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Determination of amino acids in selenium-enriched yeast by gas chromatography–mass spectrometry after microwave assisted hydrolysis

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1. Introduction

Yeast is a single cell microorganism with over 1,500 species. They can be found in association with other microorganisms as part of the normal inhabitants of soil, vegetation, marine and other aquatic environments. In ancient times, yeast had been widely used in human life such as in bread and wine making and in aid digestion. Studies of molecular genetics, ecology, physiology, generate electricity and nutriology of yeast [1] have been grown significantly in the past decades. Today, yeast is playing even more important role in brewing industry, food industry and enzyme preparation industry [2], especially in the field of nutrition. Yeast is an excellent source of protein, vitamins and essential trace elements. For an example the containing B-complex vitamins is needed for normal growth and selenium is needed in protection of cells against the effects of free radicals, the normal functioning of the immune system and thyroid gland as well as protection against various forms of cancers. As a result selenium-enriched yeast has become a popular supplement. It contains selenomethionine as the major species [3-5] which has higher bioavailability and lower toxicity than inorganic selenium. Amino acids (AAs) are fundamental units in yeast and often the determination of AAs in such matrix is desired [6-9] due to their biological and nutritional significance. As a result, the development of analytical methods for the simultaneous quantitation of AAs in yeast samples is of great importance.

For the accurate determination of AAs in yeast, hydrolysis of all the peptides and proteins to their constituent AAs is required. Hydrolysis is a first and critical step for amino acid analysis [10-11]. Enzymatic digestion, alkaline digestion and acid hydrolysis are three commonly used methods for extraction of AAs from protein. Procedures utilizing

hydrochloric acid (HCl) are most popular. In general, conventional acidic hydrolysis employs 6 M HCl for the hydrolysis of most proteins in 20-24 h at 110°C [12-13]. Unfortunately, some AAs such as cysteine, methionine and tryptophan are partially or completely destroyed under these conditions [11, 14]. Simpson *et al.* [15] and Chiou *et al.* [16] reported a procedure with use of 4 M methanesulfonic acid (MSA) at high temperature enabled a complete extraction of AAs from protein hydrolysate. Results from a previous study [17] on comparison of HCl (6 M) and MSA (4 M) for extraction in plant litter and soil have shown that better extraction efficiencies of AAs with use of MSA method were obtained. In addition, MSA method allows the determination of methionine, cysteine and S-containing amino acids such as tryptophan and threonine which are destroyed with HCl (6 M) digestion method [11, 17]. In order to improve sample throughput, efficient and fast extraction method is needed for the determination of AAs in yeast. Use of microwave-irradiation had been reported [18-19] to enhance AAs [20-23] and inorganic salts [24-25] hydrolysis. The main advantage of microwave-irradiation hydrolysis is the significant reduction in the hydrolysis time [11, 6, 27]. However, more research is still needed in this area [20, 27, 28] in order to develop efficient and fast method for the simultaneous determination of AAs.

Commonly used techniques for the separation and quantitation of AAs include: ion exchange chromatography (IEC) [17-18, 20], capillary electrophoresis mass spectrometry (CE-MS) [29-30], high-performance liquid chromatography mass spectrometry (HPLC-MS) [31-32] and gas chromatography mass spectrometry (GC-MS) [33-35]. Among these, GC-MS has high sensitivity and high resolution which allows simultaneous detection of different AAs within a single run. However, use of GC-MS detection, the AAs must be derivatized to more

volatile compounds. Hexamethyldisilazane had been used as a common derivatization agent and the trimethylsilyl derivatives have good volatility, which can be determined by GC-MS [36-37]. The disadvantage of this derivatization protocol is the draconian reaction condition that needs heating and anhydrous [38-39]. Ethyl chloroformate (MCF) on the other hand, can derivatize AAs in one step at room temperature for only 60 seconds [40-42]. As shown in recent reports, selenomethionine (SeMet) and methionine (Met) in selenium-enriched yeast were determined with use of MCF for derivatization and GC-MS for detection [5-6].

The aim of this work was to develop a fast and sensitive method for the determination of AAs in yeast by GC-MS following a microwave assisted hydrolysis. For this purpose, MSA was used as the hydrolysis reagent and microwave power, temperature, hydrolysis time and the effect of MSA concentration were investigated.

2. Experimental Section

2.1. Instrumentation

A Hewlett-Packard HP 6890 GC (Agilent Technologies Canada Inc., Mississauga, ON, Canada) fitted with HP 7683 Automatic Injector system was used for determination of amino acids in yeast extract. Detection was achieved with an HP model 5973 mass-selective detector (EI) operated at 70e. The control of components of the chromatograms system and the analysis of the chromatograms with identification and quantification of the AAs were performed with the Enhanced Chemstation software (Version B. 01.00). The column used for separation was DB-5MS (30mx0.25µmx30mx0.25µm, Iso-Mass Scientific Inc., Calgary AB, Canada). A 1-µL sample was injected in splitless mode. Helium carrier gas flow rate was 1.5

mL min⁻¹ at a pressure of 17.6 psi and injector temperature was 280 °C. The oven temperature program started at 120 °C with a hold of 2 minutes and ramped at 15 °C min⁻¹ to 290 °C with a hold of 1 min. The MS ion source temperature was 250 °C and transfer line temperature was 260 °C. The scan mode was used to monitor signals of AAs, mass range was 50-500 a.m.u. at 2.63s/scan. SIM mode was used for the final quantitation of AAs in yeast.

A CEM Focused MicrowaveTM Synthesis System, Discover/Explorer with 48-position microwave reactor attached (Matthews, NC, USA) was used for MSA digestion of yeast for AAs determination. The microwave has temperature, power and pressure monitoring device. The CEM Discover utilizes a magnetron, which operates at 2.45GHz with a maximum output power of 300 W. A 10 mL Pyrex vessels was used for microwave digestion.

2.2. Reagents and Solutions

Amino acids (AA) standards of Alanine (Ala), Arginine (Arg), Asparagine (Asn), Aspartic acid (Asp), Cystine (Cys), Glutamic acid (Glu), Glutamine (Gln), Glycine (Gly), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Homocysteine (HCys), Histidine (His), Methionine (Met), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Selenium-cystine (SeCys), Selenium -methionine (SeMet), Threonine (Thr), Tryptophan (Trp), Tyrosine (Tyr) and Valine (Val) were obtained from Sigma Aldrich (Oakville, ON, Canada). Dithiothreitol (DTT) was purchased from Fluka (Oakville, ON, Canada).

Environmental grade ammonium hydroxide was purchased from Anachemia Science (Montreal, PQ, Canada). High purity water was generated using a MILLI-Q-ADVANTAGE (A10) system from Millipore Corporation (Saverne, Alsace, France). Certified grade

chloroform was sourced from Fisher Scientific (Ottawa, Canada). Methyl chlorofomate (MCF) (99% purity) were obtained from Sigma Aldrich Canada (Oakville, ON, Canada). Methanesulfonic acid (98% purity) was obtained from Fluka (Oakville, ON, Canada), pyridine (99.9% purity) and methanol (99.9% purity) was purchased from Fisher Scientific (Ottawa, Canada).

The stock standard solutions containing tested AAs (1 mmol L^{-1}) were prepared in HCl 1% (v/v) solution and kept in a fridge until used. A stock solution of DTT (50 mmol L^{-1}) was prepared in water.

Yeast CRM SLEM-1 from National Research Council Canada (NRCC, Ottawa, ON, Canada) was used as test sample for microwave assisted MSA extraction.

2.3. Sample preparation and analysis procedure.

Various microwave temperature (120°C - 180°C), heating time (10-180 minutes) and various concentrations of MSA (1-10M) were investigated. In brief, a few mg of yeast was accurately weighed into individual 10mL dry Pyrex microwave vessels. 4.5 ml of HCl 1% (v/v) and 1.5 mL MSA were added, resulting in a concentration of 4M of MSA. The sample was hydrolyzed by the closed vessel microwave irradiation system. After cooling, the sample was centrifuged (12000 rpm, 5 min), and 1ml of the supernatant was transferred to a 15 mL vial. An appropriate amount of ammonium hydroxide was added to adjust the pH of sample to about 3. 300 μL of DTT (50 mmol L^{-1}) as the reducing reagent was added to the solution and stood for 60 minutes. The side chain on cysteine is thiol, which is susceptible to oxidization to give the disulfide derivative cystine, Dithiothreitol (DTT) as the reducing agent

is used to improve cysteine recovery.

The derivatization procedure used in the study followed that earlier procedure reported by Yang *et al.* [6]. In brief, 900 μL of pyridine-methanol (1:3 v/v) was added to the yeast extract, and 300 μL of methyl chloroformate (MCF) was dropped slowly. The sample was vortex-mixed for 1 min followed by addition of 1 mL chloroform; the vial was vortex-mixed for 1 min again. After centrifugation (2000 rpm, 10 min), the chloroform layer was transferred to a 2 mL vial (insert was 200 μL) for GC-MS analysis.

Final quantitation of AAs in the yeast was achieved by standard addition calibration with one unspiked sample and five spiked samples at five different levels of AAs standard solutions added, resulting concentration ranges of 0.01–0.50 μmolL^{-1} . Three sample blanks and three replicate subsamples of yeast were microwave digested and derivatized as described above prior to GC-MS analysis. Peak high of each AA was used to construct the calibration curve.

3. Results and Discussion

3.1. Separation of AAs.

As shown in Fig. 1a, good separation and peak profiles were obtained for most AAs obtained from a mix standard solution containing 23 AAs at 1 mmol L^{-1} which were derivatized with MCF. However, peaks for Arg, Gln and Ser were not found in the chromatogram. This may be due to either low volatilities of their derivatives and thus were not eluted at temperature program used; or poor thermal stability of their derivatives and thus decomposed during the temperature ramping of GC oven. Subsequent experiments were

conducted by analysis of this mixture of AAs standard solution which was dried to remove chloroform and diluted in 0.1% formic acid in water by HPLC-MS (with a Zorbax Eclipse Plus C18, 2.1×150 mm, 5 µm column and a guard column Zorbax stablebond C18). Peaks for Arg, Gln and Ser were found, indicating Arg, Gln and Ser were derivatized by MCF. Ala and Gly, Pro and Asn had very similar retention times and were thus not completely separated under chosen experimental conditions. However, by extracting their respective fragment molecular ions at m/z 88, 102, 187 and 95, these AAs can be determined accurately.

3.2. Analytical figures of GC-MS method for the determination of AAs.

The performance of GC-MS for the determination of AAs was evaluated by using mixed AAs standard solutions. Retention times, linear ranges, limits of detection (LODs) and limits of quantitation (LOQs) of 20 AAs are summarized in Table 1. Good linear relationships for these 20 AAs were obtained with correlation coefficients great than 0.990, in a range of 0.01–0.20 µmol L⁻¹ for Asn, Cys and Secys, respectively; 0.01–0.30 µmol L⁻¹ for Ala, Val, Leu, Ile and Secys, respectively; 0.03–0.2 µmol L⁻¹ for His and 0.01–0.50 µmol L⁻¹ for the other amino acids. The LODs and LOQs were estimated based on three and ten times of standard deviation of concentrations obtained from three blanks, respectively. LODs for all 20 AAs are in sub nM L⁻¹ range, suitable for the determination of AAs in yeast.

3.3. Optimization of microwave parameters.

A microwave digestion method with use of MSA was recently reported for the determination of Met and SeMet in yeast [43]. Thus, in order to measure all amino acids in

196 yeast, this method was thus investigated further for other amino acids potentially exist in
197 yeast. The major influence factors such as microwave temperature, power and holding time
198 as well as MSA concentration were investigated.

199 The effect of the microwave heating time in a range of 10-180 minutes for extraction
200 AAs from yeast was tested first, at microwave temperature 150 °C, power 200 W and sample
201 size of 50 mg. For simplicity, peak heights of all AAs were used and results are presented in
202 Figure 2. Clearly, peak heights of all AAs increased when heating time increased from 10 to
203 30 minutes, maintained relatively constant from 30 to 60 minutes and then decreased when
204 heating time increased above 60 minutes. It's evident that no significant change in extraction
205 efficiencies for most AAs during 30-60 minutes heating time. Extraction efficiencies for all
206 AAs decreased at shorter heating times, may be due to inefficient protein digestion.
207 Extraction efficiencies for all AAs also decreased at longer time great than 60 minutes,
208 possibly indicating break down of analytes during extended microwave exposure. A 30
209 minutes microwave heating time was chosen for subsequent experiments to ensure
210 quantitative extraction and also avoid possible break down.

211 Holding temperature of the microwave in a range of 120 to 180 °C were investigated on
212 the extraction of AAs based on 0.050 g yeast, 6 ml 4 M MSA, 200 W and 30 minutes
213 extraction. Results are summarized in Fig.3. The peaks height of all AAs increased from
214 120 °C to 150 °C and maintained relatively constant at 150 to 170 °C. When the
215 temperature further increased from 170 °C to 180 °C, peak heights of all AAs decreased. A
216 150 °C hydrolysis temperature was chosen for subsequent study to ensure quantitative
217 extraction of all AAs from yeas while avoiding possible degradation of analytes at high

temperature.

Similar to a previous study on the determination of Met and SeMet [43], it was found that microwave power on the extraction of AAs from yeast had little effect. This is because within a couple of minutes microwave heating, the temperature of the solution reaches the controlled temperature and thus power decreases significantly to maintain that temperature. A power of 200 W was chosen for subsequent study to quickly reach the desired temperature.

The effect of MSA concentration in a range of 1-10 M on extraction efficiencies of AAs from the yeast was investigated based on 0.050 g yeast, 200 W, 150 °C and 30 minutes microwave extraction. Concentrations of AAs in yeast digests were quantified by standard addition calibration with addition of 19 AAs standards. The result is presented in Fig.5. Best hydrolysis efficiencies occurred at 3 M MSA for Gly and Pro, 9 M MSA for Val and Ile, 6 M MSA for His and 4 M MSA for the rest AAs. Hydrolysis efficiencies decreased at both higher and lower acid concentration for all AAs. Concentration of 4 M MSA was chosen for subsequent experiments as highest hydrolysis efficiencies were obtained for most AAs from yeast sample.

3.4. Determination of AAs in yeast

The proposed method was applied to the determination of AAs in selenium enriched yeast sample. A standard addition method was used for the quantification of amino acids. The selenium- enriched yeast were extracted by microwave irradiation hydrolysis at 150 °C, 30 minutes, 200 W with 4M MSA and derivatized with MCF. As shown in Fig.1b, mass spectra

and retention times of AAs in yeast extract from 1 mg yeast were corresponding to the derivatives of AAs standards in Fig. 1a. Based on the above observations, maximum of 19 AAs can thus be determined without any interference from coexisting substances. Secys wasn't found in the yeast sample. For the final quantitation of all identified AAs with exception of Met and SeMet which had been reported in a previous study [44] in the yeast, were determined by standard addition calibration with least square linear regression. Results are summarized in Table 2. In general precisions of 4.32% to 12.41% RSD of AAs concentrations were obtained. Spike recovery of all 17 AAs was performed and values of 95.4% to 106.8% with RSD values of 1.36% to 8.32% were obtained. Results obtained confirm that the proposed method for the analysis of AAs in yeast sample is feasible.

4. Conclusion

A fast and simple microwave- irradiation hydrolysis method is described for the determination of amino acids in selenium enriched yeast by GC-MS detection. Total 17 AAs can be measured within one chromatography run. The sample preparation is simple and the derivatives can be analyzed directly by GC-MS. In addition, the method has low detection limits and good recoveries. Results obtained suggest that the proposed method has potential for the rapid determination of amino acids in similar materials.

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319

320

Figure Caption

Fig 1. GC-MS chromatogram a: 100 μ L mixture of 23 AA standards at a concentration of 1 mmol L⁻¹; b: AAs extracted from 1 mg Selenium enriched yeast with microwave hydrolysis.

Fig. 2. Effect of microwave heating time on the microwave hydrolysis of yeast sample

Fig. 3. Effect of microwave temperature on the microwave hydrolysis of yeast sample.

Fig. 4. Effect of MSA concentration on the microwave hydrolysis of yeast sample.

349 **Table. 1** Analytical figures of GC-MS for the determination of AAs

350

Peak Number	Analytes	Retention Time (min)	Linear Range (μM L^{-1})	correlation coefficient (r^2)	LOD (n mol)	LOQ (n mol)	Ion used m/z
1	Ala	2.70 $\pm$ 0.02	0.01- 0. 30	0.995	0.035	0.12	161
1	Gly	2.74 $\pm$ 0.02	0.01- 0. 20	0.997	0.080	0.26	116
2	Val	3.77 $\pm$ 0.01	0.01- 0.30	0.997	0.014	0.048	146
3	Leu	4.51 $\pm$ 0.01	0.01- 0.30	0.995	0.023	0.078	115
4	Ile	4.64 $\pm$ 0.02	0.01- 0.30	0.995	0.023	0.075	144
5	Thr	4.84 $\pm$ 0.11	0.01- 0.50	0.988	0.083	0.28	147
6	Pro	4.98 $\pm$ 0.01	0.01- 0.50	0.996	0.023	0.078	187
6	Asn	5.00 $\pm$ 0.05	0.01- 0.20	0.994	0.15	0.51	95
7	Asp	5.64 $\pm$ 0.03	0.01- 0.50	0.997	0.038	0.012	160
8	Glu	6.72 $\pm$ 0.02	0.01- 0.50	0.997	0.026	0.085	201
9	Met	6.87 $\pm$ 0.02	0.01- 0.50	0.996	0.013	0.043	221
10	Semet	7.42 $\pm$ 0.02	0.01- 0.50	0.997	0.013	0.044	269
11	Cys	7.61 $\pm$ 0.03	0.01- 0.20	0.996	0.025	0.083	192
12	Phe	7.80 $\pm$ 0.02	0.01- 0.50	0.996	0. 0091	0.030	162
13	SeCys	8.18 $\pm$ 0.04	0.01- 0.30	0.995	0.017	0.057	240
14	Hcys	8.56 $\pm$ 0.05	0.01- 0.20	0.996	0.026	0.088	147
15	Lys	9.93 $\pm$ 0.04	0.01- 0.50	0.996	0.050	0.17	244
16	His	10.38 $\pm$ 0.05	0.03- 0.20	0.994	0.17	0.58	210
17	Tyr	10.97 $\pm$ 0.03	0.01- 0.50	0.996	0.021	0.070	236
18	Trp	12.18 $\pm$ 0.10	0.01- 0.50	0.996	0.015	0.052	276

351

352

353 Table 2. Concentrations of AAs extracted from 1 mg yeast samples with microwave
 354 hydrolysis

Peak Number	Analytes	Regression equation ^a	coefficient (r ²)	Concentration (ug/g)	
				Mean ⁿ	RSD%
1	Ala	y = 68207x + 24874	R ² = 0.9905	32490	5.75
1	Gly	y = 42300x + 9636	R ² = 0.9903	17096	7.34
2	Val	y = 170771x + 40341	R ² = 0.9920	27674	5.11
3	Leu	y = 164701x + 43478	R ² = 0.9909	34629	4.94
4	Ile	y = 214992x + 34687	R ² = 0.9912	21165	5.53
5	Thr	y = 3663x + 780	R ² = 0.9886	25338	12.43
6	Pro	y = 219291x + 39299	R ² = 0.9904	20632	4.32
6	Asn	y = 13671x + 795	R ² = 0.9852	7683	10.53
7	Asp	y = 112107x + 42130	R ² = 0.9905	50019	5.48
8	Glu	y = 90194x + 12472	R ² = 0.9936	20345	6.65
11	Cys	y = 124230x + 2325	R ² = 0.9896	2268	8.34
12	Phe	y = 253979x + 33892	R ² = 0.9958	22044	4.36
14	Hcys	y = 100466x + 638	R ² = 0.9914	857	11.84
15	Lys	y = 57397x + 18833	R ² = 0.9919	59931	5.24
16	His	y = 15021x + 872	R ² = 0.9901	9006	10.69
17	Tyr	y = 112607x + 10075	R ² = 0.9918	16112	5.32
18	Trp	y = 69690x + 465	R ² = 0.9912	1362	9.64

355
 356 ^aY represents peak height and X represent the concentrations of spiked analytes, n=5