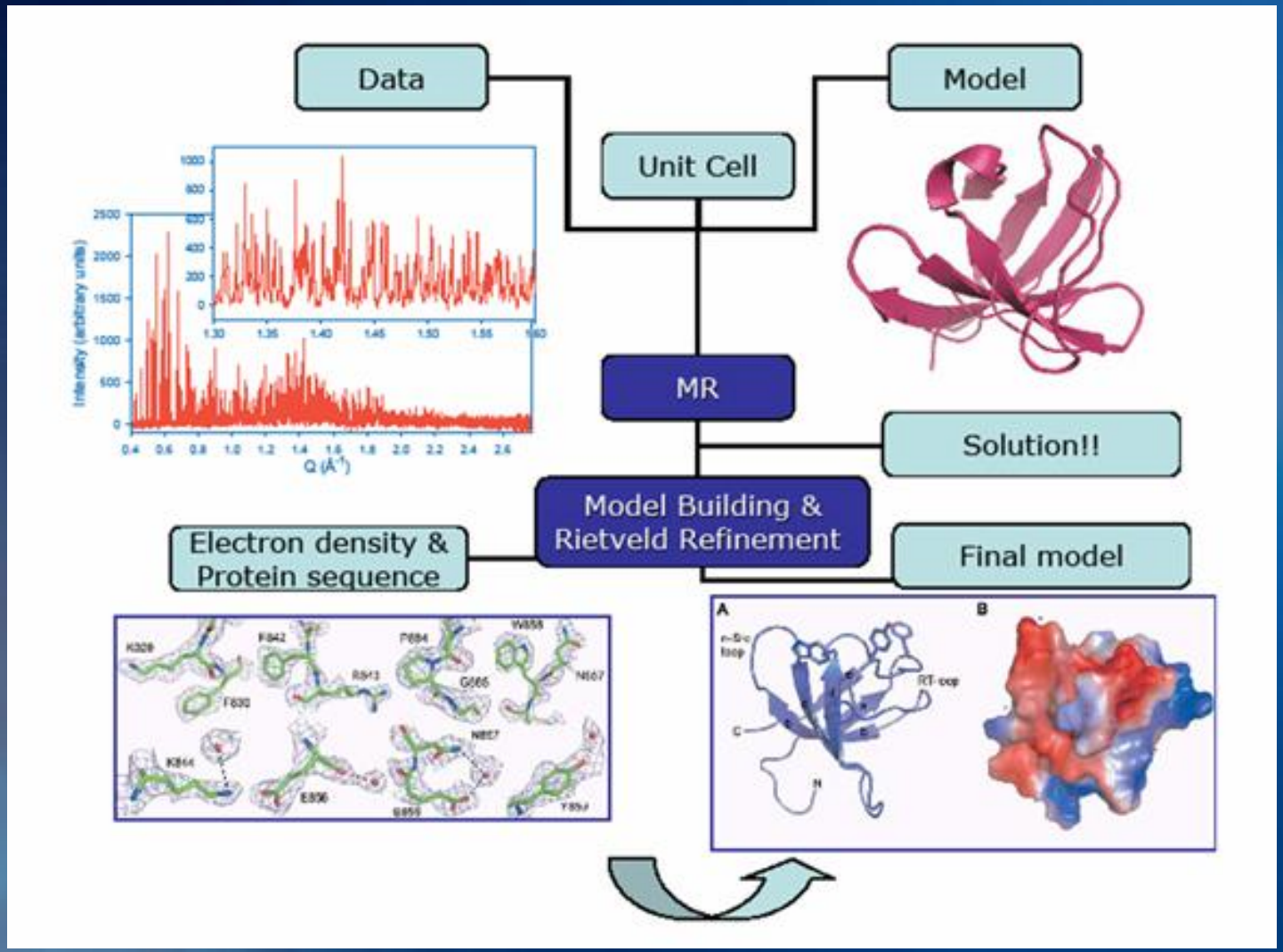




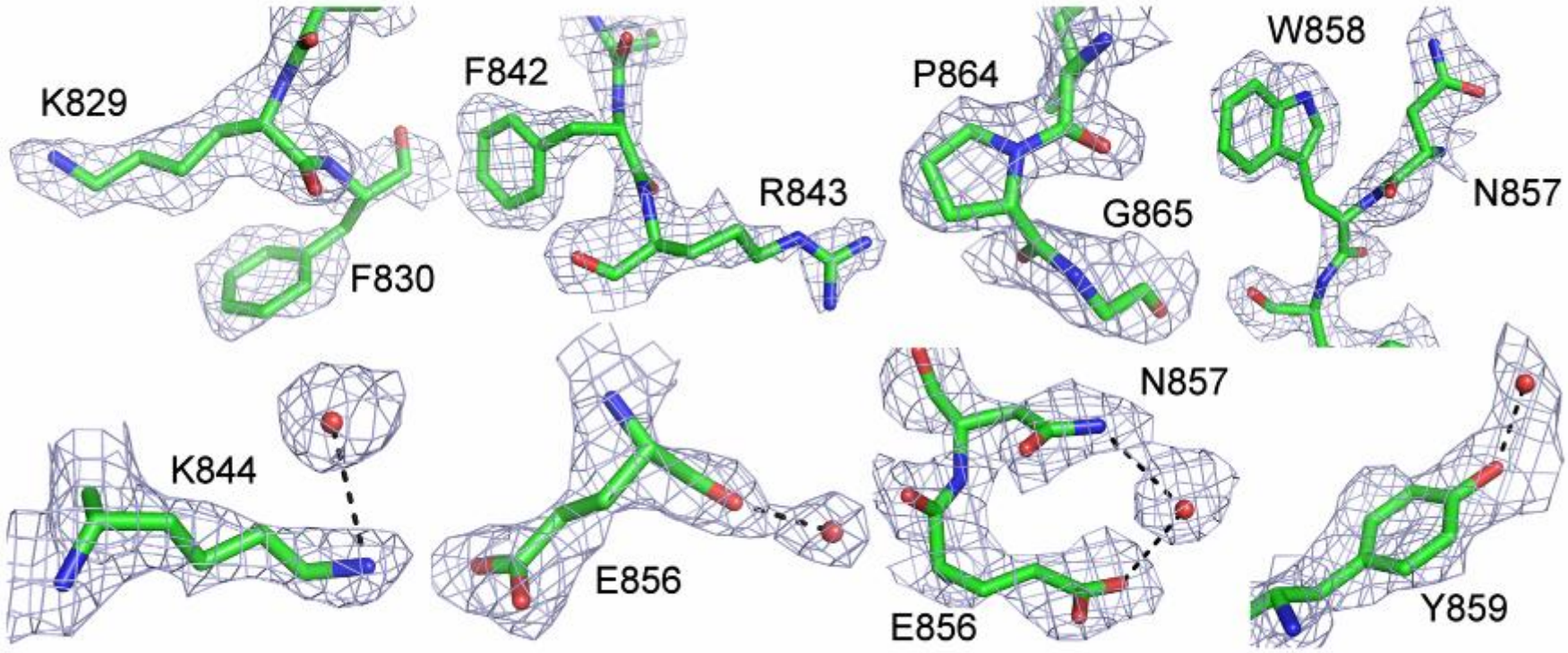
$P2_12_12_1$, a= 24.70420(9) Å, b= 36.42638(14) Å,
c= 72.09804(26) Å

I. Margiolaki, J. P. Wright, M. Wilmanns, A. N. Fitch & N. Pinotsis
J. Am. Chem. Soc. 129, 11865-11871 (2007).

Procedure followed for data analysis for SH3.2

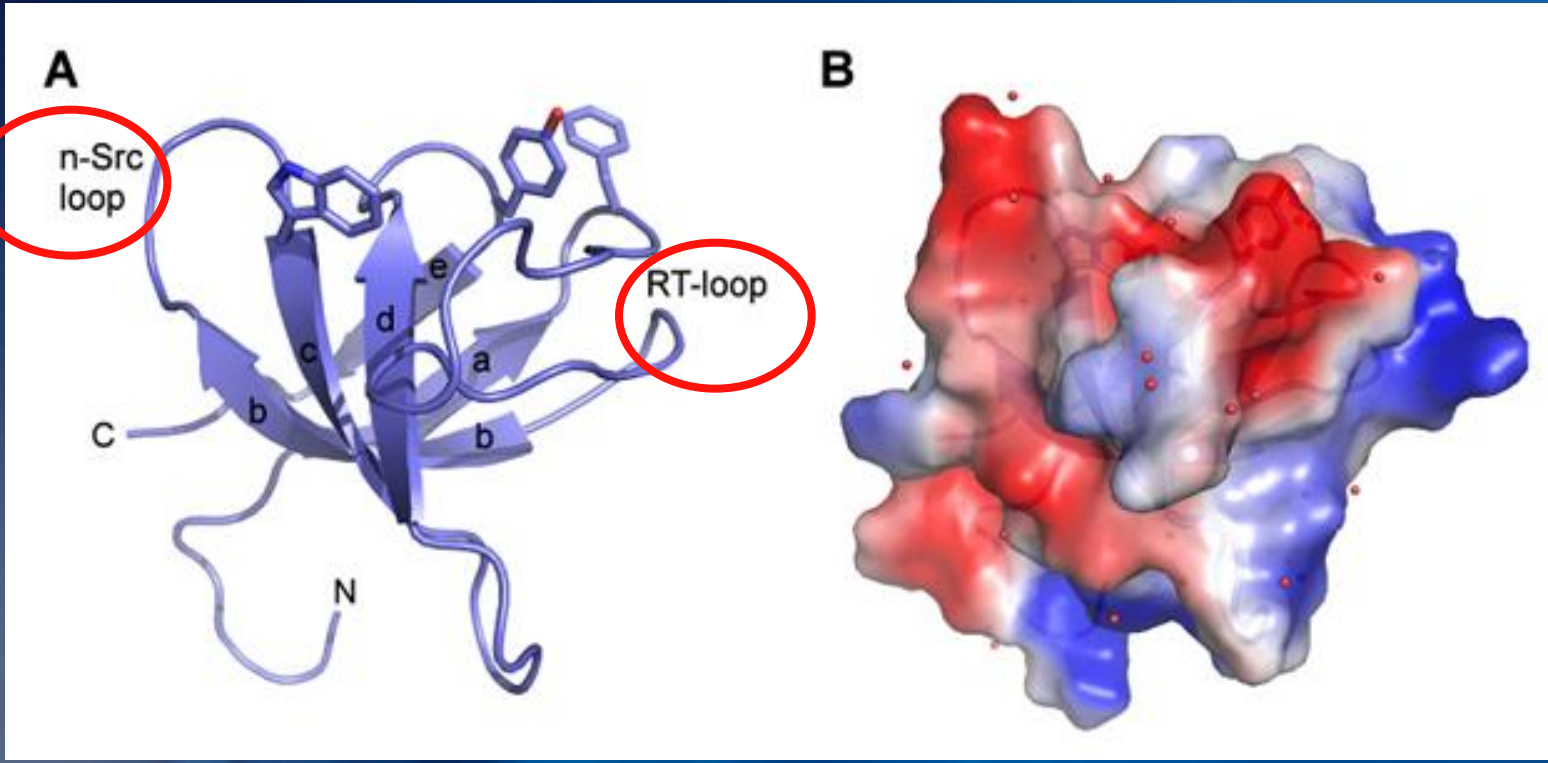


Bhat, T. N. Calculation of an OMIT map. J. Appl. Cryst., 21, 279-281 (1988,)


K829
F830
F842
R843
P864
G865
W858
N857
K844
E856
N857
E856
Y859

SH3.2: The final model

544 protein atoms and 36 water molecules were identified in total OMIT and difference electron density maps.

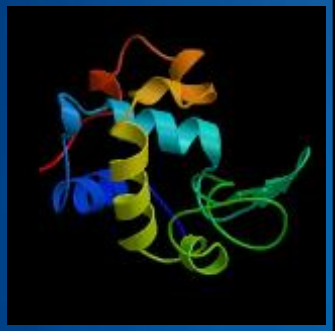


I. Margiolaki, J. P. Wright, M. Wilmanns, A. N. Fitch & N. Pinotsis.
Second SH3 *domain of ponsin solved from powder diffraction*
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY 129 (38): 11865-11871 SEP 26 2007

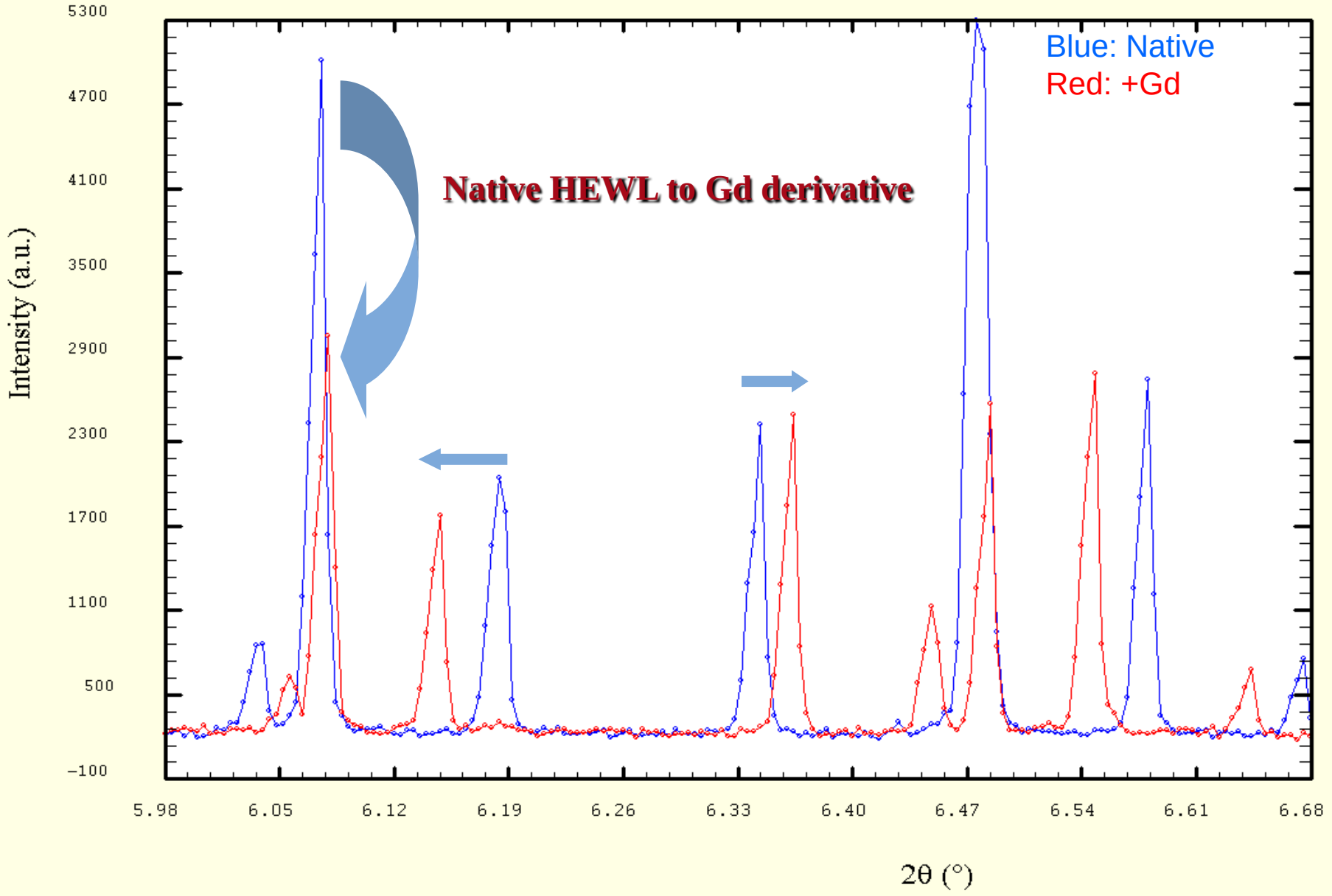
http://www.esrf.eu/news/general-old/general-2007/powder/

- Structure Solution via MIR

TEST SYSTEM

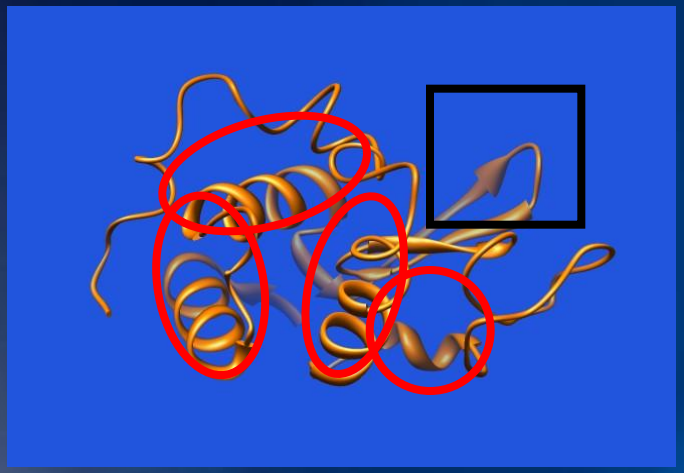
protein	Hen egg-white Lysozyme (HEWL) 
Molecular weight	14.4 kDa
Unit-cell (Å)	a=b=79.2 c=38.0
Space-group	$P4_32_12$
Relative root mean square intensity change (Crick and Magdoff)	Gd (Z=64) : 0.40

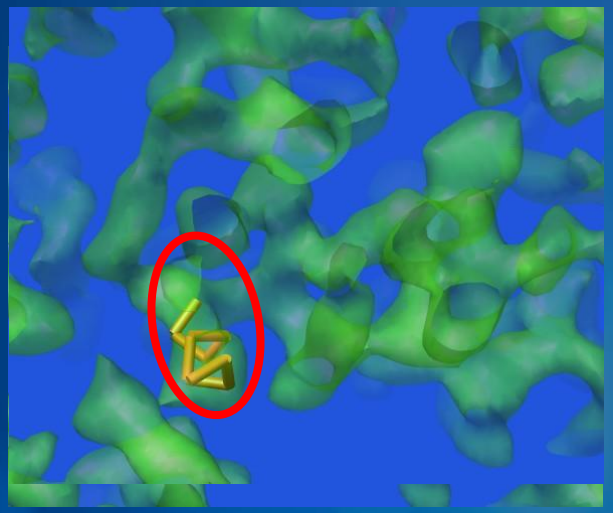
HEWL + Gd (Co-crystallisation)



Wright et al., J. Appl. Cryst. 41, 329-339 (2008) & ESRF Scientific Highlights, p. 61-62 (2006).

THE SECONDARY STRUCTURE

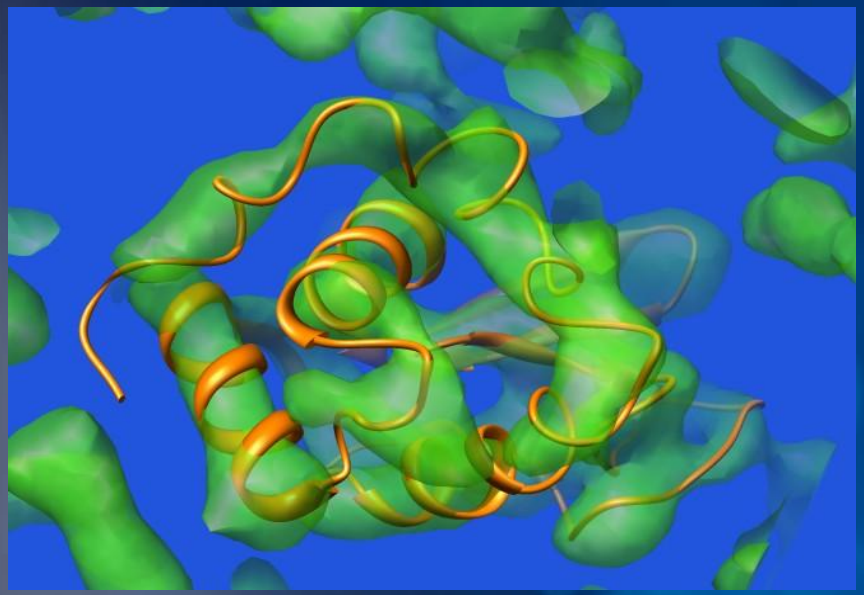


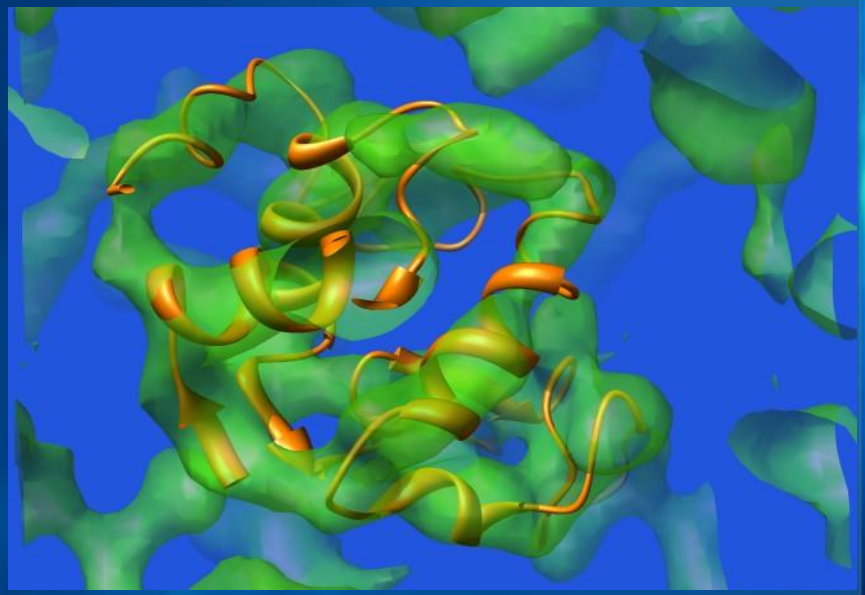


- Using FFFEAR we are able to locate all the four helices of the lyzosyme structure out of the five best fitted helices found by the program using the standard fragment library.

Basso et al. Acta Crystallogr D Biol Crystallogr. 2010; 66(Pt 7):756-61.

THE HEWL MIR DENSITY MAP





Images created by Sebastian Basso

http://infoscience.epfl.ch/record/167174

ASPECTS OF OUR STUDIES

- Polymorph Identification
- Crystal Screening

- Structure solution of novel polymorphs

3

- Structure refinement
- Ligand identification using powder data

KEYPOINTS

- COMBINED MULTIPLE PROFILES
- PERIODIC EXAMINATION OF TOTAL OMIT MAPS GENERATED FOLLOWING BHAT'S PROCEDURE
- SYNERGY OF VARIOUS SOFTWARE PACKAGES

Bhat, T. N. Calculation of an OMIT map. J. Appl. Cryst., 21, 279-281 (1988,)
I. Margiolaki & J. P. Wright, Acta Cryst. A64, 169-180 (2008)
Wright et al. Z. Kristallogr. Suppl. 26, 27-32 (2007)

SOFTWARE USED

Powder Diffraction & CCP14

Area detector data integration
Fit2D

Indexing
DASH

Indexing & Pawley fit
HighScorePLUS

Data viewer
FULLPROF

Structure Rietveld refinement
GSAS

Single Crystal & CCP4

CCP4 software package
Molecular Replacement
MOLREP
PHASER

Structure Refinement
CNS
REFMAC
PHOENIX

Visualization Tools
WINCOOT
PYMOL
CHIMERA

Home made ESRF, APS

Pawley fit
PRODD

Total Omit maps
SFCHECK
(modified version)

Data Extraction from Synchrotron
Short routines in PYTHON
Pycluster
ID31sum

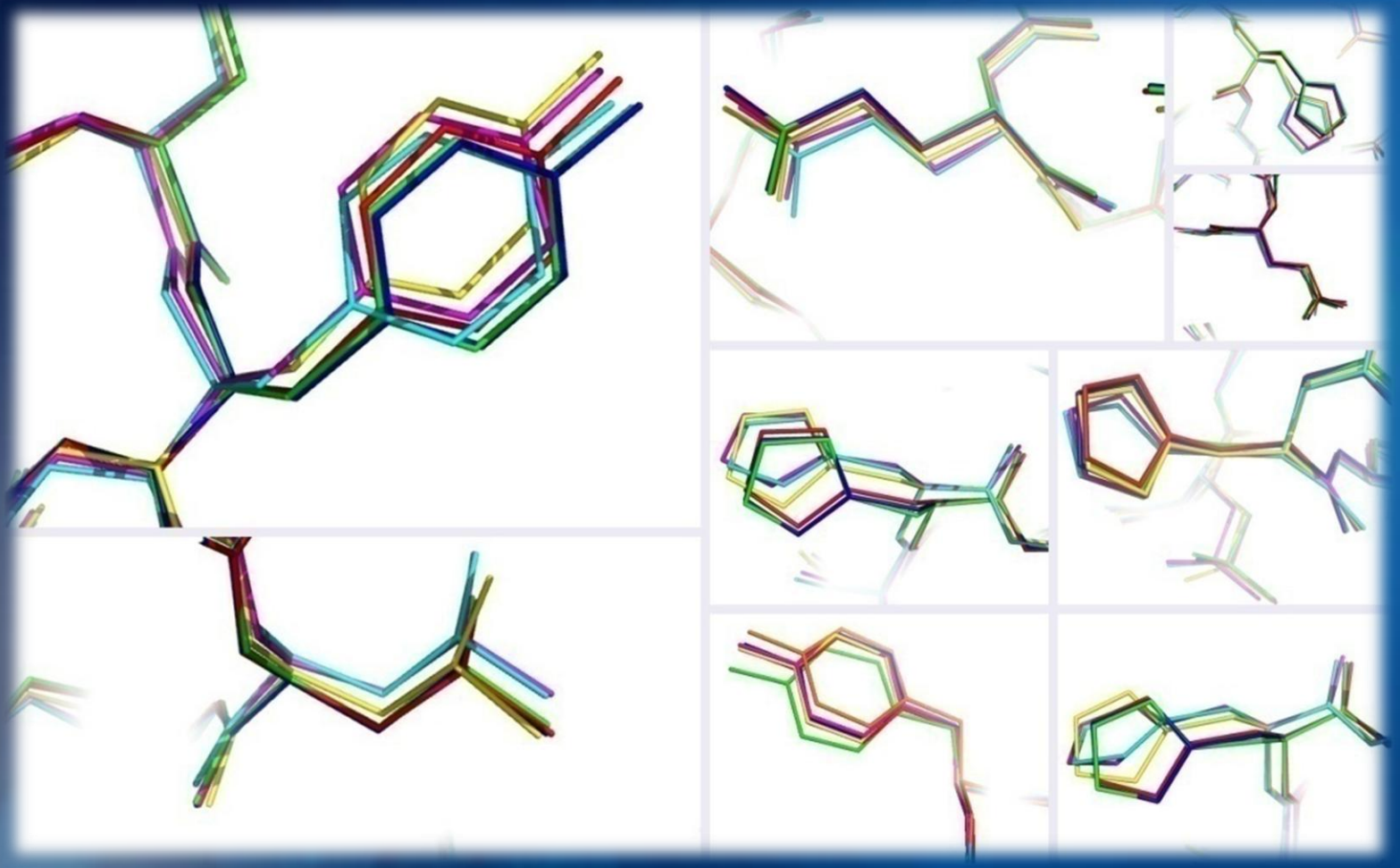
Structure Refinement

The Flexible Rigid Body Approach in GSAS

Larson A. C. & Von Dreele R. B. (2004). General Structure Analysis System (GSAS), Los Alamos National Laboratory Report LAUR 86-748.

ENHANCED STRUCTURE REFINEMENTS

Rigid body description of amino acids

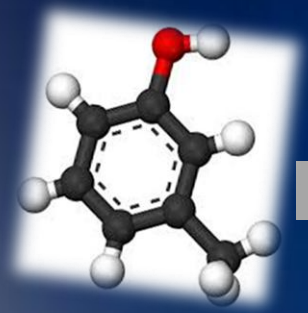


Margiolaki et al.
Acta Cryst. (2013). D69, 978–990

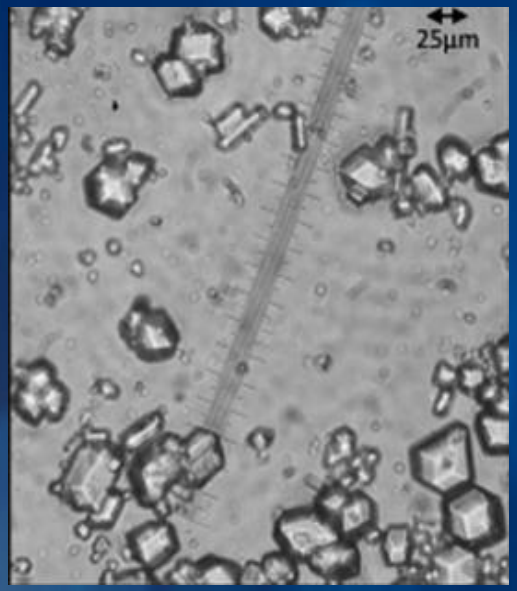
Human Insulin microcrystals

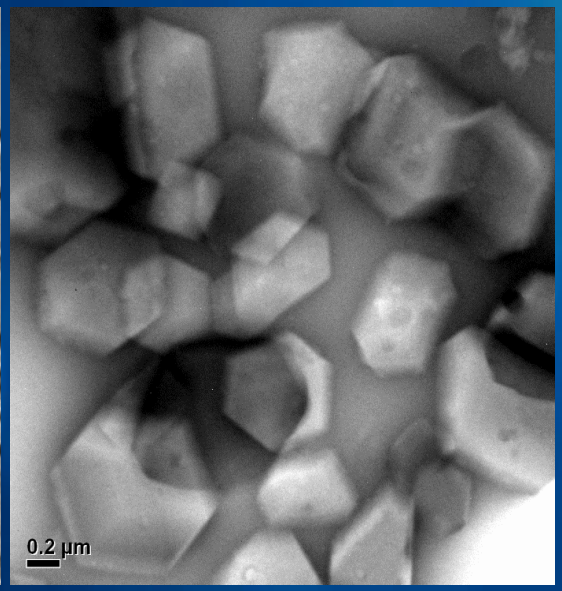
co-crystallised with **m-cresol**

THE KNOWN POLYMORPH R3 WITH ADDITIONAL LIGAND SITES (R6 CONFORMATION)



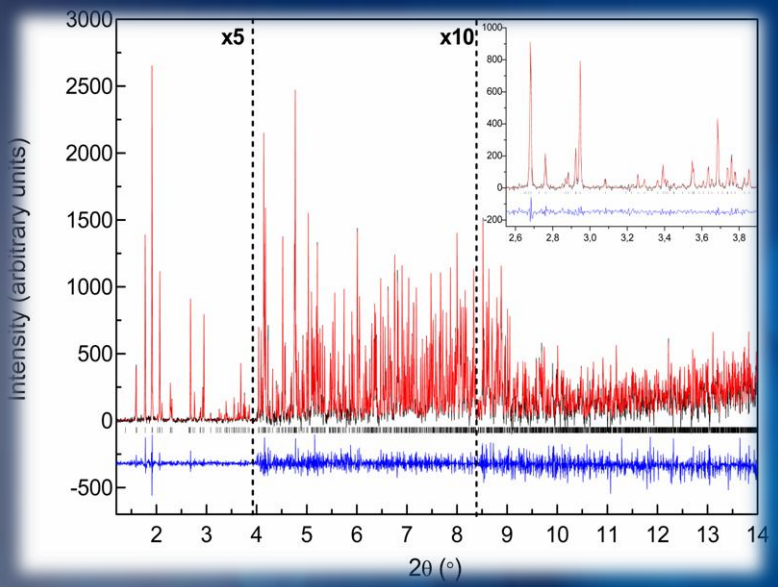
Optical Microscope	Transmission Electron Microscope

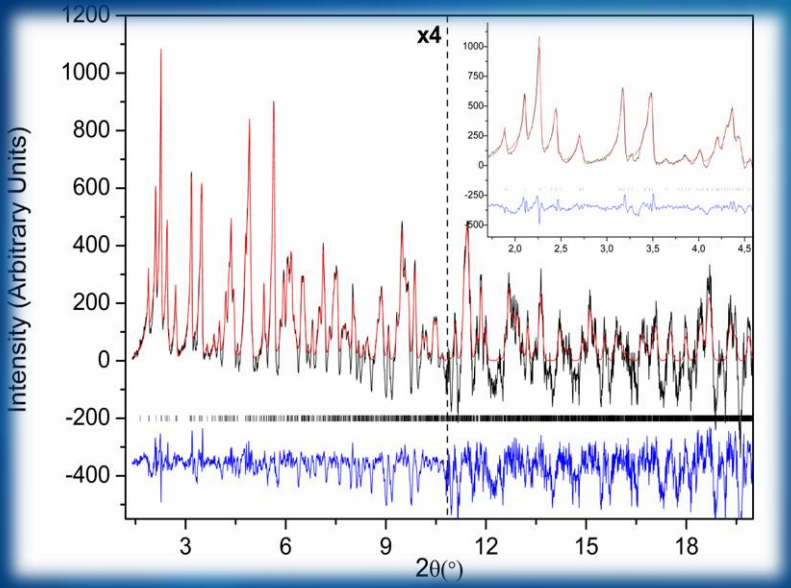


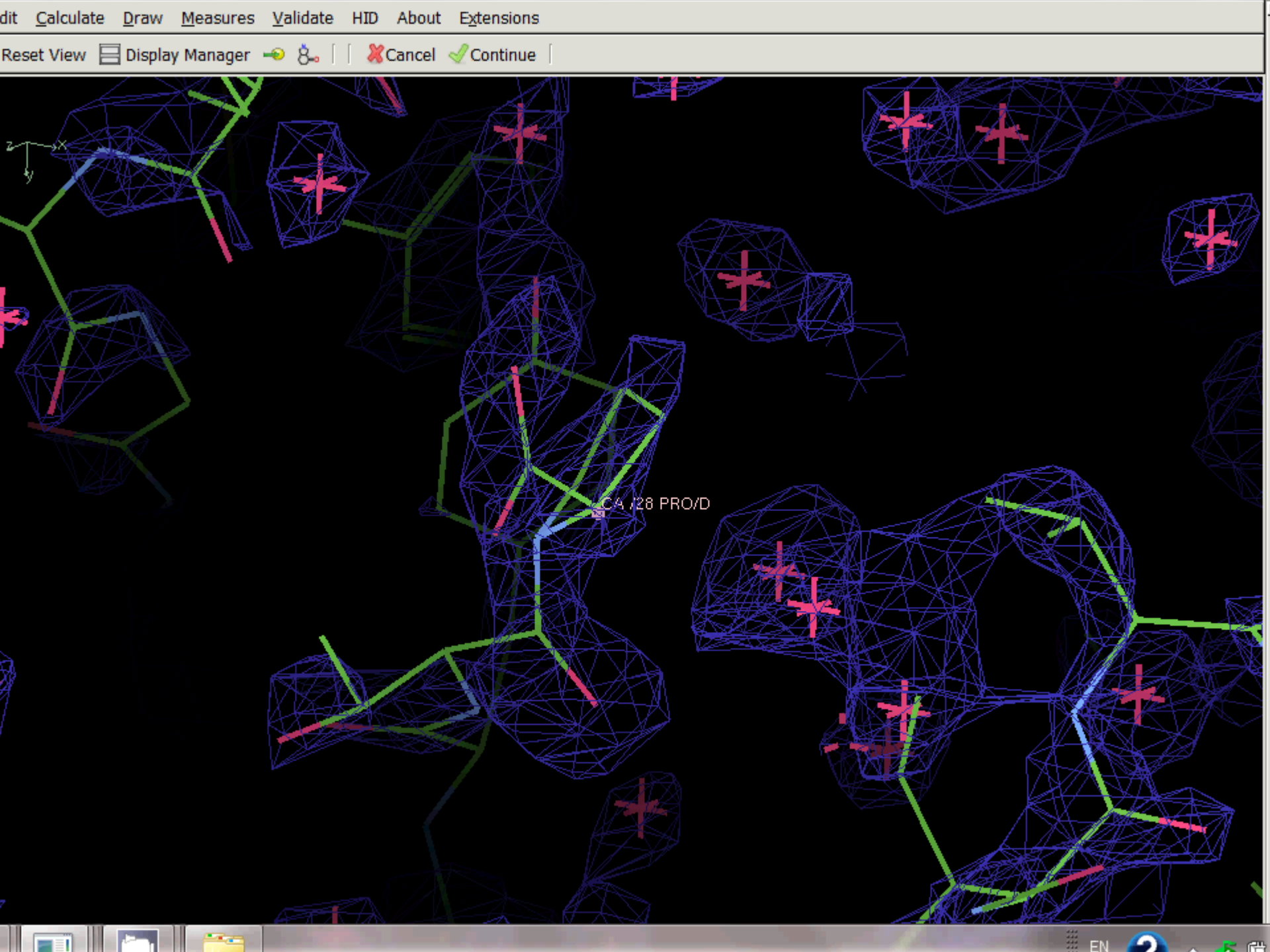


FRB RIETVELD ANALYSIS:

8 COMBINED SR-XRPD AND LAB- XRPD PROFILES



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Reset View Display Manager Cancel Continue
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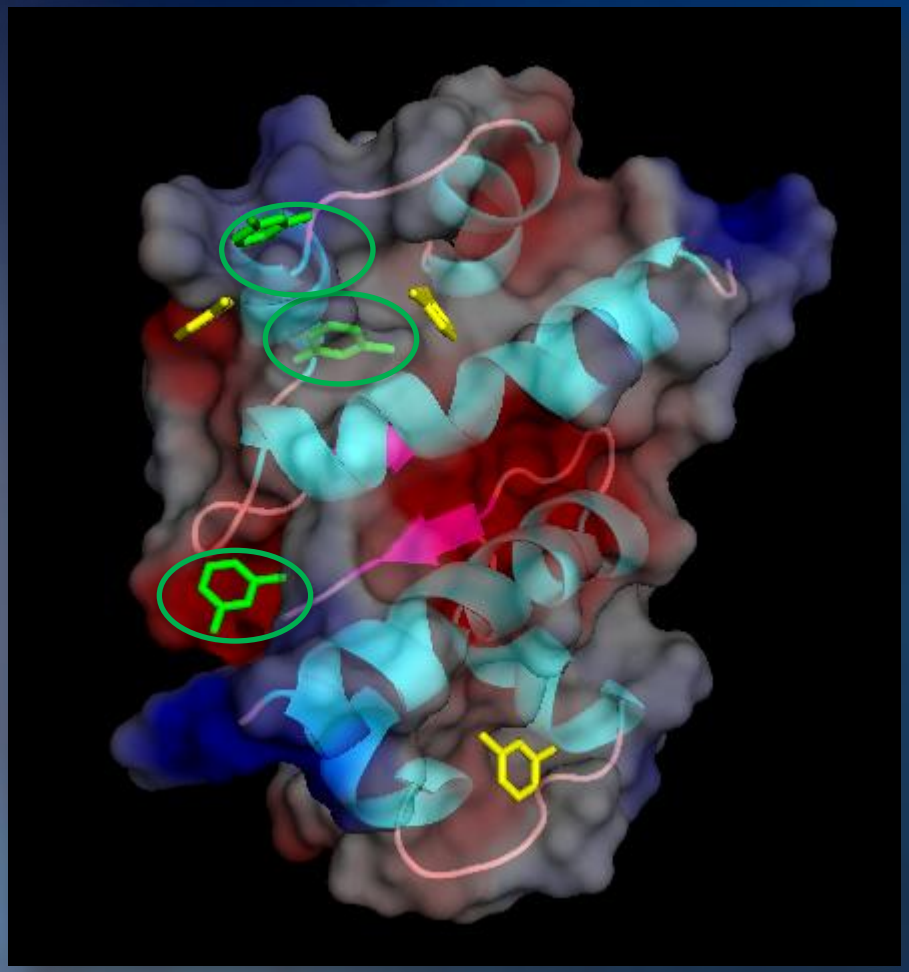
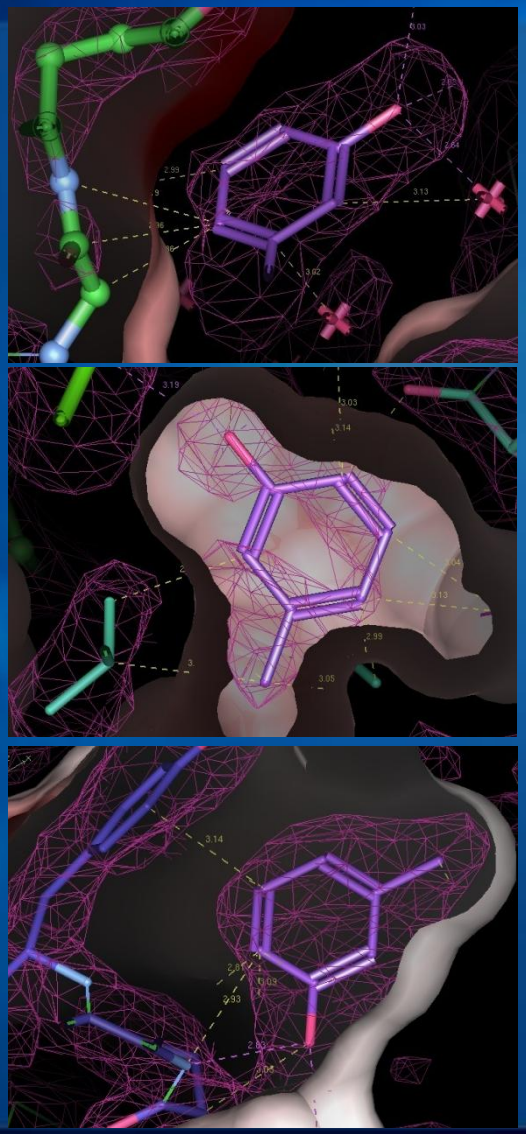
THE KNOWN POLYMORPH R3 WITH ADDITIONAL LIGAND SITES (R6 CONFORMATION)





Karavassili et al., In Preparation

- Future & Concluding Remarks

- **CRYSTAL SCREENING**

Polymorph Identification & Ligand Binding

Crystal size and morphology (XFEL)

Phase Mapping

- **COMBINED USE WITH**

XFEL measurements on nano-crystalline precipitates

Electron Diffraction on single nano-crystals

- Spence, Weierstall, Chapman , Rep. Prog. Phys. 75, 102601, 2012
Barty, Küpper, Chapman , Annual Review of Physical Chemistry 01/2013

- *Three-dimensional electron crystallography of protein microcrystals*
Shi et al., 2013, eLIFE

ACKNOWLEDGMENTS

X-RAY DIFFRACTION

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Bertrand Castro

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2012-2015
CRP code: F12024

UNESCO & L'OREAL Foundations
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Fellowship for Women in Science
2010-2012

Nanomegas
Stavros Nicolopoulos
2012-Present

EU & University of Patras
• FP7: SEE-DRUG
PI: George Spyroulias
http://www.seedrug.upatras.gr/
2012-2015

• Aristeia II
PI: Irene Margiolaki
2014-2015

• COST
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2014-2018