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LC-MS analysis of key components of the glutathione cycle in tissues and body fluids from mice with myocardial infarction

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ABSTRACT

Oxidative stress is suggested to play an important role in several pathophysiological conditions. A recent study showed that decreasing 5-oxoproline (pyroglutamate) concentration, an important mediator of oxidative stress, by over-expressing 5-oxoprolinase, improves cardiac function post-myocardial infarction in mice. The aim of the current study is to gain a better understanding of the role of the glutathione cycle in a mouse model of myocardial infarction by establishing quantitative relationships between key components of this cycle. We developed and validated an LC–MS method to quantify 5-oxoproline, L-glutamate, reduced glutathione (GSH) and oxidized GSH (GSSG) in different biological samples (heart, kidney, liver, plasma, and urine) of mice with and without myocardial infarction. 5-oxoproline concentration was elevated in all biological samples from mice with myocardial infarction. The ratio of GSH/GSSG was significantly decreased in cardiac tissue, but not in the other tissues/body fluids. This emphasizes the role of 5-oxoproline as an inducer of oxidative stress related to myocardial infarction and as a possible biomarker. An increase in the level of 5-oxoproline is associated with a decrease in the GSH/GSSG ratio, a well-established marker for oxidative stress, in cardiac tissue post-myocardial infarction. This suggests that 5-oxoproline may serve as an easily measurable marker for oxidative stress resulting from cardiac injury. Our findings show further that liver and kidneys have more capacity to cope with oxidative stress conditions in comparison to the heart, since the GSH/GSSG ratio is not affected in these organs despite a significant increase in 5-oxoproline.

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1. Introduction

Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS), and the capacity of the endogenous antioxidant defense system to deal with ROS [1]. Under physiological conditions, small quantities of ROS, which function in cell signaling, can be readily neutralized by the antioxidant defense system. However, under pathophysiological conditions, ROS production may exceed the buffering capacity of the antioxidant defense system, resulting in cell damage and ultimately cell death. This imbalance in redox state is implicated in the onset and progression of several diseases, including cardiovascular disease [1,2].

The major source of antioxidants in mammalian cells is glutathione (GSH), which is formed by the glutathione cycle, also formerly known as γ-glutamyl cycle (Fig. 1) [3]. Although the enzymes and metabolites of the glutathione cycle have been characterized extensively, only recently have they been associated with heart failure [4]. One such enzyme, 5-oxoprolinase (OPLAH), is responsible for converting 5-oxoproline, both a degradation product and an intermediate of *de novo* GSH synthesis, into L-glutamate [3,5]. 5-Oxoproline has been shown to induce oxidative stress in brain tissue and cardiomyocytes [4,6]. Furthermore, decreasing the level of 5-oxoproline by over-expressing OPLAH in mice, improves cardiac function post cardiac injury [4]. These observations suggest a major role of the glutathione cycle in heart failure.

To obtain a better understanding of the involvement of the glutathione cycle in heart failure, it is essential to decipher how key components change under physiological and pathophysiological conditions. Numerous analytical methods have been established to quantify 5-oxoproline, L-glutamate, GSH and GSSG [7–12]. Here we

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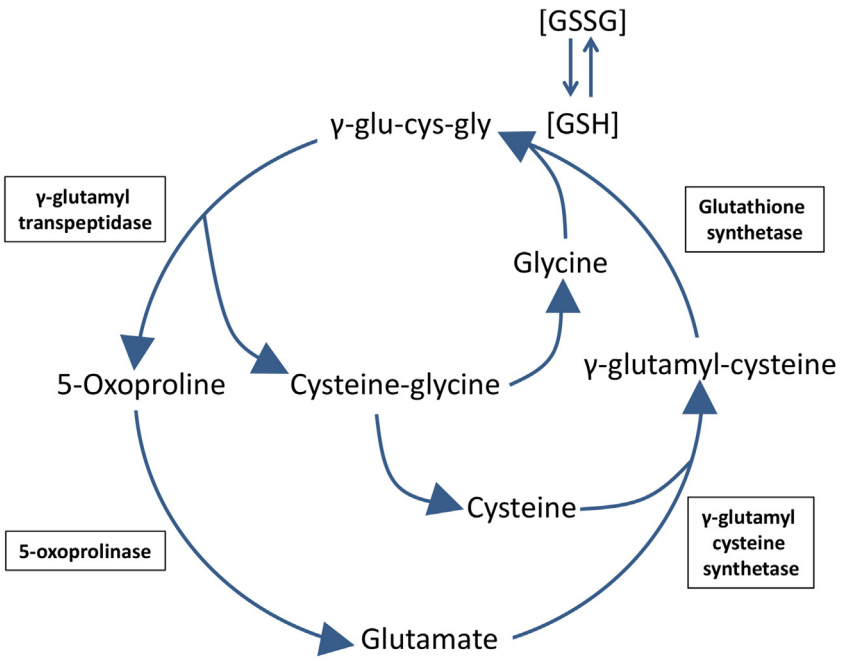

Fig. 1. Schematic representation of the glutathione cycle. 5-oxoproline, both a degradation product and an intermediate of *de novo* glutathione (GSH) synthesis, is transformed into L-glutamate via 5-oxoprolinase (OPLAH) activity. GSH can then be utilized as an antioxidant, producing oxidized glutathione (GSSG) in the process.

report the development and validation of an LC–MS method for the simultaneous quantitation of 5-oxoproline, L-glutamate, GSH and GSSG in different biological samples (heart, kidney, liver, plasma and urine) of mice with and without myocardial infarction (MI). From a methodological point of view, we show that certain matrices may lead to interferences. From a disease mechanism point of view, we show that the failing heart has limited anti-oxidant capacity compared to the kidneys and liver, making it particularly vulnerable to ROS.

2. Materials and methods

2.1. Solvents, chemicals and standards

All chemicals used had the highest purity commercially available. Methanol (MeOH, HPLC SupraGradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands). Phosphate buffered saline (PBS), bovine serum albumin (BSA), formic acid (LC–MS grade), N-ethylmaleimide (NEM) and all standard compounds (^{13}C-labeled L-glutamic acid, ^{13}C, ^{15}N-labeled GSH and non-labeled 5-oxoproline, L-glutamic acid, GSH and GSSG) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Ultrapure water was obtained from a Milli-Q Advantage A10 water purification system at a resistivity of 18.2 MΩ cm (Millipore SAS, Molsheim, France).

2.2. Permanent myocardial infarction in wild-type mice

The animal protocol was approved by the Animal Ethical Committee of the University of Groningen (permit number: DEC6632), and performed conform the ARRIVE guidelines [13]. A total of 24 C57BL/6J mice were included in the MI study. All mice were 14–20 weeks of age and 35–40 g of body weight. The mice were randomized into the SHAM-operated group and the MI group. Animals were anesthetized with isoflurane and medical oxygen, followed by the administration of 5 mg/kg of carprofen. The MI group (n = 13) underwent permanent ligation of the left anterior descending branch (LAD) of the left coronary artery. The ligation of the LAD was placed to achieve a ±30% area at risk of the left ventricle. The SHAM operated group (n = 11) underwent the same procedure without ligation of the LAD. After 4 weeks, animals were sacrificed and blood, urine, and organs were collected, immediately placed in liquid nitrogen, and stored for further sample preparation and subsequent LC—MS analysis.

2.3. Production of isotopically-labeled internal standards (IS)

5-Oxoproline internal standard (IS) was prepared from ^{13}C-labeled L-glutamic acid. Briefly, L-glutamic acid (250 mg) was dissolved in 0.1 M HCl and heated at 80 °C for 72 h, to convert ^{13}C-L-glutamic acid into ^{13}C-5-oxoproline, as previously described [12]. Later, the solution was dried under a stream of nitrogen and re-dissolved in 50 mL water.

GSSG (IS) was prepared by a controlled oxidation of ^{13}C, ^{15}N-labeled GSH. Briefly, 10 mg of ^{13}C, ^{15}N-labeled GSH were dissolved in 1 mL water. Half of the solution (0.5 mL) was mixed with of 0.5 mg NaI (final concentration 6.7 mM) and 1 μL 30% H_2O_2. The mixture was heated at 25 °C for 60 min to allow oxidation. Excess H_2O_2 was eliminated by increasing the temperature of the mixture to 65 °C for 5 min as previously described [14].

Both solutions (one containing ^{13}C-5-oxoproline and ^{13}C-L-glutamic acid, and the other containing ^{13}C, ^{15}N-labeled GSH and ^{13}C, ^{15}N-GSSG) were mixed and the solvent was evaporated. Finally, the mixture was resuspended in 1 mL water and used as IS for further experimental work. The final ratio of the components in the IS solution was 1:1,5:6:12 for ^{13}C-L-glutamic acid, ^{13}C-5-oxoproline, ^{13}C, ^{15}N-GSSG and ^{13}C, ^{15}N-labeled GSH, respectively.

2.4. Sample preparation

Murine plasma and urine were prepared by adding 200 μL of cold (−20 °C) extraction solution (0.5 μL isotopically-labeled IS and 1.25 mg of NEM in 75% methanol) to 25 μL of sample. Snap frozen murine tissues (heart, kidney, and liver) were powdered using a mortar and pestle and ± 1 mg of powdered tissue was mixed with 200 μL of cold (−20 °C) extraction solution. Plasma and urine sam-

ples were vortexed for 5 min, and tissue samples were sonicated for 5 min, followed by incubation for 45 min in a thermomixer at room temperature and 900 rpm to allow derivatization of GSH to GSH-NEM. Samples were centrifuged at 4 °C and 20,800 *g* for 20 min. The supernatant was collected and dried under a stream of nitrogen at room temperature, followed by resuspension in 100 μL water. Samples were stored at -80 °C until further LC–MS analysis. For tissue samples, pellets formed after centrifugation were homogenized in 200 μL ice-cold RIPA buffer (50 mM Tris pH 8.0, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl). Protein concentrations were determined with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific), following the manufacturer's instructions. 5-Oxoproline, L-glutamic acid, GSH-NEM and GSSG concentrations were normalized to the total protein content.

2.5. LC-MS

5-Oxoproline, L-glutamic acid, GSH-NEM and GSSG were separated in reversed-phase mode on an Acquity HSS T3 column (1.8 μm, 100 × 2.1 mm; Waters) using a 1290 Infinity LC system (Agilent). Mobile phases consisted of 0.1% formic acid in water (eluent A) and methanol (eluent B). The following gradient was applied: 0 min – 100%A, 2.5 min – 100%A, 5 min – 95%A, 6 min – 15%A, 8 min – 15%A and 10 min – 100%A. The column temperature was set at 30 °C, the flow rate was 0.3 mL/min, and the injection volume was 10 μL.

Mass spectrometry detection was performed using a 6410 Triple Quadrupole MS system (Agilent) by positive electrospray ionization (ESI+) in the Selective Reaction Monitoring (SRM) mode. The optimized MS source parameters were: ionspray voltage: +1500 V, drying gas flow (N_2): 6 L/min, drying gas temperature 300 °C, nebulizer pressure: 15 psi. The quadrupole mass analyzer was set to unit resolution and the electron multiplier to 2400 V. The run was divided into 4 segments with MS/MS transitions 130/84 for 5-oxoproline, 135/88 for $^{13}C_5$-labeled 5-oxoproline (IS), 148/84 for L-glutamic acid, 153/88 for $^{13}C_5$-labeled L-glutamic acid (IS), 433/304 for GSH-NEM, 436/307 for $^{13}C_2$, ^{15}N-labeled GSH-NEM (IS), and 613/355 for GSSG, 619/361 for $^{13}C_4$, $^{15}N_2$-labeled GSSG (IS). Fragmentor and collision energies were optimized to 100 V and 9 V for 5-oxoproline; 100 V and 13 V for L-glutamic acid; 125 V and 9 V for GSH-NEM and 200 V and 21 V for GSSG, respectively. The dwell time for each transition was 100 ms. The LC–MS system was controlled by MassHunter Workstation software (Agilent).

2.6. Analysis of surrogate matrices

Matrix effects were evaluated by spiking 5-oxoproline, L-glutamic acid, GSH and GSSG (primary standards) into murine heart, kidney, liver, plasma and urine and comparing the results with those obtained for surrogate matrices (2% BSA in PBS and PBS alone). Snap-frozen tissue samples were suspended in PBS (approximately 10% $^{w}/_{v}$) and maintained on dry ice during the experiment. Twenty-five μL of each tissue suspension (after thawing), plasma and urine were spiked with the primary standards over the concentration range of the linearity test (see below). Each sample was extracted following the procedure described above. Calibration curves were constructed based on peak area ratios of unlabeled metabolites to their corresponding ^{13}C-labeled IS.

2.7. Validation

For validation purposes 5-oxoproline, L-glutamic acid and GSSG (primary standards) were weighed, dissolved in PBS containing 2% BSA and mixed to obtain a single analyte stock solution with a concentration of 200 μM for each analyte. GSH was added to this stock solution at a final concentration of 4000 μM. The stock solution was diluted with PBS containing 2% BSA to obtain 10 calibration points ranging from 200 to 0.12 μM for 5-oxoproline, L-glutamic acid and GSSG, and from 4000 to 2.4 μM for GSH. These calibrants were subjected to the sample preparation procedure described above. The final concentrations of the calibration points were 50-0.03 μM for 5-oxoproline, L-glutamic acid and GSSG, and 1000-0.6 μM for GSH-NEM. Calibration curves were constructed based on the peak area ratios of unlabeled analytes to the corresponding isotopically-labeled standards.

Following international guidelines [15,16], method validation was performed by evaluating intra-day variability (repeatability), inter-day variability (intermediate precision), lower limit of quantitation (LLOQ), linearity, accuracy, recovery and stability. Three quality control samples (QC) were prepared by spiking a solution of PBS containing 2% BSA with 5-oxoproline, L-glutamic acid and GSSG at 40, 12 and 3 μM, and GSH at 800, 240 and 60 μM. These were defined as High, Medium (Med) and Low QC samples, respectively. The QC samples were used to evaluate accuracy, recovery and precision (repeatability and intermediate precision). Accuracy, recovery and repeatability were assessed by independently extracting the 3 QC samples and measuring them 3 times in one batch. Intermediate precision was evaluated by repeating the previous experimental procedure on 3 different days. Stability was evaluated as follows: three QC samples prepared in human plasma were prepared and measured 3 times in one batch after leaving them on the bench for 25 and 51 h at room temperature. Freeze-thaw stability was assessed by freezing the QC samples at −40 °C, thawing and LC–MS analysis. This process was repeated 5 times on different days. The LLOQ was set to the lowest point on the calibration curves where analyte responses were at least 5-times higher than a blank and the coefficient of variation (CV) was below 20%.

3. Results and discussion

3.1. LC-MS

Multiple LC–MS methods have been developed for the determination of key molecules of the glutathione cycle [7–9,12,17]. We selected reverse phase chromatography for the simultaneous determination of L-glutamate, 5-oxoproline, GSSG and GSH-NEM, since it avoids drawbacks of other chromatographic techniques, such as contamination of the ion source and short column life-time [18,19].

L-Glutamate, 5-oxoproline, GSSG and GSH-NEM were separated in 10 min with retention times of 0.90, 2.75, 5.85 and 6.90 min, respectively (Fig. S1). The closeness of L-glutamate to the dead volume of the column did not affect the quantitative response (see validation results below). To quantify GSH and GSSG a derivatization process with NEM was required to prevent oxidation during sample preparation [20]. GSH-NEM is easily detected using ESI+ compared to the nonalkylated form and displays better chromatographic properties [7].

3.2. Evaluation of matrix effects on the analytical response

An essential part of method development is the selection of a matrix to prepare calibration and QC samples [21]. Generally, the use of calibration standards in authentic matrix is preferred for accurate quantitation. However, the quantitative determination of endogenous compounds, is complicated by the lack of analyte-free authentic biological matrices [21,22]. The standard addition method, in which a calibration curve is created by adding increasing concentrations of the analyte to individual aliquots of the sample of interest, is a well-known but tedious approach to overcome this problem [21]. Using a so-called surrogate matrix is more practi-

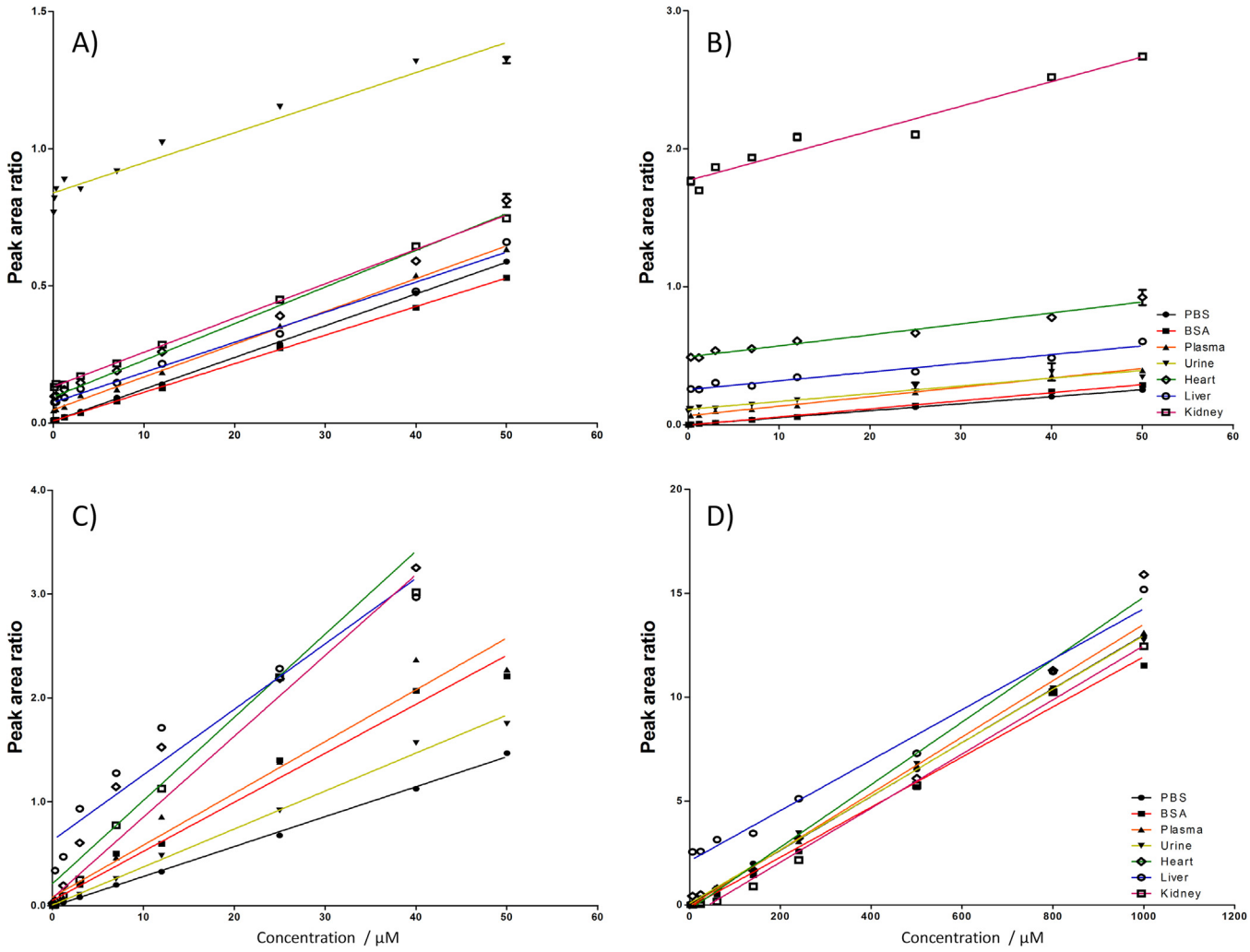

Fig. 2. Evaluation of matrix effects on the quantitative response of 5-oxoproline (A), L-glutamate (B), GSSG (C) and GSH-NEM (D) in tissues and body fluids from healthy mice. PBS 1X (black lines, black circles), 2% BSA in PBS (red lines, black squares), plasma (orange lines, black triangles), urine (yellow lines, black triangles), heart (green lines, white diamonds), liver (blue lines, white circles) and kidney (pink lines, white squares) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cal, provided a suitable matrix can be found [22]. In order to test the effect of different biological matrices on the quantitation of L-glutamate, 5-oxoproline, GSSG and GSH, we used the standard addition method in authentic matrix and compared the results with two widely used surrogate matrices, PBS containing 2% BSA and PBS alone.

The suitability of the surrogate matrices was evaluated by comparing the slopes of the calibration curves [21] and by calculating the signal suppression/enhancement (SSE) factor as previously described [23]. An SSE > 100% indicates enhancement of a particular signal, while a value below 100% indicates a suppression effect. As there are no regulatory guidelines, we set 3 thresholds for the SSE: a) analyte responses between surrogate and authentic matrices were considered identical for an SSE of 100 ± 20%, b) an SSE of 100 ± 30% was considered acceptable for quantification, although slight enhancement/suppression effects could bias the results, c) an SSE deviating more than 30% from 100% was considered unacceptable for quantitative bioanalysis.

Calibration curves in each of the tested matrices are shown in Fig. 2 and the numerical values are given in Table S1. The corresponding SSE factors can be found in Table 1. 5-Oxoproline and GSH-NEM can be measured in all biological matrices using both surrogate matrices. The optimal surrogate matrix to measure L-glutamate was 2% BSA in PBS, except for in kidneys, on which neither of the surrogate matrices proved satisfactory. For GSSG, 2% BSA in PBS proved satisfactory as surrogate matrix for plasma and urine, while none of the surrogate matrices was within an SSE of 100%±30% for the tissue extracts.

The higher y-axis intercepts of the calibration curves of 5-oxoproline in urine (Fig. 2A and Table S1) and L-glutamate in kidney tissue indicated that these matrices contain comparatively high endogenous concentrations. Furthermore, there was a considerable enhancement effect of the L-glutamate signal (SSE > 300%) in kidney tissue in comparison to both surrogate matrices (Table 1). The signal for GSSG was enhanced in all biological matrices except for urine. The reason for this enhancement is unclear, especially since we quenched the interconversion of GSH/GSSG during sample preparation.

The coefficient of determination (r^2), as a measure of linearity, is another important parameter to compare calibration curves of different matrices (Table S1). When validating analytical methods, an $r^2 \geq 0.99$ is acceptable for quantitative purposes [15,16]. Based on this, we classified calibration curves into those with an $r^2 \geq 0.99$ (bold in Table S1) and those with an $r^2 \leq 0.99$. The results show that complex matrices lead to a reduced linear fit in comparison to the surrogate matrices. Both surrogate matrices allowed

Table 1
Signal suppression/enhancement (SSE) factors for 5-oxoproline, L-glutamate, GSSG and GSH-NEM prepared in murine plasma or urine and heart, liver and kidney tissue.

SEE (%)	5-Oxoproline		L-Glutamate		GSSG		GSH-NEM	
	PBS	BSA	PBS	BSA	PBS	BSA	PBS	BSA
Plasma	**103,20**	**114,70**	134,82	**116,54**	173,13	**105,65**	**104,71**	**112,44**
Urine	**94,73**	**105,28**	**111,34**	**96,24**	***126,90***	***77,44***	**99,46**	**106,80**
Heart	**115,38**	***128,24***	157,22	**92,88**	277,78	169,52	**116,22**	***124,79***
Liver	**94,73**	**105,28**	***125,24***	**108,26**	218,81	133,53	**93,75**	**100,66**
Kidney	**108,04**	**120,08**	353,94	305,96	270,18	164,88	**100,54**	**107,96**

The signal suppression/enhancement effect was calculated as follows: SSE(%)= ($slope_{matrix\text{-}diluted}$ /$slope_{surrogate\ matrix}$) x 100.
Values in bold face fulfill the set criterion of being within ±20% of the SSE value for the authentic matrix.
Values in bold and italic face fulfill the set criterion of being within ±30% of the SSE value for the authentic matrix.

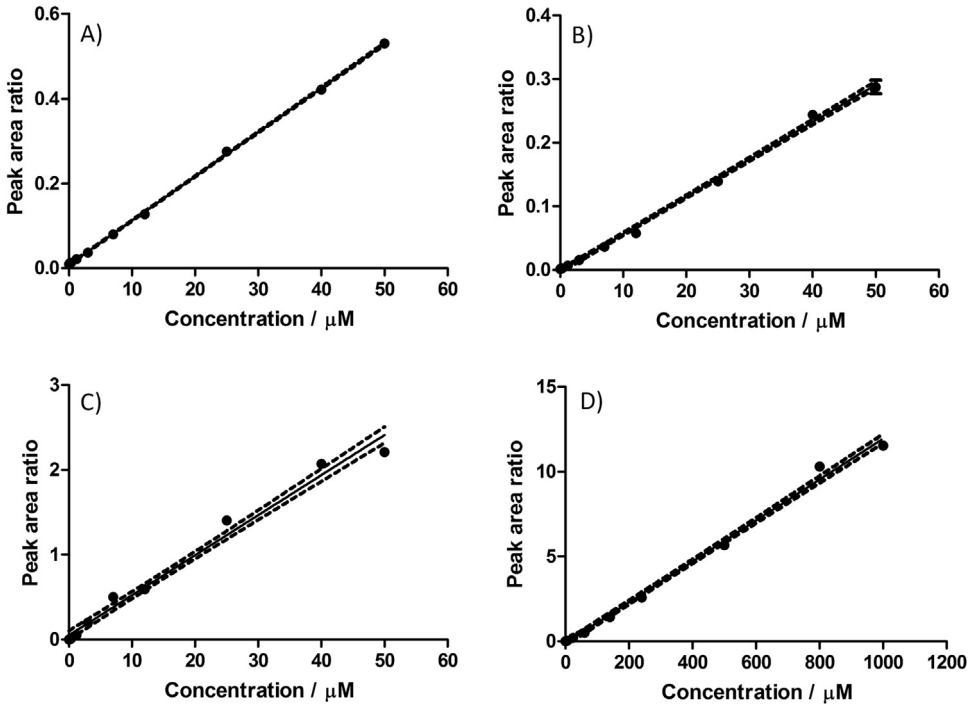

Fig. 3. Linearity of 5-oxoproline (A), L-glutamate (B), GSSG (C), and GSH-NEM (D) prepared in PBS containing 2% BSA. Calibration curves are based on peak area ratios relative to stable-isotope-labeled internal standards.

accurate quantitation of 5-oxoproline, L-glutamate and GSH-NEM, while GSSG showed a better linear fit in PBS (r^2 = 0.9986) than in 2% BSA in PBS (r^2 = 0.9833).

3.3. Method validation

Based on these results, we selected 2% BSA in PBS as the most suitable surrogate matrix, allowing reliable quantitation of 5-oxoproline and GSH-NEM in plasma, urine, heart, liver and kidney; L-glutamate in plasma, urine, heart and liver; and GSSG in plasma and urine (Fig. 2, Tables 1 and S1). Linearity was tested across a dynamic range of 0.03 to 50 µM for 5-oxoproline, L-glutamate, and GSSG, and 0.6 to 1000 µM for GSH-NEM (Fig. 3 and Table S1). The LLOQ was set to the lowest point on the calibration curves for which the analyte response was at least 5-times above the blank and the CV less than 20% in accordance with international guidelines [15,16]. Results for accuracy, precision and stability are summarized in Table 2. The bias for the quantitation of 5-oxoproline, L-glutamate, GSH-NEM and GSSG ranged from 1.2 to -9.3%, 4.4 to -10.0%, 4.1 to -10.2% and 7.0 to -4.0%, respectively. Recoveries were between 89.8–107% for all target analytes for High, Med and Low QC samples and CVs were below ±15% satisfying validation criteria for repeatability, intermediate precision and stability (Table 2). While the stability data were obtained in human plasma, they provide an indication of the stability of the metabolites in the other matrices used in this work

3.4. Analysis of the glutathione cycle in animals with heart failure

To study the effect of an induced MI on the glutathione cycle in mice, we quantitatively determined 5-oxoproline, L-glutamate, GSH and GSSG in plasma, urine, heart, kidney and liver tissues of SHAM-operated mice (N = 11) and mice subjected to MI (N = 13). 5-Oxoproline concentrations were significantly increased in all MI-mice compared to controls [plasma (6.0 vs 3.7 µM), urine (460.4 vs 191.8 µM), heart (16.3 vs 4.8 nM/µg protein) and kidney (84.5 vs 20.0 nM/µg protein)] ($p \leq 0.05$), with the exception of the liver, where the increase did not reach statistical significance (20.9 vs 11.4 nM/µg protein, $p \geq 0.05$) (Fig. 4). A similar pattern was found for L-glutamate, however, significance was only reached in kidney tissue (914.3 vs 555.3 nM/µg protein, $p \leq 0.05$) (Fig. 4). GSH ceoncentrations were not significantly different between SHAM-operated mice and MI-mice in any of the samples (Fig. 4). GSSG was elevated in all tissue samples from MI-mice (heart: 4.7 vs 0.6; p = 0.06, kidney: 8.5 vs 4.5 and liver: 20.8 vs 18.2 nM/µg protein),

Table 2
Accuracy, precision (intra- and inter-day) and stability of 5-Oxoproline, L-Glutamate, GSSG, and GSH-NEM.

		5-Oxoproline			L-Glutamate			GSSG			GSH-NEM		
		High QC	Med QC	Low QC	High QC	Med QC	Low QC	High QC	Med QC	Low QC	High QC	Med QC	Low QC
Accuracy													
Nominal Concentration	(μM)	40	12	3	40	12	3	40	12	3	800	240	60
Mean Concentration	(μM)	40,47	11,54	2,72	41,74	10,80	2,90	42,79	11,52	3,17	833,04	232,77	53,88
Bias	(%)	1,2	−3,9	−9,3	4,4	−10,0	−3,3	7,0	−4,0	5,8	4,1	−3,0	−10,2
Recovery	(%)	101,2	96,1	90,7	104,4	90,0	96,7	107,0	96,0	105,8	104,1	97,0	89,8
Precision													
Intra-day RSD	(%)	0,3	0,8	4,8	1,0	0,4	4,8	0,3	0,7	0,8	0,9	3,3	2,0
Inter-day RSD	(%)	3,0	4,3	0,6	1,9	8,8	4,7	6,0	9,0	15,5	4,5	6,0	4,9
Stability													
Bench-top (25 h) RSD	(%)	2,9	7,8	3,1	6,2	7,2	3,0	8,1	2,8	6,9	3,9	2,2	5,0
Bench-top (51 h) RSD	(%)	2,0	5,1	9,9	3,9	9,5	9,0	9,7	11,1	100	1,1	3,5	6,8
Freeze-thaw (5 cycles) RSD	(%)	5,3	5,3	6,0	8,7	4,4	9,0	6,5	5,9	8,0	2,5	3,1	3,2

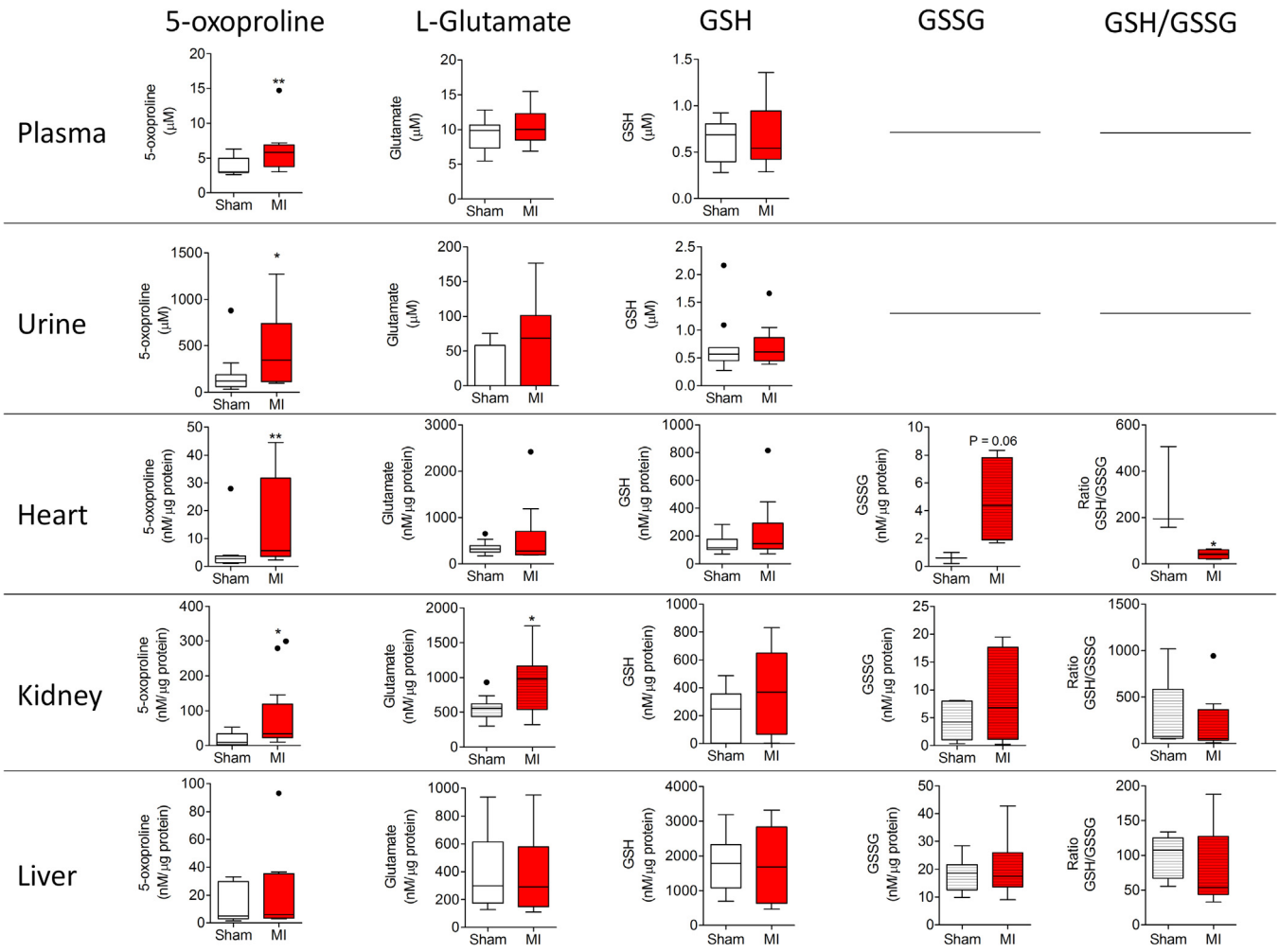

Fig. 4. Comparison of the concentration of key components of the glutathione cycle in different organs and biofluids from healthy controls (SHAM, N = 11) and mice subjected to induced myocardial infarction (MI, N = 13). Columns with lines pattern indicate the cases in which inaccurate results are obtained, according to the surrogate matrices analyses (SSE with variation>30%). Black dots indicate outlier data. The lines for GSSG and the GSH/GSSG ratio in plasma and urine indicate that the analyte was not detected. Significance level according to Student's t test: *p < 0.05, **p < 0.01.

however, this difference was not statistically significant (Fig. 4). GSSG was undetectable in plasma and urine.

So far, 5-oxoproline has been mainly measured in cell lysates and tissue samples from rats, while very few approaches have tried to establish the concentration of this metabolite in the murine setting. One particular study looked at 5-oxoproline concentration in murine kidney tissue, liver tissue, and urine following the inhibition of 5-oxoprolinase and found that in all three samples the amounts of 5-oxoproline were elevated following the inhibition of the enzyme [24]. L-glutamate concentrations have been more readily studied, however like with 5-oxoproline mainly rat or human samples have been used. As far as we are concerned, L-glutamate concentrations have not been studied in a murine heart failure model, therefore comparisons can only be made with regards to

healthy control animals. Regarding the results for this metabolite there is also discrepancies since different studies have shown higher (27 μM) [25], lower (2–4 μM) [26] and similar concentrations [27] to the ones reported here (~10 μM) in plasma samples. Although GSH, GSSG, and the GSH/GSSG ratio have been extensively studied in murine samples including models for heart failure, there are also published results supporting and contradicting our findings when it comes to absolute concentrations [28–30]. The clear differences find in absolute concentrations hinders a direct comparison of the results, however, the trends observed in our results are in line with those observed in the published literature.

The GSH/GSSG ratio is a well-established parameter to measure oxidative stress in biological systems, where a decrease is indicative of an increase in oxidative stress [9]. While differences of GSH and GSSG between the MI and control groups did not reach statistical significance, the GSH/GSSG ratio was significantly reduced in heart tissue after MI (Fig. 4).

Previously, we demonstrated that expression of OPLAH, the enzyme responsible for the conversion of 5-oxoproline to L-glutamate, is reduced in cardiac tissue after MI [4]. Reduction in OPLAH expression was linked to increased concentration of 5-oxoproline and oxidative stress [4]. This is in agreement with the current study showing that 5-oxoproline is significantly elevated in cardiac tissue and the GSH/GSSG ratio is significantly reduced. While we observed similar increases in 5-oxoproline in renal and liver tissue, there was no change in the GSH/GSSG ratio, indicating that these organs have a higher capacity to compensate for oxidative stress than the heart.

Based on our knowledge of the glutathione cycle (Fig. 1), 5-oxoproline, which is both a degradation product and an intermediate of *de novo* GSH synthesis [3], is converted back to L-glutamate by OPLAH. Therefore, a reduction of OPLAH coupled to increased 5-oxoproline concentration in heart failure would be reflected in a reduction in the availability of L-glutamate for the *de novo* synthesis of GSH. However, the current results do not support this hypothesis. It rather appears that L-glutamate and total GSH concentrations are increased upon induction of MI. This suggests that OPLAH is not a key regulator with respect to recycling L-glutamate to maintain GSH concentrations in animal tissue but rather an important enzyme controlling oxidative stress by regulating 5-oxoproline concentration. These findings shed new light on the role of the glutathione cycle in MI, further stressing the importance of 5-oxoproline in inducing oxidative stress. This in line with our observation that elevated concentration of 5-oxoproline in plasma is related to a worse outcome after heart failure in humans [4]. However, it is important to highlight that there are other sources of 5-oxoproline that are not linked to GSH metabolism. γ-Glutamyl amines that are formed by the metabolism of proteins crosslinked by transglutaminases, can also be converted into 5-oxoproline by the effect of γ-glutamylamine cyclotransferases [3].

Thus, the findings reported here provide a mechanistic link between plasma 5-oxoproline concentration as biomarker and outcome after heart failure, however, further studies are required to fully elucidate the role of 5-oxoproline in inducing oxidative stress.

4. Conclusion

We developed and validated an LC–MS method for the quantitation of 5-oxoproline, L-glutamate, GSH and GSSG, key components of the glutathione cycle, in plasma, urine and three different kinds of animal tissue (heart, kidney, liver). Using this methodology, effects on the glutathione cycle were studied following the induction of heart failure in mice. Besides the clinical usefulness of the GSH/GSSG ratio as an index of oxidative stress, our results suggest that 5-oxoproline is an easily measurable biomarker of oxidative stress related to cardiovascular disease that merits further validation.

Novelty statement

- Key metabolites of the γ-glutamyl cycle [5-oxoproline, L-glutamate, reduced glutathione (GSH) and oxidized GSH (GSSG)] are simultaneously quantified in biological samples from mice subjected to myocardial infarction.
- A thorough matrix effect evaluation for the quantitation of the analytes in heart, kidney, liver, plasma and urine samples was performed.
- The suitability of using PBS 1X and 2% BSA in PBS as surrogate matrices was established.
- Our results shed new light on the role of 5-oxoproline as an inducer of oxidative stress related to myocardial infarction and its usefulness as a possible biomarker for cardiac injury.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.08.001.

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