Method for Selenomethionine - Selenized Yeast

1.0 Foreword

1.1 The method given is a guide for sample preparation, acid digestion, derivitization and analysis of selenomethionine in selenized yeast by HPLC. In-house method

2.0 Introduction

2.1 This confidential method was developed and validated for the specific matrix of selenized yeast following a modification of standardized methodology for amino acid analysis.

3.0 Title: Test for Selenomethionine – Modified Pico-Tag Method

4.0 Warnings

4.1 Inorganic acids are highly corrosive and must be handled carefully. All acid additions are carried out in the hood and technicians are to wear protective eyewear and face shield when working with the acid.

4.2 Persons using this method should be familiar with normal laboratory practice. This method does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

5.0 Scope

5.1 This procedure is to be used for the testing of selenium enriched dry yeast products, cream samples and liquid byproducts containing 2000-5000 ppm selenomethionine on a dry basis.

6.0 Normative references

6.1 Liquid Chromatography Analysis of Amino Acids in Feeds and Foods using a Modification of the Pico-Tag Method. Rev. 1990, Millipore Corporation.

6.2 The Pico-Tag Method, A Manual of Advanced Techniques for Amino Acid Analysis. Waters Division of Millipore, WM02, Rev. 1. 1989.

6.3 SELM-1, Selenium Enriched Yeast Certified Reference Material. National Research Council Canada. Certificate issued June 2005.

7.0 Definitions

7.1 HPLC: High Pressure Liquid Chromatography

7.2 AABA: Apha amino butyric acid

7.3 SEM or SEMet: selenomethionine
7.4 PDA: Photodiode array Detector
7.5 HPLC grade water: (>18 MOhm resistance, or purchased HPLC grade)
7.6 Eluent A and B: Commercially available through Waters.
7.7 SPC: Statistical Process Control (Control chart)

8.0 Principle

8.1 Following a modification of the Pico-Tag method (Waters Division of Millipore Corp.), samples are subjected to a mild acid digestion to break the proteins down to free amino acids with minimal destruction of selenomethionine. After dilution with an internal standard and filtration, a small aliquot is used for derivatisation with phenylisothiocyanate (PITC). The phenylthiocarbamate (PTC) amino acids are analysed by reverse phase liquid chromatography and detection at 254 nm.

9.0 Reactions

9.1 The reaction of amino acids to their PTC derivatives is well documented.

10.0 Reagents and materials

10.1 Glacial Acetic Acid, HPLC grade.
10.2 Dry ice and isopropanol for vacuum trap. (reagent alcohol and ice packs work also)
10.3 Compressed nitrogen.
10.4 Concentrated HCL, Ultra grade.
10.5 HPLC grade water.
10.6 HPLC grade methanol.
10.7 D-L, Alpha aminobutyric acid , Sigma A1754
10.8 10 mM HCl,(83 ul concentrated to 100 ml volumetric.)
10.9 0.2N sodium acetate, anhydrous (1.64 g/100 ml).
10.10 200 mg/100 ml Disodium EDTA.. Sonicate to dissolve. 100 ul added to reagent A and B.
10.11 Pierce H amino acid combination standard Product 20088.
10.12 Pico-Tag sample diluent. Waters P/N WAT088119 (or make up 5mM Na_2HPO_4 pH 7.4 : acetonitrile, 95:5.)
10.13 Seleno-D,L-Methionine, Sigma S3875.
10.14 Sodium Selenate, Sigma S0882.
10.15 Sodium Selenite, Sigma S1382.
10.16 Seleno-DL-cystine, Sigma S1650

11.0 Apparatus

11.1 Vacuum pump, Pico-Tag station.
11.2 Oven, 110°C
11.3 Vortex

11.4 Volumetric flasks, 10, 50, 100 and 250 ml.
11.5 Analytical balance.
11.6 Volumetric pipettes/pipettor.
11.7 PH meter.
11.8 0.45 um sample filters.
11.9 Waters HPLC system consisting of a 2695Alliance Separations Module with a four channel solvent system, autosampler and column heater. A 2996 Photodiode Array Detector. Millenium software ver. 4.0.
11.10 Hydrolysate eluents A and B. (Waters P/N WAT088108, WAT 010983). See pg 18 of original method to prepare in-house.
11.11 25x150 culture tubes with teflon lined screw tops.
11.12 6x50 mm tubes for vacuum station.
11.13 Diamond tipped pencil.
11.14 Pasteur pipettes.
11.15 Centrifuge.
11.16 Water bath 37°C.

12.0 Sampling

12.1 Determine protein content or use 75 mg of dry selenium yeast samples and 800 mg of cream. Dry matter should be determined for conversions back to dry basis. Samples should be analyzed in duplicate. Sets of 14 may be run through the vacuum apparatus.

13.0 Procedure

13.1 Standards Preparation:

13.1.1 Stock Internal Standard, AABA. Alpha amino butyric acid. 6.25 umol/ml. Weigh 0.1613 g and dilute to 250 ml volume with 10 mM HCl.
13.1.2 Stock Standard D,L-Selenomethionine, 12.5 umol/ml. 0.0613 g/25 ml 10mM HCl.
13.1.3 Stock Standard Seleno-DL-cystine, 1.25 umol/ml. 0.0523 g/ 125 ml 10mM HCl.
13.1.4 Dilute Internal Standard. Combine 4 ml of stock AABA and dilute to 10 ml volume with 10 mM HCl. 2.5 umol/ml AABA.

13.2 Working Standards: 1.25 umol/ml. (0.625 umol/ml cystine)

13.2.1 Combination standard. Combine 0.5 ml of dilute internal standard and 0.5 ml Pierce H standard.

13.3 Mobile Phase Preparation:

13.3.1 Eluent A. Add 100 ul EDTA solution and mix adjusting the pH to 6.4 for acid hydrolyzed samples. Do not filter or degass if purchased solvent is used as this will change the organic content.
13.3.2 Eluent B. Add 100 ul of the EDTA solution and mix.

Variations of these eluents may be necessary for troubleshooting the separation of certain components. For hydrolyzed samples the chromatography is run at 46 C.

Gradient Table for Hydrolyzate Analysis – Eluent A pH 6.4, 46°C

Time	Flow	%A	%B	Curve
Start	1.0	100	0	*
1.0	*1.0*	*100*	*0*	*6*
21.0	1.0	*54*	*46*	5
21.5	1.0	0	100	6
22.0	1.5	0	100	6
26.0	1.5	0	100	6
26.5	1.5	100	0	6
39.0	1.5	100	0	6
39.5	1.0	100	0	6
44.5	1.0	100	0	6
60.0	0.2	50	50	9

*Note changes in pH, temperature, gradient times and %B. If resolution of phenylalanine and artifact peaks continues to be a problem, try a pH 6.4, 46°C gradient. See pg 42 of orginal method.

13.4 HPLC setup:

13.4.1 Log in to HPLC software with assigned name and password.
13.4.2 From the drop down menu select project *Aminoacids.*
13.4.3 Double click on *run samples* icon.
13.4.4 Select the *PDA_2695* chromatographic system.
13.4.5 The system will take a minute or two to boot, then the quick set window will appear.
13.4.6 Select the *Amino Acids IM* instrument method from the pull down menu.
13.4.7 To use a previous sample set template, go to *File, open new sample set method, based on existing sample set method*. Select a recent run to use as a template.
13.4.8 Alter the template as necessary for the current samples. Fill in *sample names*, *function*, *method set*, 20 ul *injection volume*, 44.5 minute *run time*, *weight* and *dilution* as applicable. Weights will be the weight in mg of sample, dilution will be 50 for hydrolyzed samples, 10 for oxidized samples.
13.4.9 Set the solvent system to switch over to a non-buffered storage solution (20% acetonitrile) when sample set is complete. Program a detector shut down also.
13.4.10 Standard amounts are listed in the original procedure. Amount of internal standard, AABA is 12.90, the same for both standards and samples.
13.4.11 The computer will instruct the WISP to inject samples in the exact order as they appear in the program. The first standard/sample should be run twice – the first injection is necessary to condition the column.
13.4.12 Check the component editor to ensure the internal standard is entered correctly for samples and standards. See attached table for example.

13.4.13 From the mode drop down menu, select *run only*.
13.4.14 When everything is set to go, select the green icon to initiate injection.
13.4.15 Enter a name when prompted for the sample set and then select *run*.
13.4.16 The computer will now initiate the run by starting the WISP as given in the program beginning with the vial listed on the first line.

13.5 Procedure for hydrolyzed samples: (destroys cystine and methionine partially – see validation)

13.5.1 Day 1: (approx. 2 pm)

13.5.1.1 Weigh samples corresponding to approx 32 mg protein to nearest 0.01 mg into 25 x 150 tubes. (This is a scaled version of the original procedure. Use 75 mg dry samples and 800 mg creams)
13.5.1.2 Add concentrated HCl as given in the chart and purge with nitrogen for 30 seconds. Cap immediately.
13.5.1.3 Place in 110°C oven for 18 hours. Approx. 8 am.

13.5.2 Day 2:

13.5.2.1 Remove from oven and allow to cool.
13.5.2.2 Add internal standard, 6.25 umol/ml as given in the chart for samples. Use the dilute internal standard for standards as given in the chart. Bring tubes up to 10 ml using a reference tube for comparison. Vortex for 10 seconds.
13.5.2.3 Filter an aliquot through a 0.45 um filter or microfuge for 5 minutes at 12,000 x g to clarify. Derivatize immediately or store capped in freezer.

13.5.2.4 Example set: (up to 14 samples)

Tube	Sample	Target hydrolysis amounts	Actual amounts weighed (g)
1	Typical Se cream	800 mg	
2	Duplicate cream	800 mg	
3	Typical Se yeast	75mg	
4	Duplicate yeast	75 mg	
5	Control yeast 1000 ppm Se	75 mg	
6	Control yeast 2000 ppm Se	75 mg	
7	Selenomethionine hydrolyzed	2 ml of 12.5 umol/ml	2 ml stock + 2 ml conc. HCl + 2 ml water
8	Selenomethionine no hydrolysis	"	Without the acid and heat (use 4 ml water in place of acid)

9	Selenocystine hydrolyzed	5 ml of 1.25 umol/ ml	5 ml stock + 3 ml acid + 2 ml AABA
10	Selenocystine no hydrolysis	"	As above, just use water in place of acid and no heat
11	Pierce Std		

13.5.2.5 Amounts of selenium yeast were scaled up from original procedure in order to get larger peaks.

13.5.2.6 All samples are hydrolyzed in 4N HCl.

13.5.2.7 Example hydrolysis set

Tube	Sample	H2O ml	Conc. HCl ml	Hydro 18 hr 110°C	AABA ml 6.25 umol/ml added after hyd	Bring to 10 ml with H_2O	AABA ml 2.5 umol/ml
1	Typical Se cream	1.2	1.0	√	2.0	√	---
2	Duplicate cream	1.2	1.0	√	2.0	√	---
3	Typical Se yeast	2.0	1.0	√	2.0	√	---
4	Duplicate yeast	2.0	1.0	√	2.0	√	---
5	Control yeast 1000	2.0	1.0	√	2.0	√	---
6	Control yeast 2000	2.0	1.0	√	2.0	√	---
7	Selenomethionine hydrolyzed	2.0	2.0	√	--	√	1:1
8	Selenomethionine no hydrolysis	4.0	--	√	--	√	1:1
9	Selenocystine hydrolyzed	--	3.0	√	2.0	√	
10	Selenocystine no hydrolysis	3.0	--	√	2.0	√	
11	Pierce Std						1:1

T0 = ___ pm TE = ___ am, 18 hrs.

13.5.2.8 Example Results Table:

Product	Ppm Selenomethionine As-is basis	Equivalent Se ppm (40.28% of FW)	Total Se as tested FAA	% of total Se present as selenomethionine	

13.5.2.9 Recovery of the selenomethionine standard through hydrolysis was ___ %.
Conversions to % selenomethonine may be made by dividing the ppm by 10,000. For example, 2136 ppm selenomethionine is equivalent to 0.2136% selenomethionine.

13.6 Sample Derivatization:

13.6.1 Set up Pico-tag drying system and run for 30 minutes prior to running samples. Cool acid trap with dry ice and isopropanol for 15 minutes. The drying vessel can hold up to 12 tubes. Undiluted derivatized samples are stable for at least a week if stored dessicated in the freezer. Samples should be dried quickly and thoroughly.
13.6.2 Mark 6x50 mm tubes with diamond tipped pencil / sharpie for sample identification.
13.6.3 Carefully pipet 10 ul of filtered samples into bottom of corresponding tubes. (Use gel loading tips) Place tubes into Pico-tag vessels, open valve and dry in vacuum station. Open vacuum valve slowly to prevent rapid bubbling in the tubes. Vacuum gauge should read <50 millitorr and drying should be complete in 10-15 min.
13.6.4 Run a H_20 blank to determine reagent interferences (i.e. phenylalanine) Use 10 ul. Also run 10 ul of phenylalanine and lysine working standards.
13.6.5 Prepare redry solution for 12 samples by adding 200 ul methanol, 200 ul 0.2 N sodium acetate, and 100 ul triethylamine to a vial and mixing.
13.6.6 Remove tubes from drying vessel. Add 30 ul of redry solution to tubes and vortex.
13.6.7 Dry again in vacuum station as in Step 3.
13.6.8 Prepare derivatization reagent for 12 samples by combining 350 ul methanol, 50 ul HPLC grade water, 50 ul triethylamine, and 50 ul PITC to vial and mixing. Reagent must be

fresh. Reagent should become clear on mixing. If not, methanol may have high moisture content – replace with fresh.

13.6.9 Add 30 ul of derivatization reagent to tubes and vortex as in step 5. Cap and allow to react for 20 minutes.

13.6.10 Dry in vacuum station for 15 minutes. Remove tubes from vessel and add 30 ul of HPLC grade methanol to tubes and vortex to re-suspend solution. Resume drying until vacuum gauge reads < 70 millitorr. Total time will be approximately 1 hour. The sample tubes should appear dry (white crystals) not oily.

13.6.11 100 ul of sample diluent to tubes, vortex and transfer to injection vials. Microfuge samples if necessary to clarify prior to loading autosampler vials. Inject 20 ul of standards and samples. Make two injections of the first standard. Reinject the standard every 10 samples. Diluted derivatizations are stable for 10 hours at room temperature.

13.7 Shut down

13.7.1 When finished with the vacuum pump, shut down and open to atmospheric pressure. Change oil monthly.

13.7.2 For the HPLC, flush pump A with water. Store pump and column in 10% acetonitrile. Set PDA detector to shut down when the sample set is complete.

14.0 Calculation

14.1 Processing data:

14.1.1 When the sample set is complete, the data may be processed by returning to the main screen and entering into *review data*.

14.1.2 Select *channels, OK.*

14.1.3 The injected samples will appear on a list. Highlight both the standards and samples of interest. (If they are incorrectly labeled, this may be changed under *file*, *normal mode, tools*, *alter sample*.)

14.1.4 Select *review*. Go to *file*, *open method*, select processing method needed, *open*. Integrate the standard chromatogram by selecting the integrate icon. Make changes if necessary.

14.1.5 Calibrate based on the non-hydrolyzed selenomethionine. Calibrate using the calibration icon. You may check the calibration curve by selecting the icon (make sure old calibrations are deleted). Go to *file*, *save calibration* to save the new information. Recovery should be calculated with a ratio of hydrolyzed to non-hydrolyzed sample areas, adjusting for internal standard. Recovery should be greater than 98%.

14.1.6 Unknowns may be processed in the same manner, except rather than *calibrate*, *quantitate*. Go to *peaks* tab to view the results. Results are reported in g/100 g sample. Results should be corrected to a dry matter basis. (creams).

14.1.7 Save results under file, save, all results. Results may be recorded or used to prepare a report.

14.1.8 Integration parameters are specific to the HPLC software.

14.2 Evaluation of results

14.2.1 Results are calculated by software to be grams amino acids per 100 g sample based on dilutions and amounts of each sample. Correct results to a dry matter basis as necessary (i.e. cream samples). See supplemental data section for software settings. Amounts for the selenomethionine standard should be 24.51, for selenocystine 20.88.

14.2.2 If recovery of the selenomethionine standard is above 98%, results may be reported. Ppm of selenium contained in selenomethionine may be calculated by multiplying ppm selenomethionine by 0.4026. (Selenium is 40.26% of the FW of Selenomethionine). By comparison to the total selenium results, the % selenium present as selenomethionine may be calculated as follows:

14.2.3 (ppm selenium as selenomethionine / ppm total Se)*100 = % Se as selenomethionine.

15.0 Precision

15.1 The absolute difference between two independent replicate test results obtained the same day by the same operator on the same equipment will not be greater than 10% of the arithmetic mean to accept the data.

16.0 Quality assurance and control

16.1 Standard Reference Materials:

16.1.1 SELM-1, Selenium Enriched Yeast Certified Reference Material. National Research Council Canada. Certified for 3431 ppm selenomethionine +/- 157 ppm. Recoveries must fall within certified range to use results of test samples.

16.1.2 AAFCO check sample #9923, (a layer feed.) Run this (or a similar) standard periodically to determine efficiency of the hydrolysis.

16.1.3 Control selenium yeast in-house standard are run with each set. Results for these samples should fall within the SPC range.

17.0 Special cases

17.1 Revisions: Original document, 6/07

17.2 Selenomethionine – Method revisions 4/7/06 – Alternative standard method.

17.3 A reduced level of selenomethionine standard was adapted to better match the response levels found in extracted selenized yeast.

17.3.1 100 ul of the stock 12.5 umol/ml selenomethionine (0.0613 g/25 ml 10mM HCl) is added to the hydrolysis tube. Two ml of HPLC grade water and 1 ml conc. HCL are added and the procedure followed as per samples.

17.3.2 With this new concentration, the calculations will use 2.451 as the standard amount rather than 24.51 as in the original procedure.

17.3.3 Replicates are tested (one without acid and heat) to determine loss in hydrolysis similar to the original procedure.

17.4 Calculations were changed to accommodate the new standard concentration.

17.4.1 (Area of sample SeMet/Area std SeMet) x (area Int. std in Std/area IS in sample) x (10) x (2.451) x (1/sample amount, mg) = g SeMet/100 g sample.
17.4.2 This answer may be multiplied by 10,000 to convert to ppm SeMet.
17.4.3 Ppm SeMet x 0.4026 = ppm Se
17.4.4 Ppm Se (from SeMet)/ total ppm Se = % Se as SeMet.

17.5 Selenomethionine – Method revisions 4/08

17.5.1 Validation information added to meet regulatory requirements

17.6 Selenomethionine – Method revisions 9/09

17.6.1 Compound formulations corrected for superscript and subscripts. Pages 3, 4, 7, 8, 11 and 12.

18.0 Test report

18.1 In the projects window, go to the *results* tab. Highlight the results of interest. Go to *preview*. Select the report method of interest or create a new one. Print. (for amino acids a summary report works best)

19.0 Annexes

none

20.0 Bibliography

[1] Separation of Selenium Analogues of Sulphur-containing amino acids by high-performance liquid chromatography and high resolution gas chromatography. Janak, Billiet, Frank, Luyben, Husek. *Journal of Chromatography*, 677 (1994, 192-196).