



100 Majestic Way, Bangor, PA 18013 / www.biospectra.us

MOPS TESTING METHODS

TABLE OF CONTENTS

| | |
|---------------------------------|---|
| 1. PURPOSE:..... | 3 |
| 2. SCOPE:..... | 3 |
| 3. RESPONSIBILITIES: | 3 |
| 4. REFERENCES:..... | 3 |
| 5. REAGENTS:..... | 3 |
| 6. EQUIPMENT:..... | 4 |
| 7. ANALYTICAL PROCEDURES: | 5 |

1. PURPOSE:

- 1.1. To provide the Quality Control (QC) Laboratory personnel with procedures for testing MOPS, Raw Material, In-Process and Finished Goods.

2. SCOPE:

- 2.1. Applies to the testing of MOPS, Raw Material, In-Process and Finished Goods in the QC Laboratory. Methods include testing for all types of MOPS sold by BioSpectra. Only the specific tests required for the requested type must be tested.

3. RESPONSIBILITIES:

- 3.1. The Laboratory Manager is responsible for the implementation, control, training and maintenance of this procedure.
- 3.2. The QC Analysts are responsible for compliance with the terms of this procedure. This includes notifying the appropriate personnel if any analyses fail to meet their respective specifications.
- 3.3. All QC Laboratory personnel are responsible for reviewing the appropriate SDS's prior to handling any chemicals used in this procedure.

4. REFERENCES:

- 4.1. BSI-ATM-0073, Analytical Method of Analysis: Guanidine Thiocyanate, MOPS, and Urea via ICP-MS
- 4.2. BSI-ATM-0089, Analytical Method of Analysis: Trace Metals in Finished Goods Products by ICP-OES
- 4.3. BSI-SOP-0090, Lambda 25 UV/Vis Operation and Calibration
- 4.4. BSI-SOP-0091, Portable Turbidimeter and Calibration SOP
- 4.5. BSI-SOP-0094, Muffle Furnace SOP and Calibration
- 4.6. BSI-SOP-0095, DNase (Endonuclease) Assay
- 4.7. BSI-SOP-0096, RNase (Ribonuclease) Assay
- 4.8. BSI-SOP-0098, Balance SOP
- 4.9. BSI-SOP-0126, Laboratory Notebooks
- 4.10. BSI-SOP-0138, DNase (Exonuclease) Assay
- 4.11. BSI-SOP-0139, Protease Assay
- 4.12. BSI-SOP-0140, Standardization of Titrants
- 4.13. BSI-SOP-0143, Metrohm Titrando 907 Auto-Titrator SOP
- 4.14. BSI-SOP-0254, Spectrum Two UATR SOP
- 4.15. BSI-SOP-0255, XL200 pH/mV/Conductivity Meter SOP
- 4.16. BSI-SOP-0303, NexION 350X ICP-MS SOP
- 4.17. BSI-SOP-0345, Endosafe nexgen PTS Endotoxin Reader SOP
- 4.18. BSI-SOP-0362, Operation and Maintenance of the Perkin Elmer Avio 500 ICP-OES
- 4.19. Current EP/BP
- 4.20. Current JP
- 4.21. Current *USP*

5. REAGENTS:

- 5.1. Acetate Buffer, pH 3.5 - Dissolve 62.5 g of ammonium acetate in 62.5 mL of purified water, and add 47.0 mL of concentrated hydrochloric acid. Adjust, if necessary, with 6N ammonium hydroxide or 6N hydrochloric acid to a pH of 3.5, dilute with purified water to make 250 mL.
- 5.2. Acetic Acid, 1N- Dilute 6g of glacial acetic acid to 100g with Purified water.
- 5.3. Ammonium Hydroxide, 6N-Dilute 206.9 mL of Ammonium Hydroxide 29% to 500mL final volume with purified water
- 5.4. Ammonium Peroxydisulfate- Purchased Commercially

- 5.5. Ammonium Thiocyanate, 30%-Weigh 30g and dilute to 100ml with purified water.
- 5.6. Barium Chloride TS-Dissolve 12g of barium chloride dehydrate in purified water. Filter and dilute to make a total volume of 100mL with purified water.
- 5.7. Composite 5- Purchased Commercially
- 5.8. Formamide, dry – Purchased Commercially
- 5.9. Hydrochloric Acid, Concentrated- Purchased Commercially
- 5.10. Iron Standard (10ppm)-Dissolve 863.4mg of ferric ammonium sulfate dodecahydrate to 100mL with purified water that contains 10mL of Sulfuric Acid, 2N. Pipette 10mL of this solution into a 1L volumetric flask that contains 10mL of 2N sulfuric acid, then dilute to volume with purified water.
- 5.11. Glycerin Base TS – To 200 g of glycerol, add purified water to bring the total weight to 235 g. Add 140 mL of 1N Sodium Hydroxide and 50 mL of purified water.
- 5.12. Hydrochloric Acid, 0.02N- Slowly add 20 mL of 0.1N hydrochloric acid to 80 mL of purified water to make a total volume of 100 mL
- 5.13. Hydrochloric Acid, 3N- Pipette 25.75mL of concentrated hydrochloric acid and transfer to a 100mL volumetric flask that contains a small amount of purified water. Dilute to volume with purified water.
- 5.14. LAL Water- Purchased Commercially
- 5.15. Lead Nitrate Stock Solution – Dissolve 0.1598 g of lead nitrate in 100 mL purified water and add 1 mL of nitric acid. Dilute with purified water to 1000mL. Store in glass container free from soluble lead salts.
- 5.16. Methanol, dry – Purchased Commercially
- 5.17. MOPS UATR Reference Standard: Dry an LOD vial in an oven at 105°C for 30 minutes. Cool the vial in a desiccator for 15 minutes and weigh the vial on the analytical balance. Tare the vial and weigh approximately 5.0g of MOPS from an outside source into the vial. Dry the vial at 105°C for 4 hours. Allow the standard to cool, cap, and store in a desiccator. Perform a UATR analysis on the Reference Standard and compare it to a previously approved reference scan. The correlation must be 0.95 or greater between the two scans.
- 5.18. Nitric Acid- Purchased Commercially
- 5.19. Platinum Cobalt Color Standard (10 APHA)-Dilute 2mL of Platinum Cobalt Color Standard to 100mL with purified water.
- 5.20. Potassium Hydrogen Phthalate (KHP) Standard – Prepare a vial at 120°C for 30 minutes. Allow to cool in a desiccator and weigh a maximum of 10.0g of Potassium Hydrogen Phthalate. Dry at 120°C for 2 hours. Cool and store in desiccator in a closed container. Stable for 3 months.
- 5.21. Silver Nitrate, 0.1N- Purchased Commercially
- 5.22. Sodium Hydroxide, 0.1N- Purchased Commercially
- 5.23. Sodium Hydroxide, 1N-Purchased Commercially
- 5.24. Sulfuric Acid- Purchased Commercially
- 5.25. Sulfuric Acid, 0.02N – Slowly add 20 mL of 0.1N sulfuric acid to 80 mL of purified water to make a total volume of 100 mL.
- 5.26. Thioacetamide TS – Dissolve 4.0 g of Thioacetamide in 100 mL of purified water.

6. EQUIPMENT:

- 6.1. Analytical Balance or equivalent
- 6.2. Blue M Oven or equivalent
- 6.3. EndoSafe PTS Reader, or equivalent
- 6.4. Hach Portable Turbidimeter
- 6.5. Lambda 25 UV/Vis Spectrophotometer, or equivalent
- 6.6. Metrohm Titrando 907 Auto-Titrator
- 6.7. Muffle Furnace
- 6.8. Perkin Elmer NexION350x
- 6.9. Spectrum Two UATR

6.10. XL200 pH/mV/Conductivity Meter

7. ANALYTICAL PROCEDURES:

7.1. IN-PROCESS MOTHER LIQUOR ABSORBANCE 1.500 a.u. max. at 260 and 280

nm:

- 7.1.1. Prepare at least 10 mL of a 1:1 dilution of the specified Mother Liquor sample into a small beaker.
- 7.1.2. Swirl to mix completely.
- 7.1.3. Refer to Lambda 25 UV/Vis Operation and Calibration to obtain the Absorbance of the sample.
- 7.1.4. Record results at specified wavelengths in the MOPS Analytical Procedure or In-Process MOPS ML Absorbance Log Book, whichever is applicable.
- 7.1.5. If failing results are obtained, notify the appropriate personnel immediately.

7.2. MOTHER LIQUOR ASSAY Monitor:

- 7.2.1. Perform a daily check or standardize 0.1N NaOH as per Standardization of Titrants.
- 7.2.2. Weigh 0.6g of ML sample and add 100 mL of water.
- 7.2.3. Allow sample to stir for allotted time within titration method to ensure the pH is stable before titration begins.
- 7.2.4. Titrate the sample potentiometrically, using 0.1 NaOH to the potentiometric endpoint at approximately 10.0 pH.
- 7.2.5. Assay Calculation:

$$\% \text{ MOPS} = \frac{(mL_{EP1}) \times (N_{NaOH}) \times (20.926)}{\text{Sample Weight (g)}}$$

7.3. IN-PROCESS KARL FISCHER 0.1% max:

- 7.3.1. Standardize Composite 5 as per Standardization of Titrants.
- 7.3.2. Grind the sample in a dry mortar into a fine powder utilizing a pestle.
- 7.3.3. Immediately weigh 0.8 g of sample into a glass weighing spoon and tare it.
- 7.3.4. Transfer the sample to the KF vessel by removing the rubber septum and adding the sample into the titration vessel.
 - 7.3.4.1. Do not leave the rubber septum open for long periods of time as this will allow moisture to enter the titration vessel.
- 7.3.5. Return the weighing spoon to the balance, making sure not to lose any sample that was left behind.
- 7.3.6. Once the weight stabilizes, transfer the weight to the instrument.
- 7.3.7. Check to make sure there is no residual sample stuck to the sides of the titration vessel.
 - 7.3.7.1. If there is any sample stuck to the side, gently swirl the vessel to rinse the sides of the vessel to collect residual sample. Be careful not to damage the probe. Ensure the sample is fully dissolved before the titration begins.
- 7.3.8. The moisture content will then be determined by the Metrohm Auto Titrando 907.

$$\% \text{Moisture} = \frac{(\text{mL of Composite 5}) \left(\frac{\text{mg}}{\text{mL}} \text{ of composite 5} \right) (0.1)}{\text{Sample Weight (g)}}$$

7.4. ABSORBANCE (0.1M) Refer to Summary Sheet:

- 7.4.1. Accurately weigh 0.52 g of sample.
- 7.4.2. Transfer accurately weighed sample to a 50-mL graduated cylinder and dilute to 25 mL with purified water.

- 7.4.3. Swirl to dissolve completely.
- 7.4.4. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the Absorbance of the sample.

7.5. APPEARANCE AND COLOR Refer to Summary Sheet:

- 7.5.1. Place 25-50g of the sample in a clean, dry glass beaker.
- 7.5.2. In an area with sufficient lighting, view the sample from all sides.
- 7.5.3. The sample should be white in color and characteristic of crystals.

7.6. ASSAY (Dried Basis), EQUIVALENT WEIGHT, AND PKA Refer to Summary Sheet:

- 7.6.1. Standardize 0.1N NaOH as per Standardization of Titrants
- 7.6.2. Weigh 0.6g of sample, previously dried at 105°C for 4 hours.
- 7.6.3. Dissolve in 100 mL of water.
- 7.6.4. Allow sample to stir for allotted time within titration method to ensure pH is stable before titration begins.
- 7.6.5. Titrate using 0.1 N Sodium Hydroxide to the potentiometric endpoint at approximately pH 10.0. The equivalent weight and pKa results are also reported on the printout.
- 7.6.6. Assay Calculation:

$$\% \text{ MOPS} = \frac{(mL_{EP1}) \times (N_{NaOH}) \times (20.926)}{\text{Sample Weight (g)}}$$

- 7.6.7. Equivalent Weight Calculation:

$$\text{Equivalent Weight} = \frac{(\text{Sample Weight (g)} \times 1000)}{(V_{\text{sample}})(N \text{ of NaOH})}$$

7.7. CHLORIDE Refer to Summary Sheet:

7.7.1. Sample Preparation:

- 7.7.1.1. Accurately weigh 1.0 g of sample.
- 7.7.1.2. Transfer accurately weighed sample to a 50-mL Nessler tube.
- 7.7.1.3. Add 40 mL of purified water to dissolve.

7.7.2. Standard Preparation:

- 7.7.2.1. Dilute 1.410 mL of 0.02N HCl to 100 mL with purified water and mix by inversion.
- 7.7.2.2. Pipette 5 mL of the solution to a 50-mL Nessler tube.
- 7.7.2.3. Add 40 mL of purified water.

7.7.3. Procedure:

- 7.7.3.1. To both the sample and standard solutions, add 1 mL of nitric acid and 1 mL
- 7.7.3.2. 0.1 N Silver Nitrate.
- 7.7.3.3. Dilute to 50 mL with purified water.
- 7.7.3.4. Cover with parafilm and mix by inversion.
- 7.7.3.5. Allow to stand for 5 minutes using a timer.
- 7.7.3.6. Compare turbidity of the sample and standard solutions.
 - 7.7.3.6.1. Any Turbidity produced in the sample solution should not exceed that produced by the standard to report as less than or equal to 0.005%.
 - 7.7.3.6.2. If a visible difference in the turbidity is not observed, utilize the Turbidimeter to measure the turbidity of the standard and the sample solutions. Follow Portable Turbidimeter SOP and Calibration or Bangor Portable Turbidimeter SOP and Calibration.

- 7.8. ENDOTOXIN Refer to Summary Sheet:**
- 7.8.1. Accurately weigh 25mg (0.025g) of sample in to a sterile capped conical tube or other suitable sterile vessel.
 - 7.8.2. Add 45µL of 1N NaOH
 - 7.8.3. Dissolve and dilute to 10mL with LAL reagent water.
 - 7.8.3.1. The sample preparation may be scaled to desired volume.
 - 7.8.4. Log in to the Endosafe nexgen-PTS Endotoxin Reader and follow the prompts on the instrument.
 - 7.8.5. When entering the sample information, enter sample solution concentration in g/mL.
 - 7.8.5.1. Calculation: (Sample Weight g)/10mL
 - 7.8.6. Ensure g/mL is the selected unit on the review screen.
 - 7.8.7. Enter specification of 12.5EU/g.
 - 7.8.8. Ensure all data is accurate before proceeding to load sample.
 - 7.8.9. Load 25µL of sample in to each well and run test.
 - 7.8.10. Ensure all suitability requirements meet specification.
 - 7.8.11. Report result directly from instrument printout.
- 7.9. ENZYME ACTIVITY Refer to Summary Sheet:**
- 7.9.1. RNase, DNase, and Protease as per SOPs.
- 7.10. IDENTIFICATION (UATR) Refer to Summary Sheet:**
- 7.10.1. Follow Spectrum Two UATR SOP.
- 7.11. HEAVY METALS Refer to Summary Sheet:**
- 7.11.1. Refer to 7.22 Trace Metals for primary analysis.
 - 7.11.2. Alternate Wet Method:
 - 7.11.3. Standard Lead Solution: On the day of use, dilute 10.0 mL of Lead Nitrate Stock Solution with purified water to 100.0 mL in a volumetric flask.
 - 7.11.4. Standard Preparation: Pipette 2 mL of Standard Lead Solution into a 50-mL Nessler tube and dilute with purified water to 25 mL.
 - 7.11.5. Sample preparation: Dissolve 4.0 g of sample in 25 mL of purified water in a 50-mL Nessler tube.
 - 7.11.6. Procedure:
 - 7.11.6.1. Adjust the pH between 3.0 and 4.0 using 1N acetic acid or 6N ammonium hydroxide, parafilm, and mix by inversion.
 - 7.11.6.2. To all solutions add 2 mL of pH 3.5 Acetate Buffer and 1.2 mL of thioacetamide-glycerin base TS (1mL of glycerin TS and 0.2 mL of thioacetamide TS gently heated for 20 seconds). Dilute to 50 mL with purified water and mix by inversion.
 - 7.11.6.3. Allow to stand for 2 minutes using a timer. The color of the solution from the Test Preparation is not darker than that of the solution from the Standard Preparation when viewed downward over a white surface to report as less than or equal to 5 ppm.
- 7.12. IRON (Fe) Refer to Summary Sheet:**
- 7.12.1. Refer to 7.22 Trace Metals for primary analysis.
 - 7.12.2. Alternate Wet Method:
 - 7.12.3. Dissolve 2g of sample in 20 mL of purified water.
 - 7.12.4. Prepare a standard by pipetting 1.0 mL of 9.94 ppm Fe standard and dilute to 20 mL with purified water.
 - 7.12.5. To both, add 2 mL of concentrated hydrochloric acid and dilute to 50 mL with purified water

- 7.12.6. To both, add 0.03-0.05g of ammonium peroxydisulfate crystals and 3 mL of 30% ammonium thiocyanate.
- 7.12.7. Any red color produced by the sample solution must not exceed that produced by the standard solution to report as less than or equal to 5 ppm.

7.13. LOSS ON DRYING @ 105°C Refer to Summary Sheet:

- 7.13.1. Dry an LOD vial in an oven at 105 ± 2°C for 1 hour. Cool for at least 15 minutes in a desiccator.
- 7.13.2. Place the vial on the analytical balance, record the weight, and tare the dried vial. Weigh
- 7.13.3. 1.5 g of sample and record results.
- 7.13.4. Dry for 4 hours at 105 ± 2°C. Cool for at least 15 minutes in desiccator.
- 7.13.5. Retain Sample for Assay, dried basis.
- 7.13.6. Reweigh and calculate the % LOD.

$$\% \text{ Loss on Drying} = \frac{\text{Initial}(g) - \text{Final}(g)}{\text{Initial}(g)} \times 100$$

7.14. OPTICAL DENSITY Refer to Summary Sheet:

- 7.14.1. Weigh 10 grams of sample into a beaker.
- 7.14.2. Pipette 20.0 mL of purified water into the beaker.
- 7.14.3. Heat the beaker until the sample is dissolved.
- 7.14.4. Allow the sample preparation to return to room temperature.
- 7.14.5. Measure the absorbance of the sample from 290-350 nm using the Lambda 25 UV/Vis.
- 7.14.6. Optical Density = A₂₉₀-A₃₅₀

7.15. pH (1%) @25°±2°C Refer to Summary Sheet:

- 7.15.1. Prepare a 1% solution of the specified sample.
 - 7.15.1.1. Dissolve 1.0 of sample in 100 mL of purified water.
 - 7.15.1.2. Swirl to dissolve completely.
 - 7.15.1.3. Follow the appropriate SOP for calibration and pH measurement.

7.16. pH (2.5M) @25°±2°C Refer to Summary Sheet:

- 7.16.1. Prepare a 2.5M solution of the specified sample.
 - 7.16.1.1. Accurately weigh out 13.08g of sample. Transfer sample to a 50 mL graduated cylinder and QS to 25 mL with purified water.
 - 7.16.1.2. Swirl to dissolve completely.
 - 7.16.1.3. Follow the appropriate SOP for calibration and pH measurement.

7.17. RESIDUE ON IGNITION Refer to Summary Sheet:

- 7.17.1. Turn on muffle furnace and allow it to stabilize at 600 degrees Celsius. Follow Muffle Furnace SOP.
- 7.17.2. Inspect a quartz crucible for cracks, chips and discoloration.
- 7.17.3. Utilize the 10 inch forceps to insert and remove a crucible from the furnace. Do not touch any surface of the furnace, or you will get burned.
- 7.17.4. Ignite quartz crucible at 600 ± 50 degrees Celsius for 30 minutes, cool in a desiccator for one hour and 30 minutes and weigh.
- 7.17.5. Weigh 1.0 gram of sample into the previously ignited quartz crucible. Moisten the sample with 0.2 mL of sulfuric acid.
- 7.17.6. Volatilize the sample until the sample is thoroughly charred. Heat the sample slowly, so that the sample does not boil over and no sample is lost.
 - 7.17.6.1. The rate of heating should be such that from ½ to 1 hour is required to volatilize the sample.
 - 7.17.6.2. Continue heating the sample until all the excess sulfuric acid has been volatilized.

- 7.17.7. Ignite in a muffle furnace at 600 ± 50 degrees Celsius for 15 minutes or until all carbon has been removed.
- 7.17.8. Cool in the desiccator for a minimum of an hour and a half and reweigh.
- 7.17.9. The weight of residue should not exceed 0.001 gram (0.1%). Calculate the %ROI as follows:

$$\% ROI = \frac{\text{Residue Weight (g)}}{\text{Sample Weight (g)}} \times 100$$

7.18. SOLUBILITY (5% Solution) Refer to Summary Sheet:

- 7.18.1. Dissolve 5.0 g of sample in 100 mL of purified water.
- 7.18.2. Observe under sufficient light.
- 7.18.3. Compare the solution to purified water.
 - 7.18.3.1. If the sample is not as clear as water, compare the sample to a platinum cobalt APHA 10 color standard. View vertically down against a white background. The solution must be less than the APHA 10 standard.
 - 7.18.3.1.1. Prepare an APHA 10 Color Standard by pipetting 1.00 mL of APHA No. 500 Platinum Cobalt Standard and QS to 50 mL with purified water.

7.19. SOLUTION TEST (10% in water) Refer to Summary Sheet:

- 7.19.1. Dissolve 10 g of sample in 100 mL of purified water.
- 7.19.2. Observe under sufficient light.
- 7.19.3. Compare the solution to purified water.
 - 7.19.3.1. If the sample is not as clear as water, compare the sample to a platinum cobalt APHA 10 color standard. View vertically down against a white background. The solution must be less than the APHA 10 standard.
 - 7.19.3.1.1. Prepare an APHA 10 Color Standard by pipetting 1.00 mL of APHA No. 500 Platinum Cobalt Standard and QS to 50 mL with purified water.

7.20. SULFATE Refer to Summary Sheet:

- 7.20.1. Sample Preparation:
 - 7.20.1.1. Accurately weigh 1.0 g of sample and transfer to a 50-mL Nessler tube.
 - 7.20.1.2. Add 40 mL of purified water to dissolve.
- 7.20.2. Standard Preparation:
 - 7.20.2.1. Prepare a standard solution by pipetting 0.052 mL of 0.02N H₂SO₄ in a 50-mL Nessler tube.
 - 7.20.2.2. Add 40 mL of purified water.
- 7.20.3. Procedure:
 - 7.20.3.1. To both the sample and standard solutions, add 1 mL of 3 N HCl, 3 mL of Barium Chloride TS, and dilute to 50 mL.
 - 7.20.3.2. Cover with parafilm and mix by inversion.
 - 7.20.3.3. Allow to stand for 10 minutes using a timer after the addition of the barium chloride to the sample and standard solutions. Compare the turbidity.
 - 7.20.3.3.1. Any turbidity produced in the sample solution should not exceed that produced by the standard when viewed from above and against a black surface.
 - 7.20.3.3.2. If a visible difference in the turbidity is not observed, utilize the Turbidimeter to measure the turbidity of the standard and the sample solutions. Follow Portable Turbidimeter SOP.

7.21. WATER (by Karl Fischer Titration) Refer to Summary Sheet:

- 7.21.1. Standardize Composite 5 as per Standardization of Titrants.
- 7.21.2. Grind the sample in a dry mortar into a fine powder utilizing a pestle.
- 7.21.3. Immediately weigh 0.8g of sample into the glass weighing spoon and tare it.
- 7.21.4. Transfer the sample to the KF vessel by removing the rubber septum and adding the sample into the titration vessel.
 - 7.21.4.1. Do not leave the rubber septum open for long periods of time as this will allow moisture to enter the titration vessel.
- 7.21.5. Return the weighing spoon to the balance, making sure not to lose any sample that was left behind. Once the weight stabilizes, record and transfer to the instrument.
- 7.21.6. Check to make sure there is no residual sample stuck to the sides of the titration vessel.
 - 7.21.6.1. If there is any sample stuck to the side, gently swirl the vessel to rinse the sides of the vessel to collect residual sample. Be careful not to damage the probe. Ensure the sample is fully dissolved before the titration begins.
- 7.21.7. The moisture content will then be determined by the Metrohm Auto Titrando 907.

$$\% \text{ Moisture} = \frac{(\text{mL of Composite 5})\left(\frac{\text{mg}}{\text{mL}} \text{ of Composite 5}\right)(0.1)}{\text{Sample Weight}_{(g)}}$$

7.22. Trace Metals Refer to Summary Sheet:

- 7.22.1. Primary Method: Refer to NexION 350X ICP-MS SOP and BSI-ATM-0073 for sample preparation and analysis.
- 7.22.2. Secondary Method: Refer to Avio 500 ICP-OES SOP and Trace Metals in Finished Goods Products, BSI-ATM-0089, for the determination of As, Cu, Fe, & Pb.