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Review

Measurement of homocysteine and related metabolites in human plasma and urine by liquid chromatography electrospray tandem mass spectrometry☆,☆☆

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ABSTRACT

The sulfur amino acids, methionine and cysteine play crucial roles in cells as a substrate for protein synthesis, as a methyl donor, and for the synthesis of sulfur-containing compounds, including the key intracellular tripeptide, glutathione. Homocysteine is an intermediary metabolite formed during the metabolism of methionine to cysteine. Dysregulation of homocysteine metabolism is implicated in adverse clinical outcomes such as increased risk of cardiovascular disease, stroke, Alzheimer's disease dementia and osteoporosis. While hyperhomocysteinemia is commonly observed in those conditions, the impact on other related metabolites is condition-specific. Therefore, there exists a need to establish precise and sensitive analytical techniques that allow for the simultaneous measurement of homocysteine and related metabolites in biological samples. The current review outlines the development and use of liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS) to simultaneously measure metabolites involved in sulfur amino acid metabolism. Additionally, extensions of the technique in relation to the measurement of sulfur amino acid and one-carbon kinetics *in vivo* are discussed. The LC–MS/MS technique has the capacity for unambiguous analyte identification and confirmation, due to its high specificity and sensitivity. It has the greatest potential of being accepted and utilized as a dedicated homocysteine and its related metabolite Standard reference method (SRM).

Contents

Abbreviations: ACN, acetonitrile; AR, area ratio; APCI, atmospheric pressure chemical ionization; Cys, cysteine; CysT, cystathionine; CGL, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; CGL, cystathionine-γ-lyase; DNA, deoxyribonucleic acid; DTE, dithioerythritol; DTT, dithiothreitol; ESI, electrospray ionization; ECD, electrochemical detector; GC-MS, gas chromatography mass spectrometry; Hcy, homocysteine; HPLC, high performance liquid chromatography; IS, internal standard; LC–MS/MS, liquid chromatography electrospray tandem mass spectrometry; MMA, methylmalonic acid; MRM, multiple reaction monitoring; MTHFR, methylenetetrahydrofolate reductase; NA, not available; NAC, N-acetyl cysteine; RM, remethylation; RT, retention time; SA, succinic acid; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SRM, standard reference method; TBP, tris-*n*-butylphosphine; TCEP, tris(2-carboxylethyl) phosphine; tCys, total cysteine; tHcy, total homocysteine; TM, transmethylation; TS, transsulfuration; UV, ultraviolet.

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1. Introduction

The sulfur amino acid methionine plays crucial roles in animal cells as a substrate for protein synthesis (structural, enzymes, hormones), as a methyl donor (neurotransmitter synthesis, DNA stability), and for the synthesis of sulfur-containing compounds, including the amino acid cysteine which is the rate limiting substrate for the synthesis of the tripeptide glutathione [1,2]. The latter compound is important for xenobiotic detoxification, immune modulation, and regulation of red-ox status [3]. The indispensable amino acid methionine is converted to cysteine through the sequential pathways of *Transmethylation* (TM) and *Trans-sulfuration* (TS), through the intermediate homocysteine (Fig. 1). Homocysteine lies at an important regulatory locus: It can either undergo TS, or it can be remethylated to again form methionine [4]. In the *Remethylation* (RM) pathway, homocysteine receives a methyl group through one of two routes – (a) the methionine synthase-dependent route, which requires the cooperation of several water soluble vitamins, including folate, cobalamin, and riboflavin, or (b) the betaine:homocysteine methyltransferase-dependent route, which utilizes the choline metabolite betaine as the methyl donor [5]. The RM pathway leads to the conservation of the methionine carbon chain, and likely represents an attempt by the body to maintain cellular S-adenosylmethionine (SAM) levels for methylation reactions during periods of reduced systemic methionine availability [6]. The alternative pathway for homocysteine leads to the synthesis of the sulfur amino acid cysteine, through TS, a pathway that represents the irrevocable loss of the methionine carbon skeleton. The enzyme cystathionine-β-synthase (CBS) represents the first step in cysteine formation, and it catalyses the condensation of homocysteine with serine to form cystathionine. Cystathionine is then metabolized to form the amino acid cysteine, 2-oxobutyrate and NH_4^+, through the action of cystathionine-γ-lyase (CGL) [7]. Recent reviews [8,9] have discussed the importance of the coordinated regulation of homocysteine metabolism with respect to maintaining circulating homocysteine levels in check. Dysregulation of homocysteine metabolism is implicated in a number of adverse clinical outcomes.

Hyperhomocysteinemia has been linked to increased risk of cardiovascular disease and stroke, Alzheimer's disease and dementia, and osteoporosis in humans and animal models [10]. The sheer variety of the putative associations is suggestive of impairments in key biological mechanisms. As an example, elevated tissue homocysteine supports an increase in tissue S-adenosylhomocysteine (SAH; Fig. 1) concentration, a feedback inhibitor of virtually all methyltransferase reactions [8], including those involved in DNA, protein, and lipid methylation. Homocysteine clearance is critical to avoiding this inhibition, as is an understanding of the factors regulating homocysteine metabolism. As previously reviewed [4], a multitude of factors have been implicated in disturbing homocysteine metabolism leading to hyperhomocysteinemia, including marginal or deficient nutrient status (e.g., folate, cobalamin, and B_6), pharmacological agents (e.g., nitrous oxide), genetic defects (e.g., CBS deficiency, MTHFR deficiency) and certain disease states

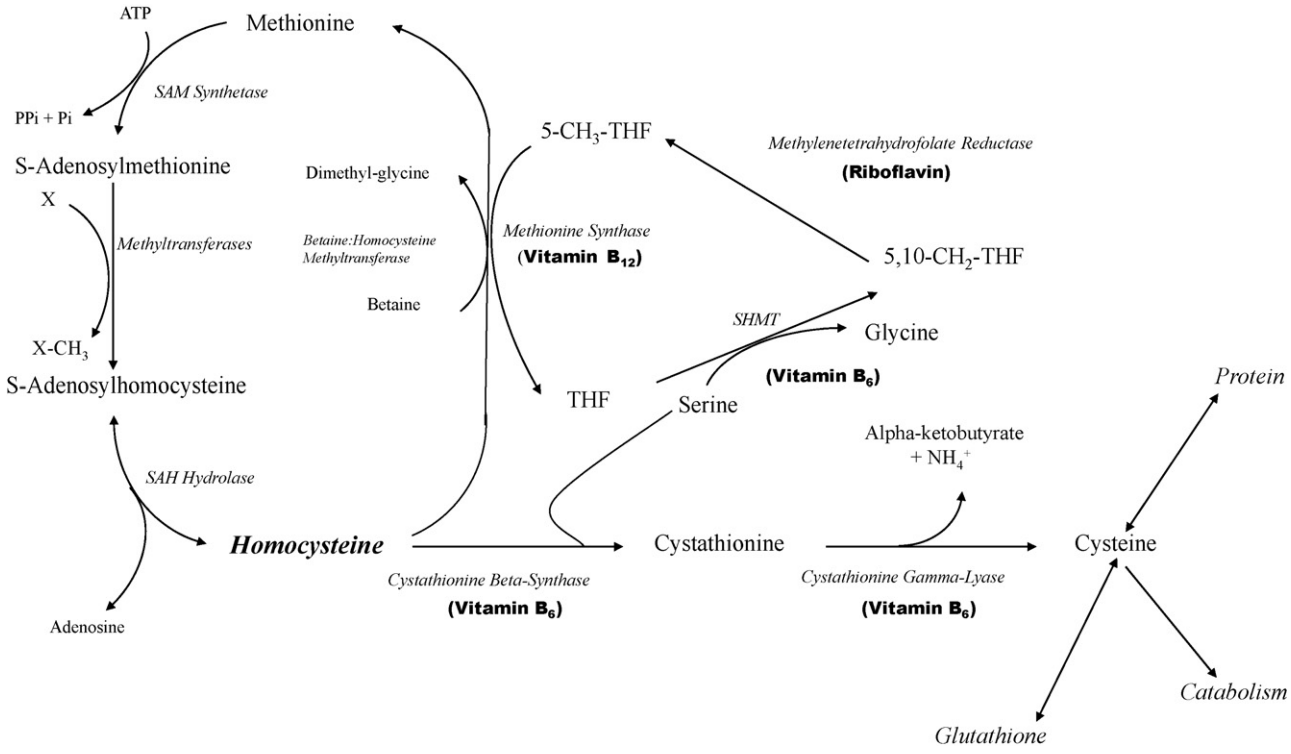

Fig. 1. Sulfur amino acid metabolism. X, methyl acceptor; SAM, S-adeonsylmethionine; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate; SHMT, serine hydroxymethyltransferase.

(e.g., renal disease, diabetes). While hyperhomocysteinemia is a hallmark of the aforementioned conditions, the impact on other related metabolites is condition-specific. As such, there is a need to establish analytical techniques that allow for the simultaneous measurement of homocysteine and related metabolites in biological samples in order to gain specific knowledge as to the nature and potential ramifications of the metabolic disturbance. The current review outlines the development and use of liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS) for just such a task. Additionally, extensions of the technique are discussed in relation to the measurement of sulfur amino acid and one-carbon kinetics *in vivo*.

2. Analytical methods for the measurement of homocysteine and related thiols

In plasma/serum, homocysteine exists in (1) *oxidized forms*, including those in which homocysteine exists in an oxidized pair with itself (homocystine), other low-molecular mass thiols (e.g., cysteine) or free thiols in proteins, in particular albumin, or in (2) *reduced form* (free homocysteine) [11]. Therefore, the first methods for measuring homocysteine, introduced in the mid-1980s [11,12], focused on total homocysteine (tHcy) where multiple unstable Hcy species in plasma or serum were treated with reductants that reduced them to tHcy. The various tHcy methods were either enzyme and immunoassay related [13], or chromatographic methods which included HPLC [14–17] with ultraviolet (UV) [18], fluorescence [19], or electrochemical detection (ECD) [20]; capillary electrophoresis with fluorescence detection [21–23]; gas chromatography–mass spectrometry (GC–MS) [12,24–30], and finally liquid chromatography tandem mass spectrometry (LC–MS/MS) [31–42]. In general, the enzyme and immunoassays were found to be highly imprecise [43,44]. Comprehensive reviews on the analytical determination of tHcy using several HPLC methodology and immunoassay have been done by Rasmussen and Moller and Nekrossova et al. [45,46]. The reader is referred to these elegant reviews for detailed understanding of these methods.

The advantages of the simple chromatography methods were the ability to analyze a wide range of analytes and the simultaneous determination of more than one compound, including tHcy, total cysteine (tCys), and glutathione. However, resolution of compound peaks with simple liquid chromatographic conditions could prove to be challenging, thus compromising compound specificity. Alternative approaches were therefore required.

2.1. Isotope dilution LC–MS/MS

Tandem mass spectrometry provides: (a) specificity, in that it is able to monitor only selected mass ions or multiple reaction monitoring (MRM) (Fig. 2), (b) increased sensitivity, due to enhanced signal-to-noise ratio, and (c) increased speed due to shorter sample cleanup time with little or no derivatization and a much shorter analysis time [39]. Magera et al. [36] were first to measure tHcy in plasma by LC–MS/MS, followed by other groups [31–35,37,38,40–42,47–50] including work by our group [39]. Our group [39] was also able to measure both tHcy and tCys simultaneously in plasma and urine. Recently, Hempen et al. [33] have validated a method for the simultaneous measurement of tHcy, methionine (Met) and methylmalonic acid (MMA) [33]. A summary of different sample preparation procedures and performance characteristics of the LC–MS/MS assay by various groups to measure tHcy is presented in Table 1.

2.1.1. Sample preparation

Almost all Hcy in freshly prepared plasma exists in a disulfide form, bound either to protein, albumin (70–80%) or to low-molecular mass sulfhydryls, mainly Cys (20–30%) and only traces (<2%) found in free sulfhydryl form [51]. Andersson et al. [52] in 1993 also showed that 50% of tCys in freshly prepared plasma was made up of protein-bound disulfide form, with 46% of free oxidized form of cysteine and about 3% was in the reduced sulfhydryl form. Perry and Hansen [53], in 1969, showed that practically all Hcy was bound to protein when plasma was stored at −20 °C for 1 week. Stabler et al. [12] also reported that tHcy increases during storage of whole blood at temperature of −20 °C. Therefore, for determination of plasma homocysteine and cysteine, it is preferable to separate and store the plasma component at −20 °C quickly.

Reductants, in general, must be prepared immediately before use and close attention should be paid to the stability of the reductant as well as the reduced compound during long automated sample processing. Ueland et al. [51] reviewed the use of several sulfhydryl reagents including dithiothreitol (DTT), dithioerythritol (DTE), mercaptoethanol, sodium or potassium borohydrate and tris-*n*-butylphosphine (TBP). The latter authors reported in their review that DTT, DTE and mercaptoethanol formed adducts with thiol-specific reagents and therefore consume the derivatizing reagents which are used in HPLC and GC–MS assays. TBP, on the other hand, did not react with thiol-specific reagents. However, it consumed fluorogenic reagent in pre-column derivatization by HPLC and was poorly soluble in water [51]. Additionally, TBP is an irritant with an unpleasant odor.

In 1997, a newer phosphine reagent, tris(2-carboxyethyl) phosphine (TCEP), was introduced by the Gilfix's group [54] in the determination of total plasma Hcy. They found TCEP to be non-volatile, stable, soluble in water and odorless and hence reliable for HPLC and GC–MS assays. Earlier in 1994, Han and Han [55] reported that unlike DTT, which readily oxidized above pH 7.5, TCEP was highly stable in both acidic and basic solutions. The ability of TCEP to reduce disulfide bonds at lower pH values (pH < 8) makes it a more effective reducing agent. Krijt et al. [56] compared TCEP to TBP and concluded that there was low agreement between the two reducing agents in the determination of tHcy and tCys. However, the TCEP method yielded better reproducibility and was a more robust method. Several other groups have used DTT as the reductant in the quantification of tHcy and other thiols in plasma by LC–MS/MS [34,36,39,41,42,50]. Recently, Huang et al. [49] made a direct comparison of DTT and TCEP for the measurement of intracellular tHcy by LC–MS/MS. They found that DTT proved to give more reliable

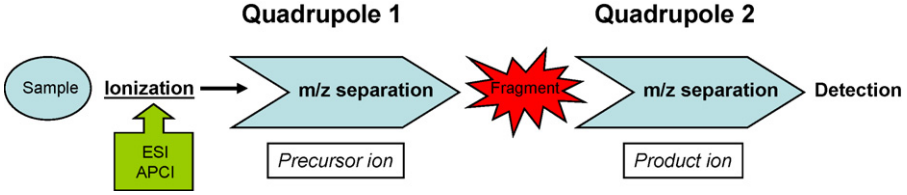

Fig. 2. Schematic of tandem mass spectrometry. LC–MS/MS: A liquid chromatograph (LC) is coupled with two quadrupole mass analyzers. The sample is ionized by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) which are subsequently separated according to their mass-to-charge ratio (m/z) and detected. Tandem mass spectrometry offers greater selectivity because it allows monitoring of the precursor (parent) ion transition to the product (daughter) ion.

Table 1
Different sample preparation methods and performance characteristics for measurement of tHcy in biological fluids by LC–MS/MS methods.

References	Biological fluid	Reducing agent	Calibration matrix	Protein removal	Run time (min)	Sample volume on column (μL)	Mean % recovery	Mean intra-day %CV (n)[k]	Mean inter-day %CV (n)[k]	tHcy + additional analytes analyzed in same run
Hempen et al., 2008 [33]	Plasma	TCEP	Plasma	Protein removal by UF[a]	4	5	97.5 (4) 102.8 (4)	2.3 (20) 2.5 (20)	5.0 (12) 5.3 (12)	tHcy MMA[i]
Li et al., 2008 [35]	Plasma	DTT[f]	Diluted Plasma	15% TCA[b]	2	1.5	98.6	3.24 (6)	4.04 (36)	tHcy; none
Huang et al., 2007 [49]	Intracellular	DTT	PBS Buffer	ACN[c]	11	0.7	102	4.9 (45)	NA	tHcy; none
Rafii et al., 2007 [39]	Plasma Urine	DTT TCEP[g]	Plasma	TFA[d] in MeOH[e]	3	0.2	100.1 (12) 97.5 (12)	4.5 (9) NA[j]	5.8 (27) NA	tHcy Cys
			Urine		3	0.2	98.5 (12) 99.6 (12)	NA NA	NA NA	tHcy Cys
Weaving et al., 2006 [42]	Plasma	DTT	Plasma	TFA in ACN	2	0.8	101.4 98.6 101.9	3.3 (60) 4.3 (60) 3.2 (60)	6.8 (60) 13 (60) 11.9 (60)	tHcy Cys, Met
	Urine	DTT	Urine		2	0.8	95.6 102.1 95.75	3.2 (60) 4.0 (60) 3.9 (60)	6.0 (60) 5.9 (60) 8.4 (60)	tHcy Cys, Met
Ducros et al., 2006 [24]	Plasma	DTT	Aqueous	TFA in ACN	3	0.23	92.6	1.5 (12)	2.5 (12)	tHcy; none
Kuhn et al., 2005 [34]	Plasma	DTT	Plasma	90% TCA	2.5	0.4	94.7	NA	NA	tHcy; none
Tuschl et al., 2005 [41]	Plasma	DTT	Plasma	70% TCA	1.2	0.5	79.8	10.2 (10)	11.5 (5)	tHcy; none
Nelson et al., 2005 [50]	Plasma	DTT	Plasma	Ascorbic Acid	15	NA	NA	2.1 (2) 1.5 (2) 1.3 (2)	6.0 (6) 1.3 (6) 7.1 (6)	tHcy Folate, 5-MT[h]
Satterfield et al., 2003 [40]	Plasma	DTT	Aqueous	TFA in ACN	15	NA	NA	0.91 (8)	1.5 (16)	tHcy; none
Nelson et al., 2003 [38]	Plasma	DTT	Plasma	NA	NA	4.8	NA	3.6 (60)	NA	tHcy; none
McCann et al., 2003 [37]	Plasma/Blood Spots	DTT	Plasma	TFA	2	0.3	NA	6.0 (10)	10.3 (10)	tHcy; none
Gempel et al., 2000 [32]	Blood Spots	DTT	Blood Spots	TFA in ACN	3	0.1	105.5	9.8 (10)	10.0 (10)	tHcy; none
Magera et al., 1999 [36]	Plasma	DTT	Plasma	TFA in ACN	2.5	0.3	96.0	4.3 (18)	4.1 (18)	None

[a] UF: ultra filtration.
[b] TCA: trichloroacetic acid.
[c] ACN: acetonitrile.
[d] TFA: trifluoroacetic acid.
[e] MeOH: methanol,
[f] DTT: dithiothreitol.
[g] TCEP: tris(2-carboxyethyl) phosphine.
[h] 5-MT: 5-methyltetrahydrofolic acid.
[i] MMA; Methylmalamolic acid.
[j] NA: not available.
[k] Mean intra- and inter-day %CV were calculated from the individual data presented in the original papers.

and reproducible results. Moreover, the stability of the reduced plasma was investigated by Magera et al. [36] over time (0, 24, 48 and 72 h). They reported stable reduction by DTT of the sulfhydryls and no significant differences in stability of the reduced tHcy and [3,3,3′,3′ $^2H_4$] Hcy (used as an internal standard) stored up to 72 h after sample preparation. We also found similar stability with DTT for plasma tHcy and tCys (unpublished data). However, due to pH differences between plasma and urine, TCEP proved to be a more efficient reducing agent for urine samples, producing peaks that were sharper, with higher intensity and less background "noise" for both tHcy and tCys. Nelson et al. [50] tested optimum conditions (concentration, incubation time and temperature) for DTT as the reducing agent for measuring tHcy. They found that the ideal DTT/serum ratio was 1×10^{-5} mol DTT/100 μL serum. This concentration of DTT was similar to the concentration used by Magera et al. [36], Gempel et al. [32], and Rafii et al. [39]. The study by Nelson et al. [50] indicated complete reduction of the Hcy disulfides almost immediately at room temperature. Hence a 15 min incubation time has been selected to allow for other manual processing steps. However, Hempen et al. [33] investigated DTT vs. TCEP as possible reducing agents and their corresponding reduction times. They found TCEP to be superior to DTT since it gave better peak shapes, less noise and higher peak intensities. They found no difference between 10 and 20 min reduction times on the results for both tHcy and MMA. Huang et al. [49] also looked at the effect of the freeze–thaw cycle on the stability of the reduced tHcy. After repeating the freezing at −80 °C for 22 h, followed by incubation at room temperature for 2 h, repeating for three cycles, they reported no significant effect on the reduced tHcy stability. From our experience in measuring tHcy and related metabolites using LC–MS/MS, for plasma/serum DTT appears to be a more suitable reducing agent, while TCEP appears to be more suitable for urine analysis.

Finally, to summarize the sample preparation steps: calibrators are prepared in the same matrices as the sample of interest. Aliquots of each sample and a standard curve made up of a number of different concentration points are then prepared. The internal standards (IS), which are isotopically labeled analogues of the analyte of interest, are then added to each individual sample and to each point of the standard curve. At this point a reducing agent (DDT or TCEP) is added, to split the dimers of the Hcy and Cys, and the tubes vortexed immediately. The mixture is left to stand at room temperature for 15 min. This time was found to be adequate to completely break the bonds of all of the analytes of cystine and homocystine. The buffer with the MeOH is finally added to precipitate the proteins. After centrifugation the supernatant is ready for LC–MS/MS analysis. Fig. 3 depicts a typical MS/MS product ion spectra of cysteine, cysteine-d_2, homocysteine and homocysteine-d_4.

Also during the analysis, all research groups working with tHcy and its related thiols, should ensure a complete reduction of the disulfide compounds, by simultaneously monitoring the parent-to-daughter transition of the disulfide forms of Hcy and Cys (homocystine and cystine).

2.1.2. Matrix effect/ion suppression

One important factor that can affect the accuracy and reproducibility in the quantitation of analytes by mass detectors is ion suppression and ion enhancement caused by coeluting undetected matrix compounds [57–59]. These refer to random compounds with the same retention times as the compounds of interest (mainly salts and detergents), which serves to increase background noise producing less well defined peaks with lower intensity. Ion suppression is caused by the presence of less volatile, endogenous compounds such as salts and detergents found in biological matrices that can change the efficiency of the droplet formation or evaporation, which in turn changes the amount of charged ions in the gas phase in the source. Matrix effects are generally not reproducible or repeatable between samples or even between repeat injections, and so can severely compromise the accuracy and reproducibility of the analysis.

2.1.3. Electrospray ionization vs atmospheric pressure chemical ionization

Mass spectrometric detection is only possible when the analyte exists in the gaseous state as ions. Since the LC is coupled to the MS, it is necessary to volatilize the molecules from the LC-eluent. The two main LC–MS interfaces that achieve this goal are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources. In ESI, a high voltage (2–5 kV) capillary in the presence of a strong electric field sprays the liquid sample to form charged droplets, which in turn move towards the MS inlet, generating analyte ions during evaporation and droplet fission. In APCI, the LC analyte is sprayed by a heated pneumatic nebulizer probe at atmospheric pressure for nebulization and a high voltage needle produces a corona discharge for ionization of the evaporated solvent. This dissociates the analyte molecule and generates ions.

All the tHcy and tCys methods by tandem MS have utilized the ESI interface. The ESI source is easy to use, consumes less solvent, has a wide polarity range and can be applied to thermally labile compounds [60]. However, King et al. [57] have demonstrated that ion suppression is much more severe with ESI than APCI. The increased ion suppression is a result of high concentrations of non-volatile materials present in the spray with the analyte. Another reason for the higher ion suppression in the ESI mode could be due to the fact that in this mode, unlike in the APCI mode, the analyte is ionized in the liquid phase inside the electrically charged droplets. As the solvent evaporates, the analyte precipitates from solution either as solid compound or as a co-precipitate with other non-volatile sample components. A more recent study by a Canadian group [61] observed ion enhancement in wastewater extracts analyzed for pharmaceuticals using LC-APCI–MS/MS as a result of interferences from the sample matrix.

2.1.4. Correcting matrix effects

Matrix effects can be minimized or corrected by enhancing sample cleanup procedures [60] and changing chromatographic conditions to better separate the analyte from matrix interferences. Recently, Hempen et al. [33] tested two different sample cleanup procedures: reduction step followed by ultrafiltration and reduction step followed by precipitation of proteins with acetonitrile (ACN). They found that the peak areas of the deuterated internal standards for both Hcy and MMA were 30% lower with the ACN precipitation method and so preferred the ultrafiltration method of sample cleanup. Still the most efficient way of correcting matrix effect is the use of standards prepared in the same matrix as the sample of interest (calibrators), plus the use of stable isotope labeled analogues of internal standards (IS) [62]. This would correct for matrix effects due to identical chromatographic conditions and ionization behavior in the source. Fig. 4A–C are calibration curves generated by our group (unpublished data), to demonstrate the matrix effect phenomena. The *y*-axis shows the area ratio (AR) of the analyte (cysteine) to the internal standard (IS) (Cys-d_2 or Hcy-d_4 or NAC). The *x*-axis is the concentration of the analyte cysteine. Fig. 4A shows calibration curves of Cys in plasma and urine compared to the Cys curve in water using Cys-d_2 (stable isotope labeled IS). The equations for the plasma, urine and water curves were $y = 0.00146x + 0.454$, $y = 0.00183x + 1.698$ and $y = 0.0014x + 0.013$, respectively. The slopes of the three lines are similar. Fig. 4B displays the calibration curves of Cys in the three matrixes using Hcy-d4 as the internal standard (stable isotope labeled IS for Hcy). Again the slopes of the three curves were similar, showing that Hcy-d_4 would also have been an ideal IS in obtaining reliable results correcting for any matrix effect. On the other

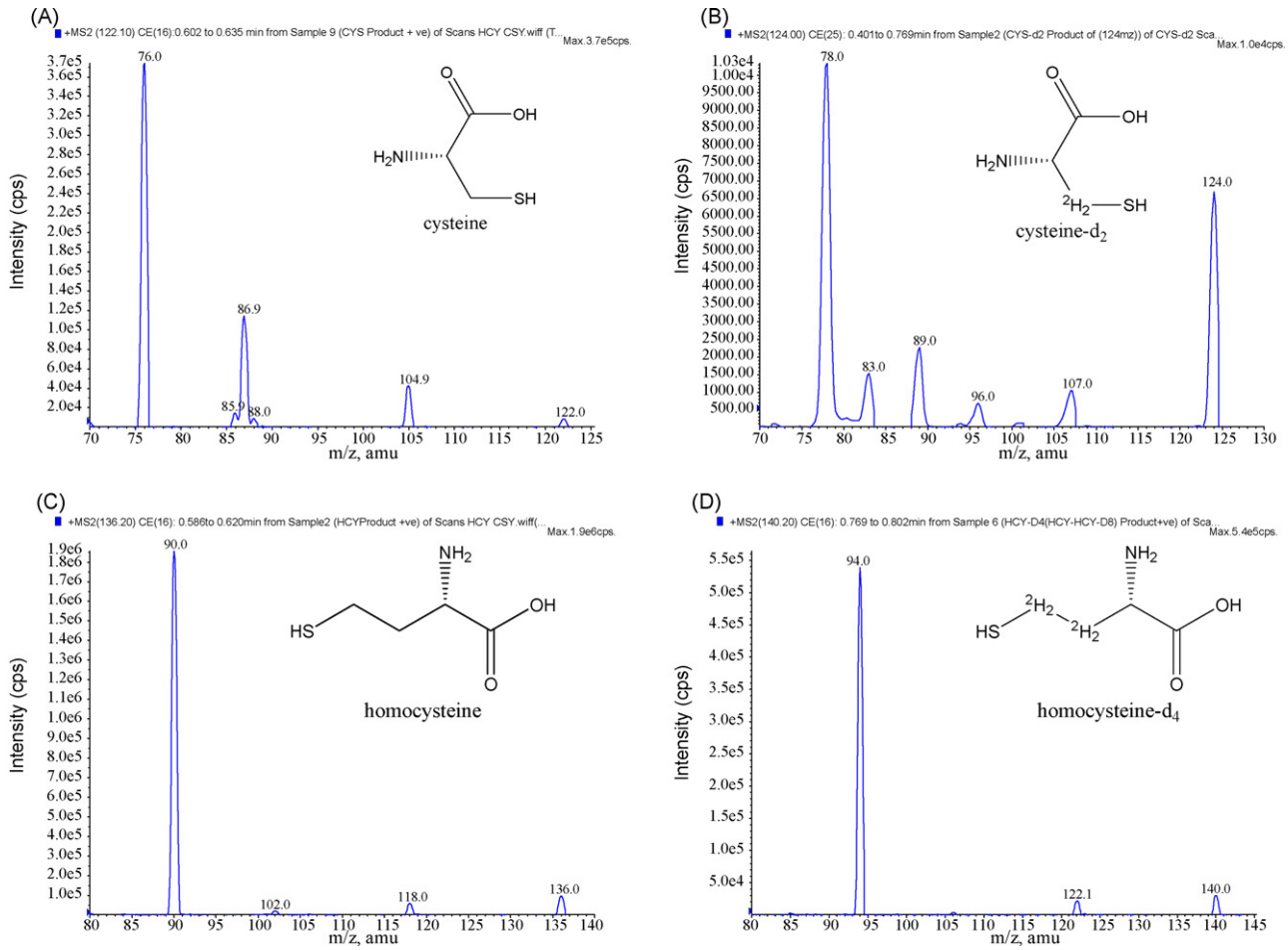

Fig. 3. MS/MS product ion spectra of (A) cysteine, (B) cysteine-d_2, (C) homocysteine and (D) homocysteine-d_4. The chromatograms show the fragmentation of $[M+H]^+$ molecular ions producing daughter fragments; m/z 136.1 for tHcy, m/z 122.1 for tCys, m/z 140.1 for Hcy-d_4 and m/z 124.1 for Cys-d_2 producing optimum fragments at m/z 90.0, 76.0, 94.0 and 78.0 for tHcy, tCys, Hcy-d_4 and Cys-d_2, respectively.

hand, in Fig. 4C, calibration curves of Cys in plasma, urine and water using NAC as the IS show the slopes of the three lines, especially the plasma curve, are different. The equations for the plasma, urine and water curves for this figure were $y = 0.015x + 3.82$, $y = 0.0082x + 1.83$ and $y = 0.0044x + 0.072$, respectively. The slope for the plasma curve was three-fold higher than for the water curve using NAC as the IS. Thus inaccurate quantification of Cys was observed when we used NAC, which is not a stable isotope analogue of Cys. These findings highlight the importance of using stable isotope labeled IS and calibrators as a means of correcting for the matrix effect observed when quantifying compounds in biological samples. Zhao and Metcalfe [61] have also shown that, using stable isotope IS, they were able to correct the overestimations in their results that had occurred as a result of interferences from the sample matrix in the APCI source.

When using matrix derived standard curves for quantitating the tHcy and tCys concentrations, the endogenous tHcy and tCys amounts, using area ratios (analyte/internal standard) have to be corrected for dividing the y-intercept by the slope of the calibrator curve [37]. Li et al. [35] developed a new sample preparation procedure to produce zero blank, free of endogenous background of Hcy. Calibrator standards were prepared by diluting the pooled deproteinated plasma samples three times with water to obtain a zero blank, a solution free of endogenous Hcy. However, the unknown, undiluted plasma samples were quantified against the diluted calibrant standard curves. In doing so, the calibrators were not comparable to their physiological samples from all perspectives, creating a bias. This is evident in their mean tHcy values for healthy controls being approximately 1.6 times higher than those reported by other groups.

3. Multiple, simultaneous analyses in one injection

It is advantageous to be able to determine the concentration of tHcy and related metabolites in both plasma and urine. So far different groups have improved their LC–MS/MS methods to be able to analyze tHcy, tCys and Met simultaneously in one short run in both plasma and urine [39,42] (Fig. 5). Nelson et al. developed a simultaneous quantification method of measuring Hcy and folate in human serum or plasma [50]. Hempen et al. [33] developed an LC–MS/MS method for the quantitative determination of tHcy, Met and MMA. All the analytes were measured in a 4 min run without the need for derivatization. Since negative ionization was more advantageous for MMA and succinic acid (SA), an isomer of MMA that needed to be separated by LC, potentially increasing the run time, the MS determination was split in three intervals, reducing the run time. In the 1st period of 1.49 min, in positive ionization mode, the Hcy MRM transition of m/z 136.1 > 90.1, Hcy-d_4 MRM of m/z 140.1 > 94.1 and homocystine (Hcy–Hcy) MRM of m/z 269.0 > 136.2 at retention time (RT) = 1.3 min were monitored. In the 2nd interval both positive ionization and negative ionization modes were applied in the next 0.6 min in an alternating way in order to detect Met in the positive mode (MRM m/z 150.1 > 104.1, RT = 1.7 min) and SA in the negative mode (MRM m/z 116.9 > 72.8, RT = 1.9 min.). In the 3rd interval, MMA and MMA-d_3 were measured in the negative mode to

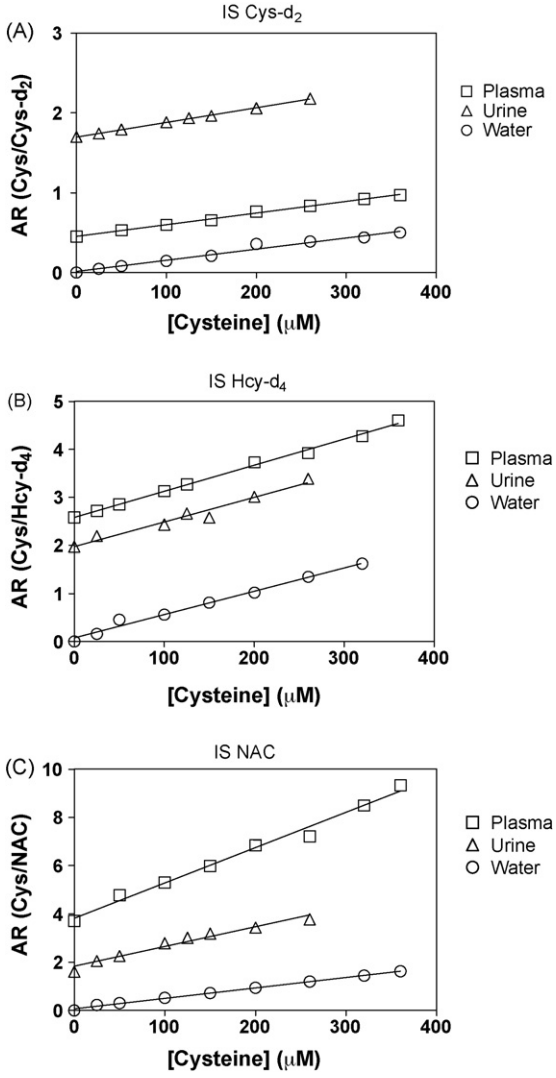

Fig. 4. Choice of calibrators, internal standard and the effect of matrix effect in LC–MS/MS methods. (A) The *y*-axis shows the area ratio (AR) of the Analyte (cysteine) to the internal standard (IS) (Cys-d_2 or Hcy-d_4 or NAC (*n*-acetyl cysteine)). The *x*-axis is the concentration of the analyte cysteine. (B) Calibration curve of Cysteine concentration from plasma, urine and water using Cys-d_2 (30 μM in plasma and water, 15 μM in urine) as the internal standard (IS). (C) Calibration curve of Cysteine concentration from plasma, urine and water using Hcy-d_4 (30 μM in plasma and water, 15 μM in urine) as the internal standard (IS). (D) Calibration curve of cysteine concentration from plasma, urine and water using *n*-acetyl cysteine (NAC) (30 μM in plasma and water, 15 μM in urine) as the internal standard (IS).

separate it from its isomer SA which had the same MRM transition with a RT of 2.3 min.

Recently we have also been able to detect Met (MRM *m*/*z* 150 > 61, RT = 0.91 min), cystathionine (CysT) (MRM *m*/*z* 226.3 > 134, RT = 0.77 min), serine (MRM *m*/*z* 106 > 60, RT = 0.79 min), glycine (MRM *m*/*z* 76 > 30, RT = 0.77 min) and creatinine (MRM *m*/*z* 114.2 > 44, RT = 0.69 min) in urine simultaneously along with tHcy (MRM *m*/*z* 136 > 90, RT = 0.78 min) and Cys (MRM *m*/*z* 122 > 76, RT = 0.82 min), all within 1 min using the LC–MS/MS method (Fig. 6, unpublished data). The optimization conditions of the measurement of Hcy and its metabolites in biological fluids by LC–MS/MS is shown in Table 2. There was no need for separation of the analyte peaks since the parent-to-daughter transitions for each were unique. Regardless, more work needs to be done to validate the quantification of all the analytes in different biological fluids with appropriate internal standards and calibrators with similar biological matrices. In our preliminary work, interference of the reducing

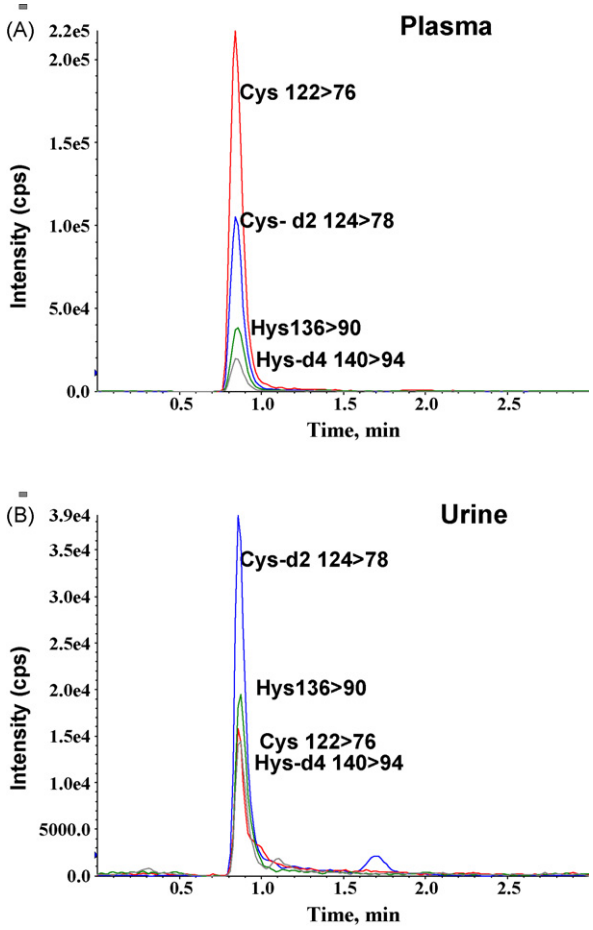

Fig. 5. Total ion chromatogram of a typical (A) plasma and (B) urine sample. The total run time between samples was 4.5 min with an acquisition time of 3 min.

agent, TCEP, with the chromatography of creatinine and CysT in the urine was observed. Since the entire run time is only 3 min, it would be feasible to have a separate run without TCEP, using the same LC–MS/MS method for quantitating CysT and creatinine in urine.

3.1. Further application of the current LC–MS/MS method

3.1.1. Modeling metabolite kinetics

Young and co-workers developed an isotopic tracer model of methionine metabolism, in which the rates of TM, RM and TS can be estimated during the primed-continuous infusion of L-[$^2H_3$-methyl-1-^{13}C]-methionine [63]. These authors used the model to measure the impact of sulfur amino acid intake [63,64], age [65], and burn trauma [66] on methionine kinetics. In the original approach, isotopic enrichments of methionine corresponding to *m* + 1 (carboxyl carbon), and *m* + 3 (methyl group) were determined by GC–MS, and this method has continued to be the predominant method utilized for measuring enrichment [67]. Here the *m* + 1 refers to the extra mass due to the presence of 1-^{13}C in the carboxyl group. The *m* + 3 refers in this case to the extra mass due to the presence of three ^{2}H in the methyl group. While the original model provided significant insight into the metabolic disposition of methionine, it was not without its limitations. First, the choice of precursor enrichment pool to use for calculating flux can significantly impact the quantitative estimates derived. Methionine metabolism occurs intracellularly, but most studies have access to peripheral blood only. As a result, the use of methionine enrichment in blood is unlikely to represent the true enrichment at the site of metabolism, a critical assumption in models designed to measure

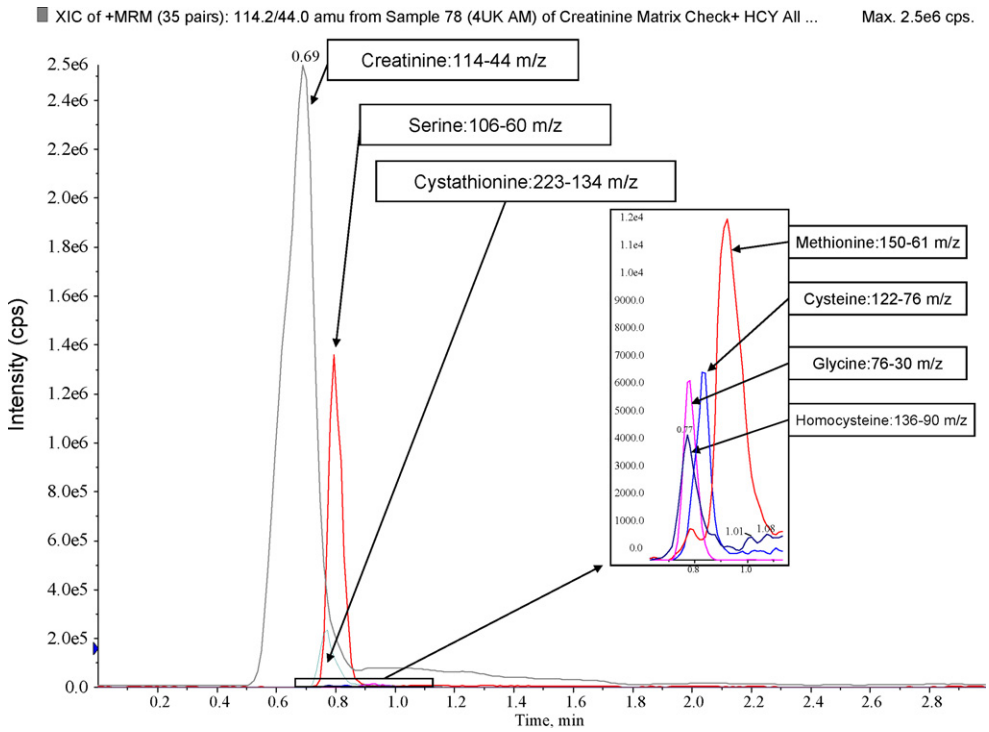

Fig. 6. Total con chromatogram from a single urine injection displaying simultaneous and multiple analytes involved in sulfur amino acid metabolism.

Table 2
Optimization conditions in measurement of Hcy and related metabolites in biological fluids by LC–MS/MS methods.

References	Analyte	Ionization mode	MRM[g] transition	DP[a]	EP[b]	CE[c]	CXP[d]	LC column
Hempen et al., 2008 [33]	Hcy	+ESI	136.1 > 90.1	25	5	15	3	Reverse-phase C_{18} (100 mm × 3 mm; 5 μm)
	Hcy-d_4	+ESI	140.1 > 94.1	25	3	17.5	3	
	Hcy-Hcy	+ESI	290.0 > 136.2	16	7.5	15	4	
	Met	+ESI	150.1 > 104.1	21	5	13	4	
	SA[e]	−ESI	116.9 > 72.8	−20	−2	−14	−1	
	MMA[f]	−ESI	116.9 > 72.8	−20	−2	−14	−1	
	MMA-d_3	−ESI	119.9 > 75.9	−20	−4	−14	0	
Rafii et al., 2007 [39]	Hcy	+ESI	136.1 > 90.1	42	3	16	6	Waters symmetry C_8 (100 mm × 2.1 mm; 3.5 μm)
	Hcy-d_4	+ESI	140.1 > 94.1	42	3	16	6	
	Hcy-Hcy	+ESI	269.1 > 136.0	46	5	15	7	
	Cys	+ESI	122.0 > 76.0	35	5	20	13	
	Cys-d_2	+ESI	124.0 > 78.0	35	5	20	13	
Rafii and Pencharz et al. unpublished data	Cys–Cys	+ESI	241.1 > 152.0	47	7	19	8	
	Met	+ESI	150.0 > 61.0	40	4	25	10	
	CysT	+ESI	226.3 > 134	40	8	20	11	
	Ser	+ESI	106.0 > 60.0	35	4	14	3	
	Gly	+ESI	76.0 > 30.0	29	6	25	13	
	Creatinine	+ESI	114.2 > 44.0	35	5	29	3	
Weaving et al., 2006 [42]	Hcy	+ESI	136.0 > 90.1	26	−7.5	17	4	LC–CN column (330 mm × 4.6 mm; 4.6 μm)
	Hcy-d_4	+ESI	140.1 > 94.1	26	−7.5	17	4	
	Cys	+ESI	122.0 > 76.0	11	−7.5	19	4	
	Cys-d_2	+ESI	126.0 > 80.0	11	−7.5	19	4	
	Met	+ESI	150.0 > 103.8	11	−6	15	16	
	Met-d_3	+ESI	153.0 > 107.1	11	−6	15	16	

DP and EP were used to optimize the parent fragments and the CE and CXP were used to optimize the daughter fragments.
[a] DP: declustering potential (V).
[b] EP: entrance potential (V).
[c] CE: collision energy (V).
[d] CXP: collision exit potential (V).
[e] SA: succinic acid.
[f] MMA: methylmalonic acid.
[g] MRM: multiple reaction monitoring.

metabolite kinetics. This problem was addressed by MacCoss et al. [68], who used the enrichment of plasma homocysteine, instead of methionine, as the precursor pool for flux determinations, due to the fact that homocysteine is derived intracellularly from methionine. The results from this research group indicated that previous estimates of methionine kinetics may have been underestimated by approximately 40%.

The approach used by the latter authors relied, again, on GC–MS methods [25], and required separate injections for the determination of methionine and homocysteine enrichments. Our group has used this methodology to provide evidence that the rate of TS is significantly reduced when total sulfur amino acid intake is provided as a balance of methionine and cysteine as compared when the same level of total sulfur amino acid intake is supplied by methionine alone [69]. However, we were unable to detect sufficient enrichment in homocysteine with GC–MS, and therefore relied on the original model which was based on methionine enrichments alone. This fact, coupled with the need to perform multiple injections for the measurement of methionine and homocysteine enrichment, necessitates the examination of alternative analytical approaches to measuring methionine metabolism. The availability of the LC–MS/MS method discussed above now provides such an alternative, and may offer enhanced sensitivity, decreased analytical time, and simultaneous analysis of metabolite enrichment profiles. We have recently extended the LC–MS/MS methodology to determine the impact of vitamin B_6 deficiency on cysteine and serine kinetics. Vitamin B_6 deficient piglets were infused with L-[3-^{13}C] cysteine and L-[2,3,3-$^{2}H_3$] serine for 6 h, with blood samples drawn hourly. Within a single run and with no sample derivatization, we are able to simultaneously determine enrichments, in plasma, for $m+1$ cysteine, $m+3$ serine, $m+1$ glycine (from serine) and $m+1$ methionine (from serine) (unpublished data). The established LC–MS/MS method now permits the examination of the quantitative impact of factors such as nutrient status, physiological and pathological states on measures of methionine and one-carbon metabolism *in vivo*.

3.1.2. Normalization of urinary thiols and related metabolites to creatinine

Urinary measurements of metabolites including thiols need to be 'normalized' in order to account for the variability in lean body mass, glomerular filtration rate and incomplete 24 h urinary collections. Creatinine excretion from the body is relatively constant, therefore analyte concentrations are normalized to urinary creatinine [69]. Recently methods have been developed to measure creatinine using LC–MS/MS in urine [69,70], serum [71,72] and other biological matrices [73,74]. In our LC–MS/MS method to measure thiols, we were able to detect creatinine simultaneously with the use of creatinine-d_3 as an internal standard (Fig. 6). This method therefore removes the extra step in fractionating urine samples to be sent to a different laboratory for creatinine measurement. Furthermore, our method using LC–MS/MS is more specific and sensitive for accurate creatinine analysis and can also be used for serum/plasma matrices as well. Therefore this method can be utilized in a high-throughput clinical application to measure creatinine clearance (Ccr) in patient populations who are being screened for kidney disease [75].

4. Summary

With the increasing clinical importance of homocysteine and its related products as health markers, there is a growing need for verification of the LC–MS/MS method among multiple laboratories internationally, and a need to set a reference method across the board. The isotope dilution LC–MS/MS method has the greatest potential of being accepted and utilized as a dedicated Hcy and its related metabolite SRM. The dual MS coupled to a suitable chromatographic separation technique has the capacity for unambiguous analyte identification and confirmation due to its high selectivity and sensitivity. Due to the complexity of the biological matrices, sample pre-treatment, use of standards prepared in the same matrices as the sample of interest, as well as the use of isotopically labeled IS are crucial parts of the LC–MS/MS methods to correct for ion suppression.

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