DETERMINATION OF CHELATED MINERAL CONTENT IN A METAL PROTEINATE (Bioplex Zn 15%) USING FT-IR SPECTROSCOPY

1. Principle

1.1. Overview

Fourier Transform Infrared (FTIR) spectroscopy can be used to monitor the modification in the vibrational absorption bands of a ligand due to metal-ligand complex formation. When a metal ion forms a bond with a ligand, the vibrational frequencies of the functional groups involved in the bond formation are altered. The simple stretching vibrations in the 1600 cm^{-1} to 3500 cm^{-1} region are the most characteristic and predictable, and absorptions in this region are used to identify functional groups in molecules. The region of an IR spectrum from approximately 1600 cm^{-1} to 500 cm^{-1} is called the fingerprint region and contains a unique set of absorptions for a given molecule. Specifically, by comparing the spectra of both free and complexed ligands in this region, the occurrence of complexation may be verified.

Generation of a linear series of metal-proteinate standards containing known amounts of chelated mineral can be effected by combining and blending metal-proteinates containing either fully unbound metal or fully bound metal. The generation of metal-proteinates with either fully unbound or fully bound metal is carried out by controlling the pH during reaction of hydrolysed soy and metal. At very acidic pH the metal will be in the free 2^{+} form, whilst as the pH increases the mineral will react and bind to negatively charged functional groups on the amino acids and peptides present in the hydrolysate.

Principal component regression (PCR) is used to develop calibration and prediction models for determination of chelated metal content of complex metal mixtures by utilizing attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra.

2. Materials and methods

2.1 Reagents:

Use only reagents of recognized analytical grade, unless otherwise specified, and deionized or demineralized water or water of equivalent purity (18,2 MΩ/cm at 25 °C).

Warning: Preparation of the metal-proteinate samples utilizes hydrochloric acid and sodium hydroxide. Adequate personal protection (minimum of safety glasses, gloves and laboratory coat) should be used when handling acids and bases. All solutions should be prepared in a suitable fume cupboard equipped with scrubbing facilities.

2.1.1 Hydrochloric acid

ACS reagent, 37% (Product # 320331 SIGMA-ALDRICH)

2.1.2 Sodium hydroxide solution

6 M NaOH (from Product # S5881 SIGMA-ALDRICH)

2.1.3 Soy Flour (Kaysoy Flour 064-100 Industrial Grade, Archer Daniels Midland Company)

2.1.4 Alkaline protease (Alcalase® 2.5 L, Declared Activity 2.5 AU-A/g)

2.1.5 Zinc(II) sulfate heptahydrate

Reagent Plus®, ≥99.0% (product # Z4750 SIGMA-ALDRICH)

2.2 Apparatus

2.2.1 Glass beakers (1000 ml x 3)

2.2.2 Graduated cylinders (500 ml x 2 and 100 ml x 2)

2.2.3 Water bath (Grant OLS200 or similar)

2.2.4 Agate mortar and pestle (Product # Z112518 SIGMA-ALDRICH)

2.2.5 Sieve Standard, traceable to NIST SRM certified reference material, 45 µm (325 mesh)

2.2.6 Spraydrying unit (Buchi Mini Spray Dryer B-290 or similar)

2.2.7 High density polyethylene (HDPE) airtight containers (100 g capacity)

2.2.8 Analytical balance (4-point fine balance Adam PW 254 or similar)

2.2.9 Micropipettes

2.2.10 pH meter (Mettler Toledo FiveEasy pH Meter Complete with LE409 pH

Electrode or similar)

2.2.11 Timer

2.2.12 Double-Safe™ PTFE coated thermometer L 200 mm, -10 to 110 °C temperature, immersion level, total (Product # Z676403 SIGMA-ALDRICH)

2.2.13 PerkinElmer Spectrum 100 FT-IR spectrometer with a 9-bounce Diamond/ZnSe Attenuated Total Reflectance (ATR) sampling accessory.

2.2.14 Ultrafreeze (New Brunswick U410 HEF, -86°C High Efficiency Upright Freezer or similar)

2.2.15 Aluminium foil

2.2.16 Polyethylene weighing boats

2.2.17 High density polyethylene (HDPE) airtight containers (5 g capacity)

2.3. Preparation of metal-proteinate standards

2.3.1 Soy hydrolysis

2.3.1.1 Enzymatic hydrolyses are carried out in the laboratory under alkaline conditions by preparing an aqueous suspension of soy flour and distilled water. Hydrolyses are carried out at pH 8.5 and 55 °C.

2.3.1.2 Weigh 50.0g defatted soy flour (2.1.3) with precision to 2 decimal places into glass beaker (2.2.1)

2.3.1.3 Add distilled water (500 mL) to previously weighed soy flour (2.3.1.2). **2.3.1.4** Mix soy flour water mixture (2.3.1.3) thoroughly to prepare a smooth suspension. Stirring is to be performed manually

2.3.1.5 Place soy flour water suspension (2.3.1.4) into water bath (2.2.3) previously warmed to 55°C. Add thermometer (2.2.12) to soy flour water suspension and monitor temperature to ensure soy flour water suspension equilibrates to 55°C.

2.3.1.6 Once equilibrated to temperature, adjust pH to 8.5 using sodium hydroxide solution (2.1.2) and allow to re-equilibrate to temperature once again

2.3.1.7 Add 1ml alkaline protease (2.1.4) to the equilibrated soy flour suspension (2.1.4).

2.3.1.8 Add soy flour (50 g) to the soy flour suspension (2.3.1.6)

2.3.1.9 Add 1.5 mL of the alkaline protease (2.1.4) to the soy flour suspension (2.3.1.7).

2.3.1.10 Monitor pH continually and maintain pH between pH 8.5 - 9.0 by adding sodium hydroxide (dropwise) as required.

2.3.1.11 Maintain the pH between pH 8.5 - 9.0 for 2 hours by adding sodium hydroxide (dropwise) as required.

2.3.1.12 Hydrolysis is allowed to continue for a further four hours without pH adjustment.

2.3.1.3 Allow soy hydrolysate (2.3.11) to cool to room temperature (20°C) before reacting with metal (2.1.5). Hydrolyzed soy should be reacted with metal within 2 hours of cooling.

2.3.2 Formation of metal proteinates

2.3.2.1 The sulphate form of the required metal is used to prepare batches of metal proteinates with a metal content of 15.1 % (w/w) dry weight. Selective pH adjustment is used to influence metal binding whereby material adjusted to pH 2.0 contains only metal in the free 2^+ form and material adjusted to pH 6.0 contains no metal in the 2^+ form

2.3.2.2 Aliquot 2 x 180 mL of the cooled soy hydrolysate (2.3.14) into 2 x 500 ml glass beakers (2.2.1)

2.3.2.3 Add 59.4 g of metal salt (2.1.5) with precision to 4 decimal places to each of the soy hydrolysate aliquots (2.3.2.2)

2.3.2.4 The metal soy suspensions (2.3.2.3) are mixed manually with stirring at 5 minute intervals for 1 hour at room temperature.

2.3.2.5 After 1 hour the final pH of one of the metal soy suspensions (2.3.2.4) is adjusted to pH 2.0 using hydrochloric acid (2.2.1) and transferred to an HDPE airtight container (2.2.7)

2.3.2.6 The remaining metal soy suspension (2.3.2.4) is adjusted to pH 6.0 using sodium hydroxide (2.2.2) and transferred to an HDPE airtight container (2.2.7)

2.3.2.7 The pH adjusted metal soy suspensions (2.3.2.5 and 2.3.2.6) should be frozen overnight at -70 °C.

2.3.2.8 The frozen pH adjusted metal soy suspensions (2.3.2.7) are subsequently spray-dried (2.2.6). The drying conditions are: Inlet 140°C, Outlet 75°C, Aspirator 100% and pump 20%.

2.3.2.9 The spray-dried powders (2.3.2.8) are homogenized using an agate pestle and mortar (2.2.4) and transferred to fresh HDPE airtight containers (2.2.7) prior to further use.

2.3.2.10 Each homogenized sample (2.3.2.9) is ground individually to a very fine powder using an agate pestle and mortar (2.2.4)

2.3.2.11 Individually ground samples (2.3.2.10) are subsequently sieved manually by horizontal shaking through a 45 µm aperture sieve (2.2.5) onto aluminium foil (2.2.15).

2.3.2.12 Particles of size >45 µm which are not retained by sieving (2.3.2.11) are subsequently reground with an agate pestle and mortar (2.2.4) and transferred to fresh HDPE airtight containers (2.2.7).

2.3.2.13 The sieved and ground pH 2.0 and pH 6.0 adjusted metal-soy proteinates (2.3.2.12) can then be used to generate metal-proteinate standards for subsequent quantitative FTIR analysis

2.3.3 Preparation of metal-proteinate standards

2.3.3.1 A linear series of standards are generated ranging from 0% bound mineral (as represented by the pH 2.0 adjusted proteinate) to 100% bound mineral (as represented by the pH 6.0 adjusted material) by combining specific gram weights of pH 1.0 and pH 5.0 sieved metal-soy proteinates (2.3.2.12).

2.3.3.2 Specific weights of pH adjusted metal-proteinates are weighed into polyethylene weighing boats (2.2.16) and combined. Table 2.1 outlines the gram weights required to generate the linear calibration standards. Ensure precision to 4 decimal places when weighing individual samples:

Table 2.1 Metal-proteinate standards

Sample number	**1**	**2**	**3**	**4**	**5**	**6**
Gram weight of pH 2.0 adjusted proteinate	1.0g	0.8g	0.6g	0.4g	0.2g	0g
Gram weight of pH 6.0 adjusted proteinate	0g	0.2g	0.4g	0.6g	0.8g	1.0g
% Bound metal after blending	**0%**	**20%**	**40%**	**60%**	**80%**	**100%**

2.3.3.3 Combined metal proteinate standards are once again blended to homogeneity using an agate pestle and mortar (2.2.4) before transfer to HDPE airtight containers (2.2.17)

2.3.3.4 Prior to further use, the metal content for each of the metal-proteinate standards (2.3.3.3) is quantified to ensure uniformity in terms of total content. This is performed as outlined by European Standard EN 15510:2007 using ICP-MS.

2.4. Quantitative Fourier Transform Infrared (FTIR) Spectroscopy

2.4.1 Infrared spectroscopy is carried out using a PerkinElmer Spectrum 100 FT-IR spectrometer with a 9-bounce Diamond/ZnSe Attenuated Total Reflectance (ATR) sampling accessory.

2.4.2 Version 6.3.2 of the PerkinElmer software package "Spectrum" is used for all spectral collection

2.4.3 Powdered samples (5-10 mg) are placed onto the ATR crystal with force applied at 40 N.

2.4.4 FTIR spectra are collected using the parameters outlined below

Abscissa: (cm^{-1})	
Start:	1800.00
End:	650.00
Interval:	-1.000000
Accumulations :	16
Detector:	LiTa03
Source:	MIR
Beamsplitter:	OptKBr
Resolution:	4.00 cm^{-1}
Beam type:	Ratio
Phase Correction:	Magnitude
OPD Velocity:	0.20 cm/sec
J-Stop Size:	8.94 mm
Igram Type:	Double
Scan Direction:	Combined
Filterwheel Position:	1
IR Type:	FT
IR-Laser Wavenumber:	15798.00 cm^{-1}
Scan Range / cm^{-1}:	1800 – 650

2.4.5 Nine replicate spectra were acquired for each calibration standard and 6 replicate spectra for each sample.

2.4.6 Data analysis on all spectra is performed using principal component regression (PCR) which allows for the generation of calibration and prediction models for determination of chelated metal content.

2.5 Data processing by principle components regression (PCR)

2.5.1 Spectrum Quant + version 4.6.0.0151 is used for quantitative FT-IR analysis. PCR+ is the selected algorithm for analysis of data. All quantitative methods are developed using the Method Wizard function in Quant+.

2.5.2 Calibration standards method development in Quant+ is as follows:

- Open Quant +
- Select "File" and then "New" from the drop down menu
- Enter method name, analyst name and method description in the "Method Wizard – Description" window and proceed by pressing OK.

- A new window called “Method Wizard – Standards” appears
- Standard spectra are selected by clicking on the browse option.
- Once selected and added press OK to proceed.
- A new window called “Method Wizard – Concentrations” is now displayed
- The Property being investigated by the Calibration curve and its units of measurement are entered into the Property 1 box by right clicking on this box and filling in the required parameters.
- Standard concentrations are entered in the appropriate boxes below the Property 1 box
- Press OK to proceed to the “Method Wizard – Calculations” window. All pre-processing parameters to be applied to standard and sample spectra are selected in this window.
- The following pre-processing parameters are to be selected and applied

Data range
Start: 1800.0
End: 650.0
Interval: 1.0

Analyze Data In: Absorbance

Scaling (Spectra): Mean

Pre-Processing
Smooth: No
Normalization: Standard Normal Variate (SNV) + de-trending

Baseline Correction: Offset

Ordinate Threshold
Upper: 1.5
Lower: None

No. of Factors
Min: 1
Max: 35

- Press OK to proceed and save the method
- A method summary will now appear
- Select calibrate from the “Method Wizard” calibrate from the toolbar at the top of the screen
- Select”Expert Assist”
- Quant + now carries out the PCR analysis of the standards and a graphical representation of the data generated is displayed for review.

- Select “Summary” from the Method Wizard toolbar to review all reports on the calibration which outline all iterations carried out and the resulting statistical information from the calibration

2.5.3 Prediction of the chelated mineral content of unknown samples using Quant+ is as follows:

- To predict an unknown sample against the calibration curve select “Predict” from the Method Wizard tool bar.
- Use “Browse” to select the spectra of the unknown sample you wish to predict
- Select “Statistics” and “Residual Spectra”, these will be displayed in the prediction report
- Press Ok to proceed to the prediction
- A prediction report for the selected spectra is then displayed indicating the chelated mineral content of the sample.