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Quantitative analysis of sulfur-related metabolites during cadmium stress response in yeast by capillary electrophoresis–mass spectrometry

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Abstract

The objective of this research is to establish an evaluation system of metabolites by capillary electrophoresis–mass spectrometry (CE–MS) in response to chemical stress using the unicellular genome model, yeast (*Saccharomyces cerevisiae* strain S288C). A method previously reported by Soga et al. was modified and validated for the determination of sulfur-related metabolites, 23 cationic metabolites, in yeast extract. A mixture of 5 mM formic acid in 50% (v/v) 2-propanol was used for the sheath liquid to improve the detection sensitivity. Moreover, washing of CE capillary with 0.1 M ammonia solution between successive runs enhanced the reproducibility. After analytical validation, the method was applied to the metabolomic analysis of yeast cells in response to cadmium (Cd) stress. Under Cd exposure, some interesting observations were obtained, particularly the depletion of glycine and the strong accumulation of L-γ-glutamylcysteine in yeast cells.
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1. Introduction

Metabolomics is an emerging new omics science analogous to genomics, transcriptomics and proteomics, and can be regarded as the end point of the “omics” cascade [1,2]. The advantage of metabolomic analysis is that the biochemical consequences of mutations and stress response mechanisms can be observed directly. As the metabolome represents a wide variety of chemical compounds, it is logical that numerous high-throughput analytical techniques are being used for metabolomics. Being non-destructive, nuclear magnetic resonance (NMR) spectroscopy is highly beneficial as a metabolomics technique [2–4], but it also possesses one major disadvantage, which is that it is relatively insensitive compared to mass spectrometry (MS) [5]. In the rapidly growing field of metabolomics, MS coupled to a chromatographic separation technique is a useful method used to profile low molecular weight compounds [2,6–9]. Capillary electrophoresis–mass spectrometry (CE–MS) has been considered a highly promising technique for comprehensive metabolomics analysis because most of the metabolites are polar and ionic compounds. It gives high-resolution separations of cationic metabolites, anionic metabolites and nucleotides/CoA in a reasonable time, and requires a minimum amount of samples using three different separation modes [10,11].

The aim of this study is to investigate qualitative and quantitative difference of metabolites in response to chemical stress using model organisms such as yeast cells by CE–MS. For example, in yeast it is well known that cadmium (Cd) triggers a strong induction of enzymes of the sulfur metabolic pathway, consistent with a strong increase of glutathione synthesis for the detoxification of the toxic metal [12]. The global changes in gene and protein expression underlying the yeast stress response to Cd have been analyzed by DNA microarray technology and two-dimensional gel electrophoresis, respectively [13–16]. Recently, a combined proteomic and metabolomic study using LC–MS was carried out for investigating Cd stress in yeast [17]. The study, however, examines only seven target analytes, namely, homocysteine, cystathionine, cysteine, methionine, L-γ-glutamylcysteine, reduced glutathione (GSH), *S*-adenosyl-L-homocycteine. Therefore, to investigate the global

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metabolic pathway of sulfur metabolism in yeast, a CE–MS method for quantitative analysis of 23 sulfur-related cationic metabolites was developed and validated. The proposed analytical method was applied to the investigation of qualitative and quantitative difference of metabolites in response to Cd stress.

2. Experimental

2.1. Chemicals and solutions

DL-Homocysteine and *O*-acetyl-L-serine were obtained from MP Biomedicals (Irvine, CA, USA), L-γ-glutamylcysteine ammonium salt and L-cysteinylglycine were obtained from Bachem AG (Bubendorf, Switzerland), *O*-acetyl-L-homoserine hydrochloride was obtained from Toronto Research Chemicals (North York, ON, Canada), and *S*-adenosyl-L-methionine chloride was from Sigma–Aldrich Japan (Tokyo, Japan). All other standard materials as its free forms were obtained from Wako Pure Chemical (Osaka, Japan) or Sigma–Aldrich Japan. L-Methionine sulfone as internal standard was purchased from Sigma–Aldrich Japan. Formic acid and methanol were of LC–MS grade, and 2-propanol was of HPLC grade. Water was purified with a Milli-Q water purification system (Nihon Millipore, Tokyo, Japan). All other chemicals and reagents were of analytical grade and were obtained from commercial sources.

2.2. Apparatus

CE–MS experiments were performed using a P/ACE MDQ system (Beckman Coulter, Tokyo, Japan). An ESI interface (Agilent Technologies Japan, Tokyo, Japan) was used to couple the CE instrument to a Bruker Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics, Ibaraki, Japan). Sheath liquid consisting of 5 mM formic acid in 50% (v/v) 2-propanol was applied coaxially at a rate of 10 μL/min by using a syringe pump (model 74900, Cole-Parmer, Vernon Hills, IL, USA).

2.3. Sample preparation

Yeast (*Saccharomyces cerevisiae* strain S288C) cells were exposed to 300 μM cadmium chloride ($CdCl_2$) in yeast extract peptone dextrose (YPD) medium for stress treatment; YPD medium without ($CdCl_2$) served as control. After filtration with a 0.45 μm membrane filter, the cells were frozen in liquid nitrogen, and then crushed to a very fine powder using a chilled mortar and pestle. Yeast metabolites were extracted from the freeze-dried yeast cells (ca. 100 mg) in 700 μL of cold chloroform and 500 μL of cold methanol followed by the addition of 500 μL of cold Milli-Q water containing a known amount of methionine sulfone as an internal standard. The resulting mixture was centrifuged at 15,000 rpm for 15 s. The supernatant was then filtered through an ultrafiltration membrane (Ultrafree-MC filter, 5000 NMWL, Millipore, Bedford, MA, USA). The filtered solution was stored in the deep freeze at −80 °C until analyzed.

2.4. CE–MS conditions

Analytical conditions were modified based on a previously reported method [11]. An untreated fused-silica capillary (50 μm i.d., 375 μm o.d. and 100 cm long, Polymicro, Phoenix, AZ, USA) was used for CE separation. The CE capillary was conditioned for 20 min with 0.1 M ammonia solution prior to initial use, and was washed for 3 min with 0.1 M ammonia solution and for 5 min with a running solution of 1 M formic acid solution (pH 1.8) before each run. A small volume of sample was hydrodynamically injected into the capillary by applying 1 psi (6.9 kPa) pressure for 10 s. A constant voltage of 30 kV was applied across the capillary for the electrophoretic separation. For detection, the mass spectrometer was operated in the positive-ion ESI mode in the range 70–650 mass-to-charge ratios (*m*/*z*) at a maximum accumulation time of 100 ms with a target of 30,000 ions. The target mass was set to 200 for tuning optimization.

3. Results and discussion

3.1. Estimation of extraction method from yeast cells

Quenching of metabolism is generally achieved by rapidly freezing samples in liquid nitrogen or cold-methanol [18–20]. In addition, high extraction efficiency from yeast cells must be obtained with an appropriate extraction solvent. Different extraction methods have been employed for the extraction of yeast metabolites, such as chloroform–methanol-buffer, perchloric acid, potassium hydroxide, boiling ethanol, methanol–water and cold-pure methanol extractions [20,21]. Recently, a more reliable procedure has been reported for quenching yeast cells with cold methanol or chloroform–methanol-buffer solution by GC–MS analysis [20]. In CE, however, the sample matrix, especially its ionic strength and contaminants adhering to the capillary wall, affects the separation efficacy. The overall performance of the different sample preparation methods was examined using chloroform–methanol–water, methanol–water and cold-pure methanol extractions because a high ionic strength in the sample often gives poor peak shapes and current disruption. The results showed that the extraction efficiency could be increased by using chloroform–methanol–water extraction compared to methanol–water extraction for most of our target metabolites. Excess amount of methanol in sample solution by pure-methanol extraction caused current disruption in the CE separation.

3.2. Change in analytical conditions for CE–MS

A comprehensive and quantitative analysis of charged metabolites by CE–MS has been already reported by Soga et al. [10,11]. Because our research interests of this study mainly involve sulfur-containing amino acids, we first attempted to analyze the yeast extracts spiked with potential sulfur-related metabolites using the separation conditions for cationic metabolites [11]. However, poor detection sensitivity was obtained without freeze concentration of yeast extracts. The analytical

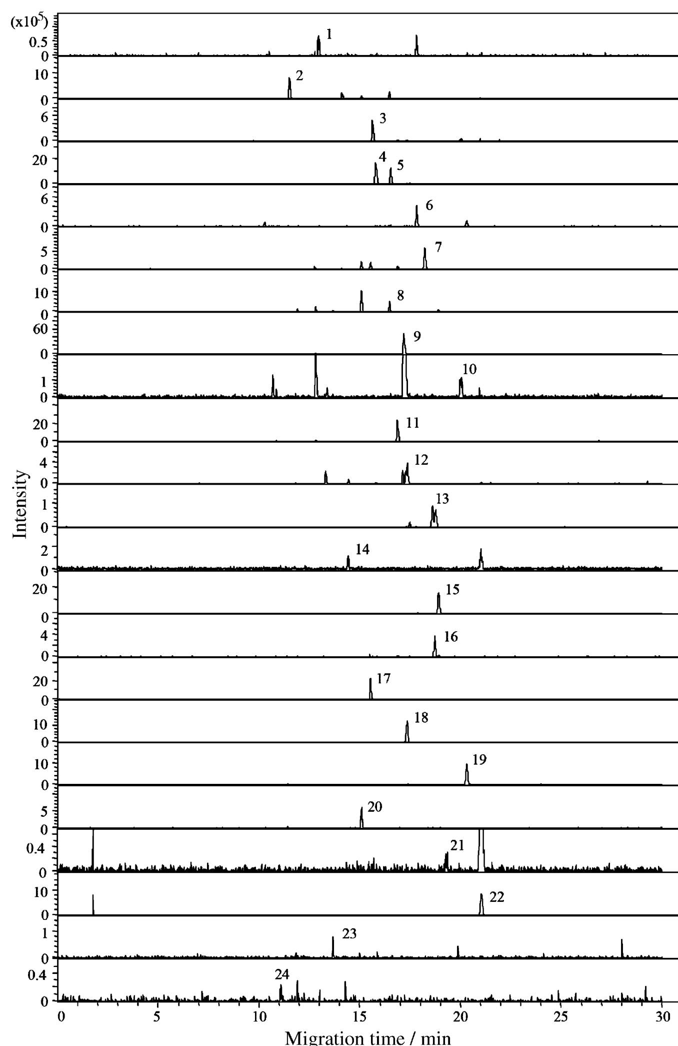

Fig. 1. Selected ion electropherograms obtained from a yeast extract spiked with 23 potential sulfur-related metabolites. Spiked concentrations: 50 μg/mL for each target metabolite, 150 μg/mL for glutamic acid and reduced glutathione (GSH). 1: glycine (m/z = 76), 2: alanine (m/z = 90), 3: serine (m/z = 106), 4: homoserine (m/z = 120), 5: threonine (m/z = 120), 6: cysteine (m/z = 122), 7: aspartic acid (m/z = 134), 8: homocysteine (m/z = 136), 9: glutamic acid (m/z = 148), 10: *O*-acetyl-L-serine (m/z = 148), 11: methionine (m/z = 150), 12: *O*-acetyl-L-homoserine (m/z = 162), 13: methionine *S*-oxide (m/z = 166), 14: cysteinylglycine (m/z = 179), 15: methionine sulfone (internal standard) (m/z = 182), 16: *O*-succinyl-L-homoserine (m/z = 220), 17: cystathionine (m/z = 223), 18: cystine (m/z = 241), 19: L-γ-glutamylcysteine (m/z = 251), 20: homocystine (m/z = 269), 21: oxidized glutathione (m/z = 308), 22: GSH (m/z = 308), 23: *S*-adenosyl-L-homocysteine (m/z = 385) and 24: *S*-adenosyl-L-methionine (m/z = 399).

conditions were partly modified, resulting in the 3.3–11.5-fold increase in peak intensity (1.0–5.3-fold increase in signal to noise) provided by using a mixture of 5 mM formic acid in 50% (v/v) 2-propanol for sheath liquid instead of 5 mM ammonium acetate in 50% (v/v) methanol. Furthermore, additional washing of CE capillary with 0.1 M ammonia solution between successive runs enhanced the reproducibility without delay in migration times. The CE–MS method was employed for a yeast extract spiked with potential sulfur-related metabolites. Fig. 1 shows 23 metabolites as extracted ion electropherograms.

Table 1
Percent recovery of potential sulfur-related metabolites spiked in yeast extract

Components	Spiked concentration	
	50 or 150 μg/mL[a]	100 or 300 μg/mL[a]
Glycine	94.9	104.4
Alanine	98.8	102.7
Serine	76.8	100.9
Homoserine	104.1	93.2
Threonine	125.6	104.1
Cysteine	92.8	112.8
Aspartic acid	94.7	113.3
Homocysteine	101.0	106.4
Glutamic acid	126.1	108.3
O-acetyl-L-serine	99.2	101.0
Methionine	104.4	107.9
O-acetyl-L-homoserine	99.1	98.3
Methionine *S*-oxide	100.8	108.5
O-succinyl-L-homoserine	101.4	112.4
Cystathionine	97.8	115.5
Cystine	103.5	90.6
L-γ-Glutamylcysteine	99.4	102.4
Homocystine	90.2	93.3
Reduced glutathione (GSH)	92.7	104.8

Samples were prepared by adding a known amount of the analytes to an aliquot of yeast extract. The recovery was calculated against each standard calibration curve.

[a] Spiked with higher concentrations of glutamic acid and GSH at 150–300 μg/mL in yeast extract.

Table 2
Repeatability and reproducibility of CE–MS analysis for the measurement of yeast metabolites

Components	Repeatability % (n = 6)[a]	Reproducibility % (n = 6)[b]
Glycine	10.5	11.4
Alanine	11.6	nd
Serine	9.3	11.5
Homoserine	10.3	23.8
Threonine	9.4	11.4
Cysteine	11.2	nd
Aspartic acid	9.6	8.8
Homocysteine	10.7	nd
Glutamic acid	13.8	11.0
O-acetyl-L-serine	9.3	nd
Methionine	10.5	9.6
O-acetyl-L-homoserine	10.4	22.7
Methionine *S*-oxide	11.9	nd
Cysteinyl-glycine	7.2	nd
O-succinyl-L-homoserine	9.3	22.2
Cystathionine	7.6	15.6
Cystine	12.0	nd
L-γ-Glutamylcysteine	8.4	nd
Homocystine	8.4	nd
Reduced glutathione (GSH)	9.4	10.6
Oxidized glutathione	14.6	nd
S-adenosyl-L-homocysteine	12.8	nd
S-adenosyl-L-methionine	11.1	nd

[a] The values were obtained with six replicate injections from a standard addition sample spiked with 100 μg/mL of each component.

[b] The values were obtained by analyzing six sample preparations from the same yeast powder.

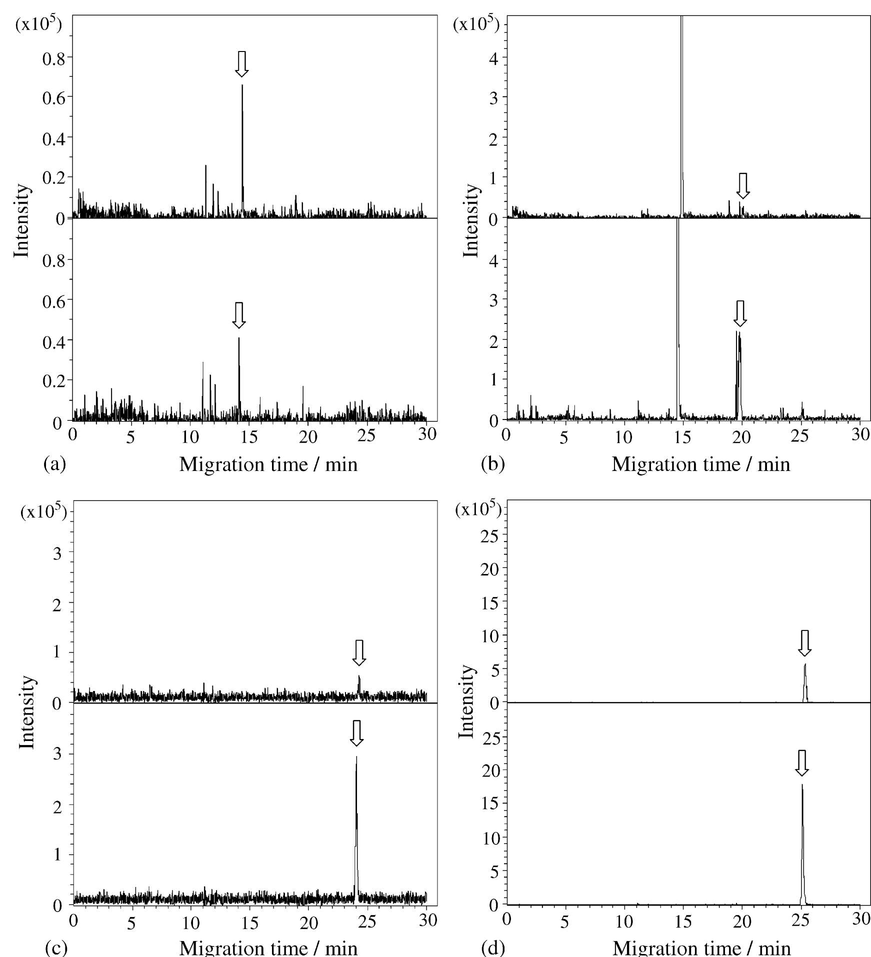

Fig. 2. Representative electropherograms showing differences in Cd stress response. (a) glycine (m/z = 76), (b) *O*-acetyl-L-homoserine (m/z = 162), (c) L-γ-glutamylcysteine (m/z = 251) and (d) GSH (m/z = 308). Upper electropherograms: control for 2 h, lower electropherograms: Cd stress treatment for 2 h. The arrows indicate the peaks of the target analytes.

3.3. Analytical validation

The analytical method was validated in terms of linearity, accuracy and precision. Standard addition is particularly appropriate to account for matrix effects in quantitative analysis. For the assessment of linearity, standard stock solutions were prepared by dissolving each standard substance at the concentration of 5 mg/mL (except for glutamic acid and GSH at the concentration of 15 mg/mL) with water or 0.1 M hydrochloric acid, followed by mixing and diluting to an appropriate concentration with 50% (v/v) methanol. Standard addition samples at six different concentrations were prepared by mixing an appropriate concentration of the standard stock solution with an aliquot of yeast extract (1:1). Aqueous standard solutions were prepared by mixing an appropriate concentration of the standard stock solution with methanol–water (1:1) instead of yeast extract. The calibration curves showed linearity in the additive concentration ranges of 6–300 μg/mL for glutamic acid (r = 0.9974) and GSH (r = 0.9955), and 2–100 μg/mL for the other components (r = 0.9893–0.9998). Matrix effects can be revealed by comparing the parameters of the standard

Table 3
Differences in sulfur-related metabolites during Cd stress response in yeast cells

Components	Stress treatment with 300 μM cadmium chloride		
	1 h	2 h	3 h
Glycine	0.653 ± 0.072	0.441 ± 0.074	0.375 ± 0.042
Serine	0.276 ± 0.062	0.237 ± 0.016	0.527 ± 0.057
Homoserine	0.712 ± 0.079	0.921 ± 0.087	1.024 ± 0.129
Threonine	0.489 ± 0.062	0.343 ± 0.004	0.310 ± 0.021
Aspartic acid	1.540 ± 0.135	1.222 ± 0.070	1.214 ± 0.163
Glutamic acid	1.043 ± 0.050	1.241 ± 0.053	1.316 ± 0.098
Methionine	0.273 ± 0.037	0.326 ± 0.002	0.386 ± 0.057
O-acetyl-L-homoserine	5.562 ± 2.788	14.776 ± 6.964	16.288 ± 6.784
O-succinyl-L-homoserine	1.333 ± 0.371	1.668 ± 0.196	1.326 ± 0.108
Cystathionine	0.802 ± 0.137	0.588 ± 0.020	0.487 ± 0.068
Reduced glutathione (GSH)	2.089 ± 0.411	2.660 ± 0.569	2.584 ± 0.517

The average (mean ± S.D.) values obtained from the difference in stress/control concentration ratio of the metabolites at the same time point.

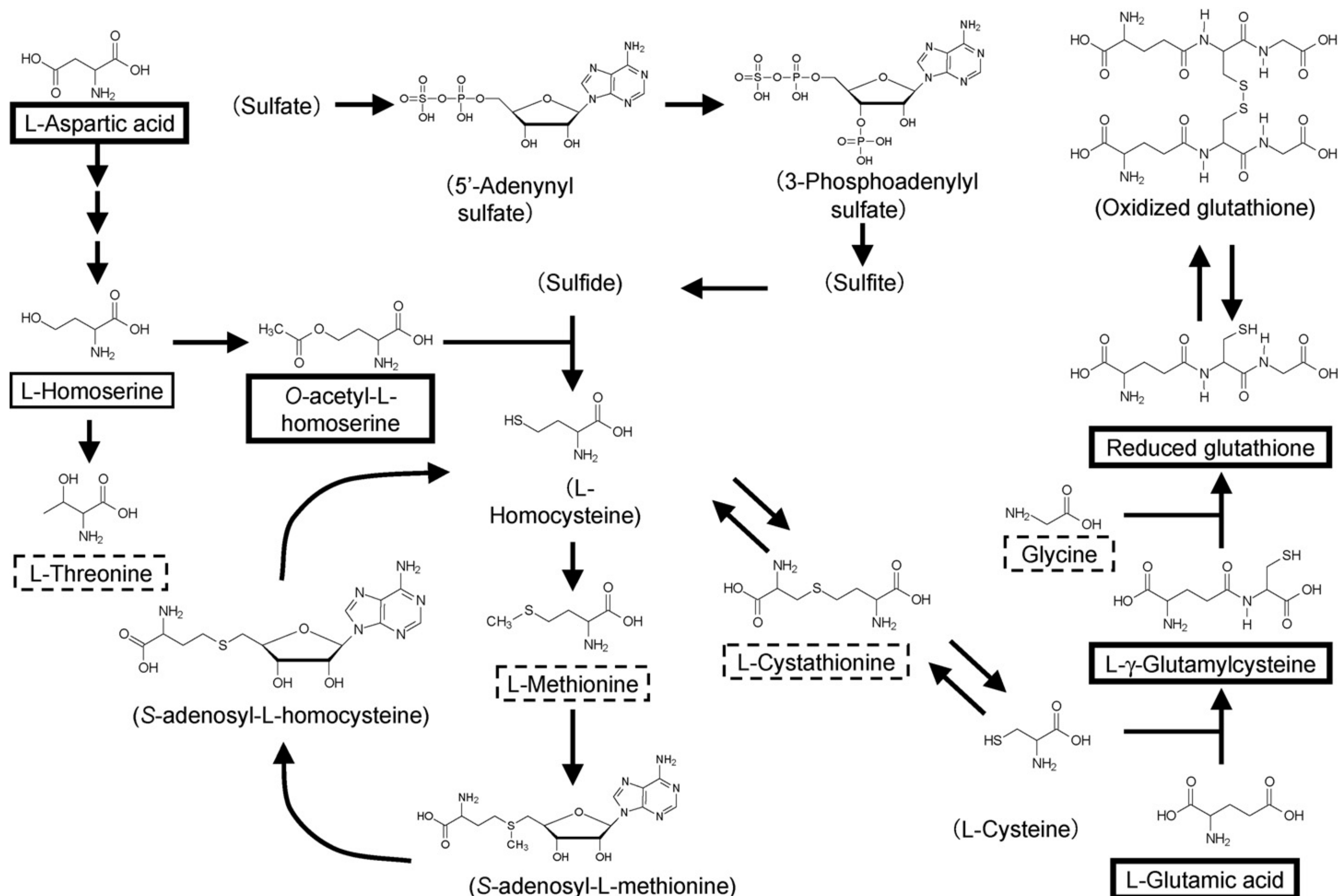

Fig. 3. An overview of sulfur metabolism pathway and the differences in Cd stress response in yeast cells. The frames indicated in bold, narrow and dash lines on the compound name show the changes in stress response as increase, unchanged and decrease metabolites, respectively. Undetected metabolites are indicated in parentheses.

addition line with those of an aqueous standard calibration line. The slope ratio obtained by dividing the slope of the linear regression line for the standard addition should be close to 1.0 if the matrix did not interfere. The ratios for 18 metabolites were obtained in the range of 0.9365–1.1453. Five to six calibration points in the concentration range of 2–100 μg/mL were not obtained for cysteinylglycine, homocystine, oxidized glutathione, *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine due to the low detection sensitivity. The results of the percent spike recoveries are given in Table 1.

Repeatability was evaluated by analyzing six replicate injections of a standard addition sample. The overall reproducibility was verified by measuring yeast extraction solutions prepared from six aliquots of the same powdered yeast cell sample. As given in Table 2, it was found that repeatability and reproducibility values were acceptable in our study. Although the only exception was for the comparatively poor reproducibility of homoserine (23.8%), *O*-acetyl-L-homoserine (22.7%) and *O*-succinyl-L-homoserine (22.2%), these analytes were detected at low concentrations near the LODs.

3.4. Determination of sulfur-related metabolites in response to cadmium stress

The modified and improved CE–MS method was now employed for the metabolomic analysis of yeast cells in response to Cd stress. Three replicate sample preparations under the same conditions (initial, control incubation for 1–3 h, stress incubation for 1–3 h) and two replicate injections of each sample were performed. MS electropherograms of representative yeast metabolites in Cd stress-induced and control yeast extracts (2 h) are shown in Fig. 2. Several metabolites showed remarkable differences between control and stress-treated yeast cells. The peak area ratios of the analyte/internal standard were calculated from their corresponding selected ion electropherograms of the target metabolites. In Table 3, the indicative values of the Cd stress response were obtained by dividing the peak area ratio for the control condition to each stress condition at the same time point. We briefly discuss the sulfur metabolism pathway based on the CE–MS measurements as shown in Fig. 3. In this study, five cationic metabolites (i.e. cysteine, homocysteine, oxidized glutathione, *S*-adenosyl-L-homocysteine and

S-adenosyl-L-methionine) were below detection limits. The results showed that a common feature of the Cd stress response is obtained from the accumulation of GSH and *O*-acetyl-L-homoserine. A very interesting observation of this investigation was that Cd stress induced the depletion of glycine and the strong accumulation of L-γ-glutamylcysteine. This observation has lead to the speculation that glycine may be rate-limiting for the production of glutathione. However, further investigation in biological experiments is needed to verify the speculation.

4. Conclusion

This paper demonstrated a validated CE–MS method for quantitative analysis of 23 sulfur-related cationic metabolites. To improve the detection sensitivity, a previously reported CE–MS method for cationic metabolites [11] was modified by using a mixture of 5 mM formic acid in 50% (v/v) 2-propanol as sheath liquid. The CE–MS method was applied to the investigation of qualitative and quantitative difference of yeast metabolites in response to Cd stress. The depletion of glycine and the strong accumulation of L-γ-glutamylcysteine as well as the accumulation of GSH and *O*-acetyl-L-homoserine in yeast cells was a novel finding of this study. CE–MS is a promising approach to investigate the global changes in relevant metabolites to study stress responses in biological organisms.

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