# Analysis of Microwave-Assisted Enzymatic Digests of Hemoglobin by Mass Spectrometry

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# Background

- Increasing numbers of applications to quantify peptides and proteins in clinical and environmental chemistry created a demand for rapid identification and quantification of proteins and protein modifications. Most of these applications focus on modifications of the N-terminal valine of hemoglobin.
- The standard procedure for obtaining information about these hemoglobin modifications is digesting the protein with endo-proteases and analyzing resulting peptides by mass spectrometry.
- The digestion time depends on the nature of the proteins and enzyme, and varies from hours to days. Thus, digestion time has become a limiting factor in the speed of the protein identification and quantification processes.
- Previous research using other proteins indicated microwave energy is a viable technology in accelerating protein digestion and increasing sample throughput. However, its applicability to protein quantification still remains unknown.

# Objective

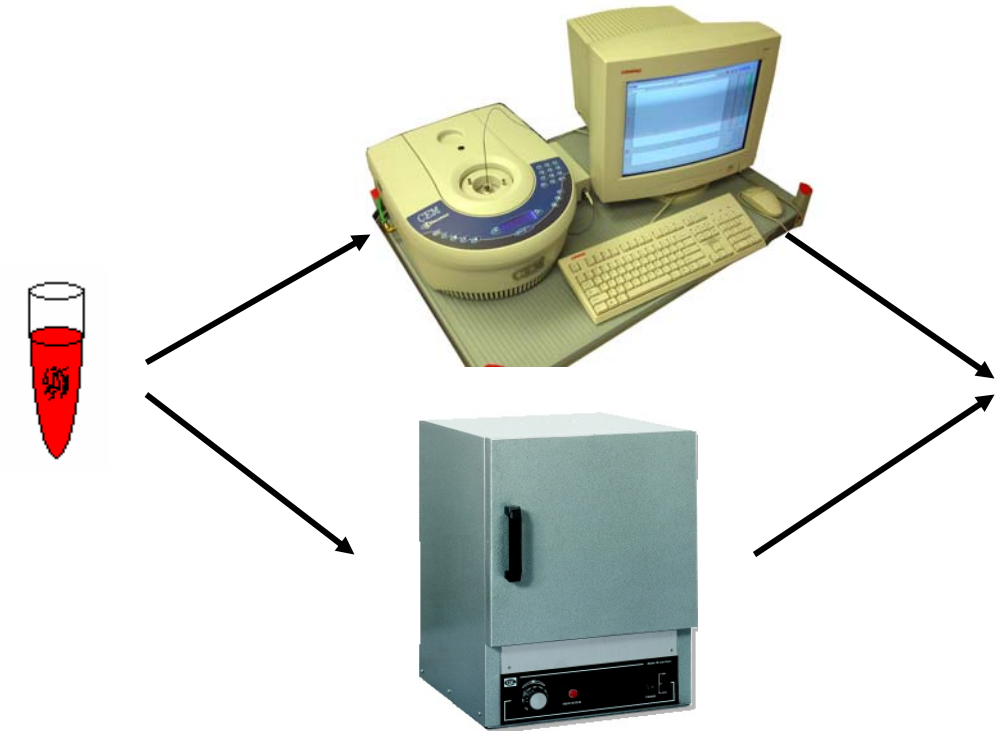
- Fully-controlled, programmed, and focused microwave energy may greatly accelerate enzymatic digestions. This study assesses this technology for its applicability to protein quantification study.
- The main objective is to reduce digestion time of hemoglobin using this new technology while maintaining the digestion efficiency and reproducibility of conventional digestion.

# Methods

- Enzymatic digestion of hemoglobin was carried out with trypsin.
- Microwave digestion was performed with a CEM® Discover™.
- Reaction products were analyzed on a Finnigan LCQ™ DECA ion trap mass spectrometer.
- Hexapeptide (VHLTPE, N-terminal hexapeptide of the β-chain of hemoglobin) was used as the internal standard (IS) since it is not affected by trypsin.
- The same amount of IS and protein were used in all experiments, so the digestion efficiency can be estimated as the area ratio between the N-terminal octapeptide of the β-chain of hemoglobin and the IS. The doubly-charged ions for both peptides were selected to assess the extent of the digestion due to their better S/N ratio.

# Different Digestion Methods

Microwave-Assisted Digestion



Conventional Digestion

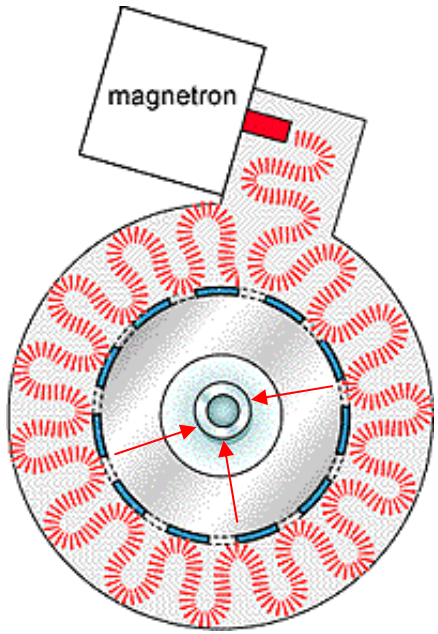
# LC/MS Details

- Samples were injected onto a 2-mm i.d., C-12, reverse phase column (Phenomenex Jupiter 4μ Proteo 90 Å).
- Gradient: from 100%A (A = 0.025% TFA in water and B = 0.025% TFA in acetonitrile) to 40%A over 100 min., flow rate: 200 μL/min.
- Full MS scan was selected for all experiments.
  - Mass range: 150-2000 m/z
  - Spray voltage of ESI source: 5.5 kV
  - Sheath gas: 80 (arbitrary unit)
  - Auxiliary gas: 20 (arbitrary unit)
  - Inlet capillary temperature: 175 °C
  - Inlet capillary voltage: 25 V

# Microwave Instrument

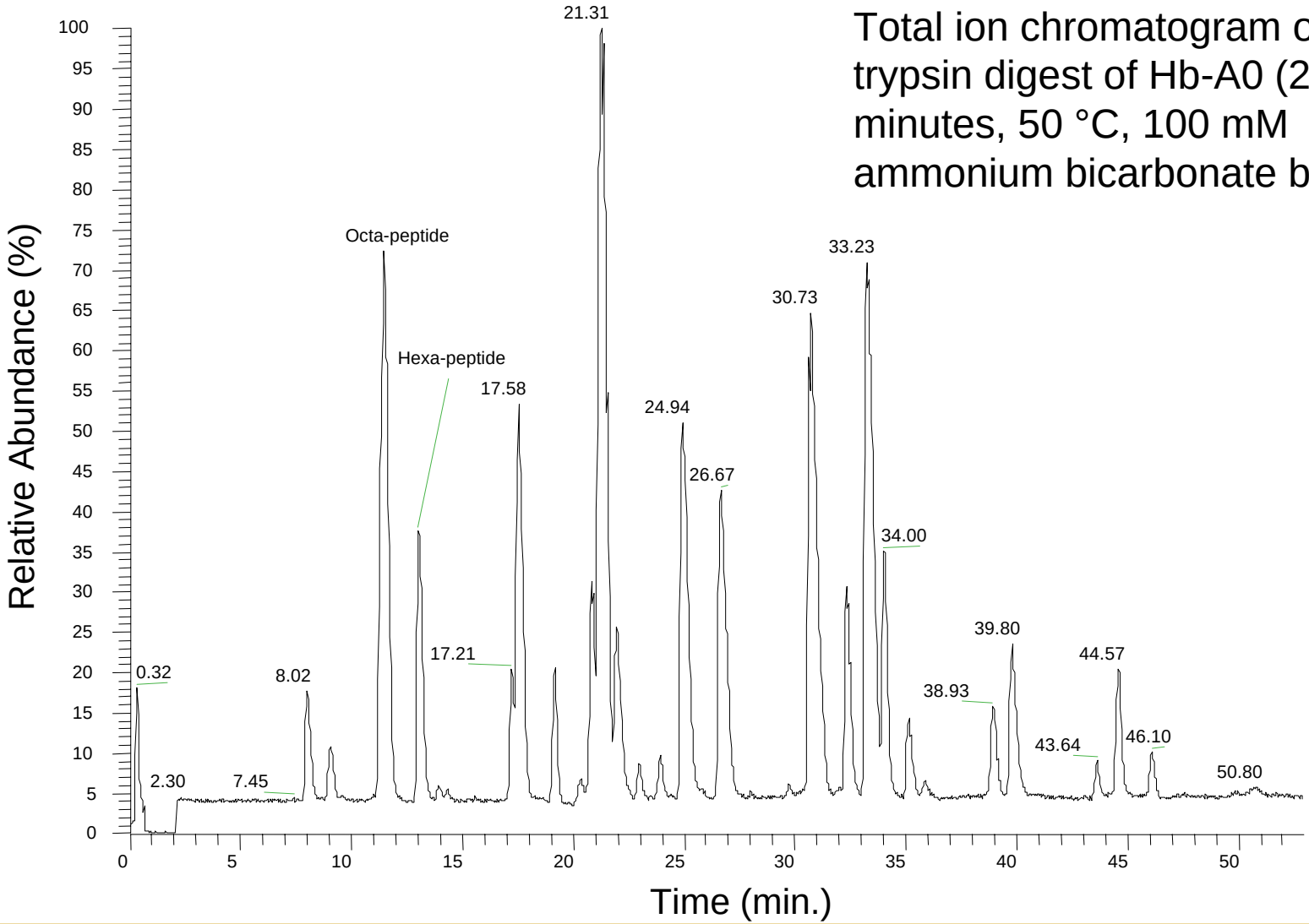
Key Features:

- Single-mode cavity design
- Temperature and pressure feedback control
- Vessel flexibility
- Reaction quenching




Microwaves couple directly with the molecules present in the reaction mixture, leading to a rapid rise in temperature and increasing the reaction rate.

# Example Chromatogram

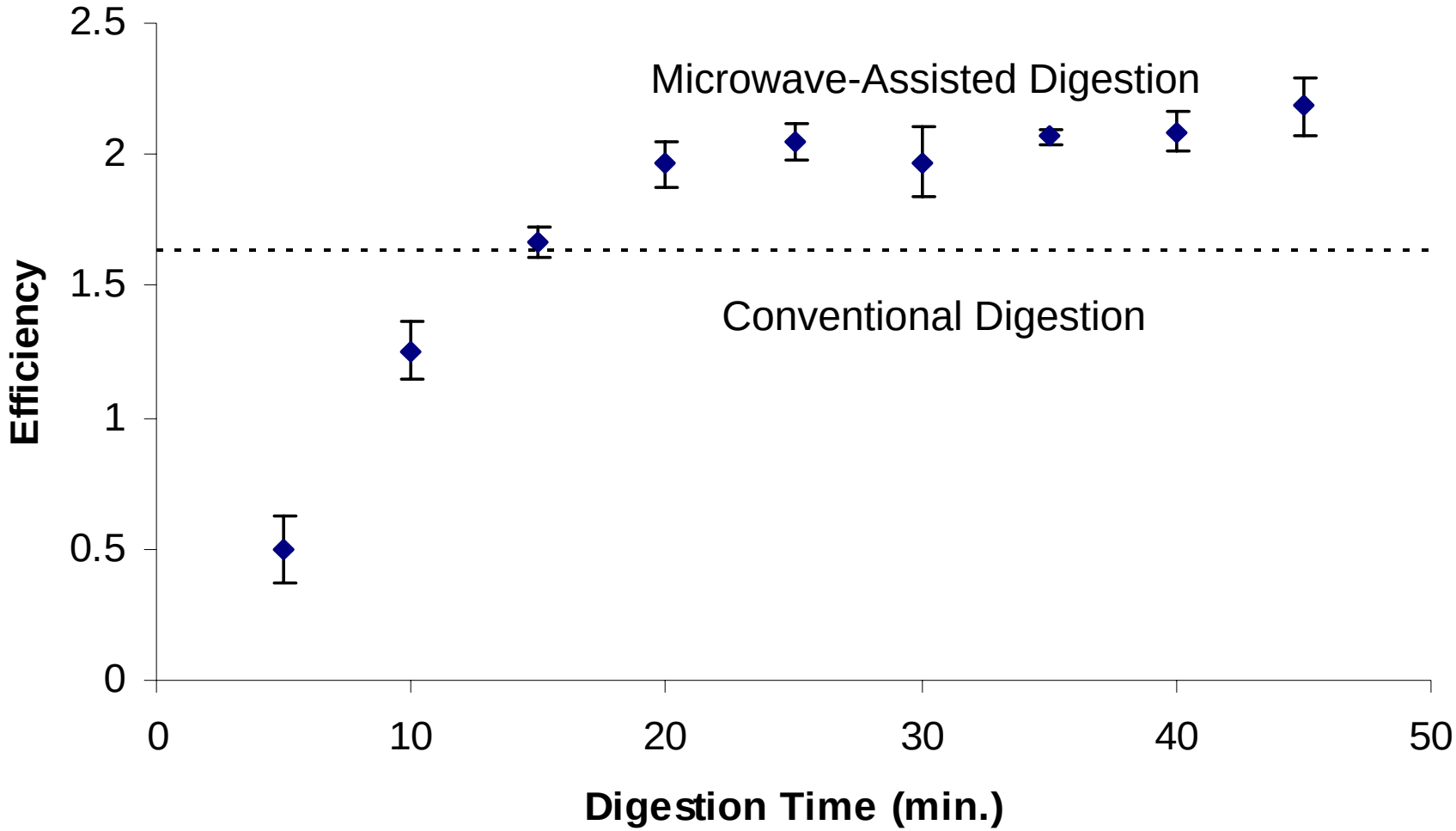



Total ion chromatogram of trypsin digest of Hb-A0 (20 minutes, 50 °C, 100 mM ammonium bicarbonate buffer)

# Experiment Details

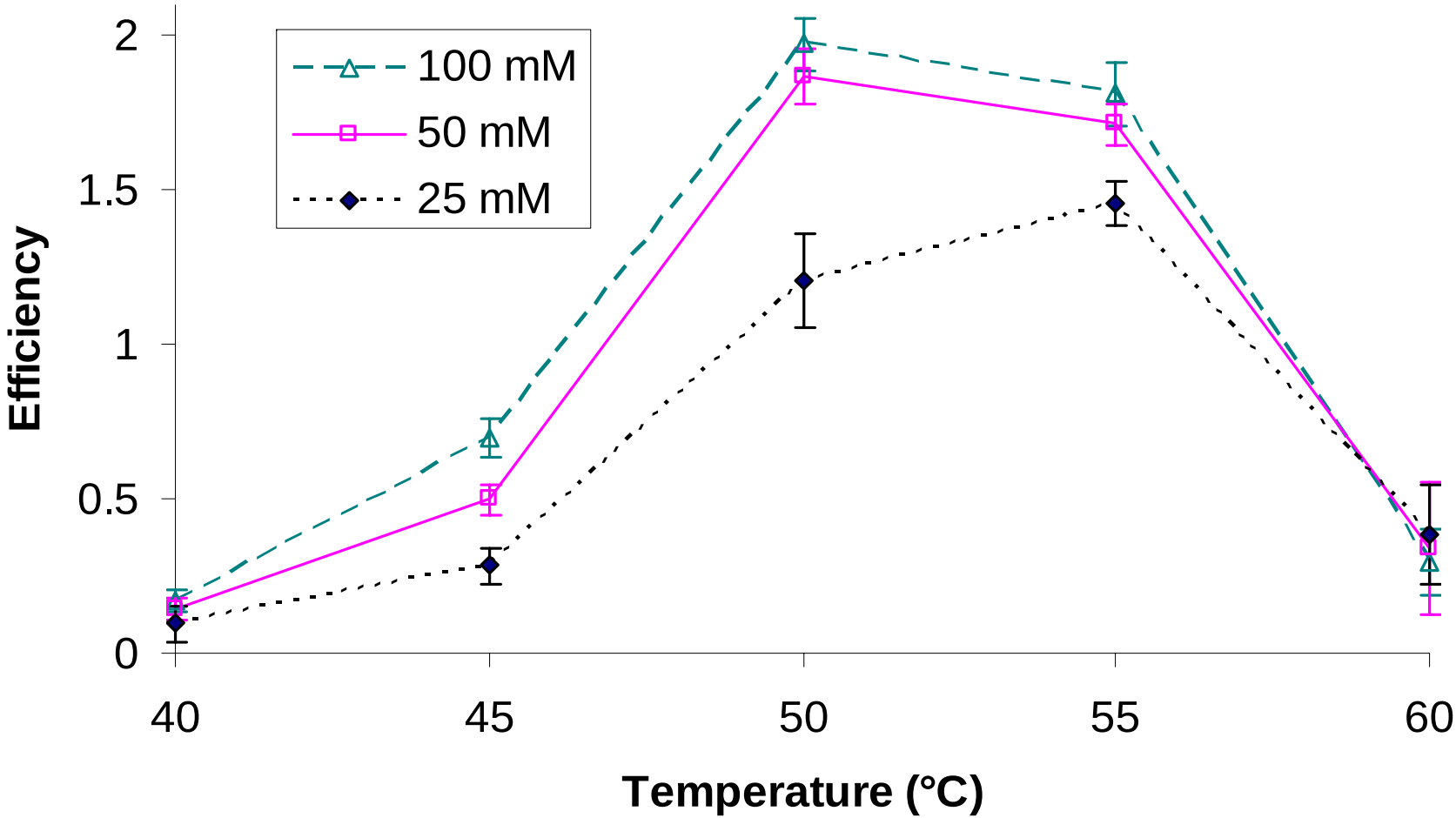
- Hemoglobin-A0 was treated with trypsin at a protease-to-protein ratio (w/w) of 1:25, 1:50, 1:100, and 1:200.
- Ammonium bicarbonate buffer concentrations were 25, 50, and 100 mM (pH: 8.5).
- Microwave irradiation was performed at controlled temperatures of 40, 45, 50, 55, and 60 °C.
- Microwave irradiation was performed for a duration of 5 to 45 minutes, in steps of 5 minutes.
- Conventional digestion was performed at 37 °C for 18 hours.

# Efficiency vs. Digestion Time



# Effect of Temperature & Buffer Conc.

## (20-min. digestion time)



# Results

- Digestion efficiency of hemoglobin-A0 reached plateau after 20 minutes.
- The highest degree of digestion occurred at 50 °C.
- Increasing buffer concentration from 25 mM to 50 mM resulted in a significant efficiency gain (50% at 50 °C), but increasing it further to 100 mM resulted only in a minor increase in efficiency ( 6%).
- Increasing the protease-to-protein ratio resulted only in a small increase in digestion efficiency (13 % gain from 1:200 to 1:25 ratio).
- The observed digestion efficiency at optimal conditions was higher than that of conventional digestion (2.0 vs. 1.6).
- The reproducibility at optimal conditions was 5% CV.

# Discussion

- Microwave-assisted protein digestion is described in literature mainly for peptide-mapping and qualitative protein analysis[1,2,3]. The described optimal digestion conditions are similar to those found in this study (60 vs. 50 °C, 10 vs. 20 min.). Differences may mainly be due to the use of different proteins.
- The reproducibility was found to be comparable to conventional methods.
- The digestion time is profoundly reduced with this technology (20 min. vs. 18 hours) at increased efficiency, making this technology an interesting alternative to conventional digestion.
- This study focused on N-terminal octapeptide of the β-chain of hemoglobin-A0 only. The digestion efficiency with regard to other peptides remains to be assessed.

# Conclusion

**Microwave-assisted digestion of hemoglobin A0 with trypsin is an alternative to conventional digestion technology. The main advantages seem to be:**

- Shorter digestion time (20 minutes vs. 18 hours)
- Increased efficiency (2 vs. 1.6)
- Good reproducibility (5% CV)

# References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2. J. L. Rutherford; J. Bonapace; M. L. Nguyen; T. Pekar; J. Pirro High Throughput Protein Identifications Utilizing Microwave Protein Digests and Off-line Nano-spray Chip Technology. Keystone Symposium for Mass Spectrometry in Systems Biology, February 14-19, 2004 in Santa Fe, NM
3. J. L. Rutherford; J. Bonapace; M. L. Nguyen; T. Pekar; D. Hinerfeld; J. Pirro Enhanced Protein Identifications Utilizing Microwave Protein Digestions and Off-line Nano-spray Chip Technology. Keystone Symposium for Mass Spectrometry in Systems Biology, February 14-19, 2004 in Santa Fe, NM

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Questions? Comments?


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