FULL PAPER
THE ANALYST
www.rsc.org/analyst

Combined cysteine and homocysteine quantitation in plasma by trap and release membrane introduction mass spectrometry

Adriana Paula Vellasco,[a] Renato Haddad,[a] Marcos N. Eberlin*[b] and Nelci F. Höehr*[a]

[a] *Medical Science Faculty, State University of Campinas – UNICAMP, PO Box 6111, 13083-970 Campinas, SP, Brazil. E-mail: nelci@fcm.unicamp.br; Fax: +55-19-3289-3273; Tel: +55-19-3289-3273*

[b] *Institute of Chemistry, State University of Campinas – UNICAMP, PO Box 6154, 13083-970 Campinas, SP, Brazil. E-mail: eberlin@iqm.unicamp.br; Fax: +55-19-37883073; Tel: +55-19-37883073*

Received 19th April 2002, Accepted 18th June 2002
First published as an Advance Article on the web 23rd July 2002

Recently, a new and efficient method for total homocysteine (tHcy) quantitation in plasma using trap and release membrane introduction mass spectrometry (T&R-MIMS) with a versatile removable direct introduction membrane probe (DIMP) was described [R. Haddad, M. A. Mendes, N. F. Hoehr and M. N. Eberlin, *Analyst*, 2001, **126**, 1212]. Herein we report on the use of the DIMP-T&R-MIMS technique for total cysteine (tCys) quantitation; hence combined tCys and tHcy quantitation in plasma or serum can be achieved. The method employs Cys and Hcy derivatization with ethyl chloroformate (after disulfide bond reduction with dithiothreitol and protein precipitation with trichloroacetic acid), preconcentration in a capillary silicone membrane, and their thermal desorption to the gas phase inside the ion source region of a mass spectrometer, at a point exactly between the two ionization filaments. Thermal desorption uses the uniform heat radiation provided by the two ionization filaments. The analytes are then ionized by electron ionization and both Cys and Hcy are quantitated by mass spectrometry using selected ion monitoring. For tCys quantitation, good linearity and reproducibility was observed for concentrations ranging from 5 to 350 μM, recovery was near 95%, and the limit of detection (LOD) was of 2 μM. This LOD is well below the mean Cys concentration in plasma, and serum samples from a large group of healthy people showed a mean tCys concentration of 132 ± 45 μM.

Introduction

The amino acid cysteine (Cys) belongs, together with glutathione (GSH) and homocysteine (Hcy), to a group of natural biological aminothiols present in blood and other physiological fluids.[1] These three essential thiols are involved in crucial human physiological processes, such as cellular homeostasis.[2] In plasma, Cys and GSH form an extracellular antioxidant defense system, whereas Hcy is an extracellular oxidant that generates reactive oxidant species.[3,4]

Cys is also involved in many important cellular functions, such as protein synthesis, cellular metabolism, and detoxification.[2] In metabolic pathways, Cys is a precursor of GSH and participates in two synthetic steps: the first, γ-glutamylcysteine synthetase couples glutamate to Cys to form γ-glutamylcysteine and then second, GSH is synthesized by the coupling of γ-glutamylcysteine to glycine catalyzed by glutathione synthetase.[5,6] Cys forms important metabolites such as pyruvate, which can be converted to phosphoenolpyruvate and glucose;[7] and also to taurine. Cys is predominantly oxidized to inorganic sulfate by cysteine dioxygenase *via* cysteine sulfinic acid, and this pathway is usually thought to promote organic detoxification.[8]

Abnormally high level of Hcy, hyperhomocysteinemia, is a valuable marker of common diseases, and a risk factor of increasing concern in cardiovascular disease.[1,9] Cys is directly involved in the phatogenic mechanisms of hyperhomocysteinemia since it catalyzes Hcy auto-oxidation in the presence of metals ions, and promotes the formation of reactive oxygen species, particularly hydrogen peroxide and superoxide anion.[10,11] An increased Cys blood concentration in patients with cardiovascular disease has indicated that Cys is also an important factor in cardiovascular risk, and that hypercysteinemia is independently associated with cardiovascular disease and atherosclerotic injury.[11a] It is also likely that high blood concentrations of Cys, Hcy and LDL cholesterol act synergistically.[11a]

Several methods for tCys quantitation in biological samples are available, using mainly spectrophotometry,[6,12] chemiluminescence,[13] spectrofluorimetry,[14] high-performance liquid chromatography (HPLC),[15,16] and gas chromatography-mass spectrometry (GC-MS).[17] The drawbacks of these methods are associated either with interferences, laborious and time-consuming analyte extraction or separation procedures, or relatively high analytical costs. Therefore, the development of new alternative analytical techniques for combined tCy and tHcy quantitation in biological samples may offer more advantageous procedures.

Membrane introduction mass spectrometry (MIMS)[18] is a powerful analytical technique for monitoring and quantitation of volatile organic compounds (VOC)[19] and some semi-volatile OC (SVOC)[20] in water, soil, and air, displaying high sensitivity, speed and simplicity. For biological samples,[21] however, application of MIMS has been limited because of the poor detection limits for the more polar analytes, normally the targets in clinical analysis. But this limitation has been minimized considerably with the development of trap and release strategies in MIMS (T&R-MIMS),[22] which has greatly expanded the application of MIMS to an increasing series of polar and biologically important analytes. We recently developed a more advantageous new design for a T&R-MIMS system using a direct introduction membrane probe (DIMP) with increased simplicity and sensitivity, and reduced memory effects,[23] and have demonstrated its application to tHcy quantitation in human plasma.[24] We now report that this technique is also applicable with satisfactory speed, sensitivity, linearity and reproducibility

 DOI: 10.1039/b203832c

to the determination of tCys, hence that the method is efficient for combined tHcy and tCy quantitation in plasma (or serum), two increasingly important markers of cardiovascular disease.

Materials and methods

Instrumentation

Mass spectrometric measurements were performed using 70 eV electron ionization (EI) and an Extrel (Pittsburgh, PA) mass spectrometer fitted with a single high transmission quadrupole mass analyzer. A standard EI ion source was used with just a minor modification; the id of one of the two gas entrance lines was enlarged to 1.27 cm.[23] A T&R-MIMS system using a removable DIMP was used. The system[23] differs from the original T&R-MIMS system[22] as it uses a removable DIMP to place the capillary membrane loop inside the ion source block at a point exactly between the two parallel filaments. Hence, uniform heating and maximum sensitivity and reproducibility are achieved, while reducing memory effects. Analyte solutions at room temperature (23 ± 1 °C) were pumped through the DIMP by an eight-roll peristaltic pump at a rate of 2 mL min^{-1}. The capillary membrane was provided by Dow Corning Co. (Silastic Medical-grade tubing) with a wall thickness of 0.056 cm, an id of 0.063 cm, and an od of 0.12 cm. An HP 5890 GC-MS was used for the GC-MS analysis.

Sample collection and preparation

Blood samples were collected by venipuncture from apparently health subjects, cooled on ice and centrifuged at 800*g* within 20 min of collection. Samples must be centrifuged as fast as possible after collection since in whole blood an increase of thiol concentration may occur owing to ongoing metabolism and time-dependent release from erythrocytes.[25] The supernatants were kept at −20 °C to prevent tCys alteration.[26] To measure tCys and to decouple the Cys from proteins and Hcy, the disulfide bonds were reduced by the addition of 250 µL of dithiothreitol (DTT) to 1 mL of the resulting serum (or 1.5 mL of Cys aqueous solutions). The mixture was then incubated for 30 min at 36 °C. The proteins were precipitated by the addition of 1 mL of 5% aqueous solution of trichloroacetic acid (TCA) under vigorous vortexing followed by centrifugation at 3000*g* for 15 min.

Derivatization

The aqueous solutions as well as the serum (or plasma)[26] samples were derivatized with ethyl chloroformate (Scheme 1)[27] by adding 250 µL of a 4 : 1 ethanol : pyridine solution and 50 µL of ethyl chloroformate (ECF). The resulting mixture was then vortex mixed for 1 min, and the volume adjusted to 2.0 mL with deionized water.

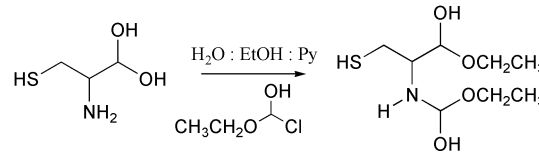
tCys

Standard Cys solutions (D,L-cysteine from Sigma Corp.) were prepared in de-ionized water by serial dilution of a 500 µM aqueous solution. After derivatization, the samples were pumped continuously through the DIMP for quantitation (for a detailed description of the system see ref. 23), using 15 min of trapping time. After trapping, a 2 min air plug was pumped through the lines, and the analytes were then thermally desorbed from the membrane, ionized by EI, and quantitated using selected ion monitoring (SIM) MS .

Results and discussion

Selected ion monitoring (SIM)

Cys was derivatized with ECF thus forming ECF-Cys, and its 70 eV EI mass spectrum (Fig. 1) was first collected using GC-MS. The mass spectrum of ECF-Cys displays the $[M - H]^+$ ion of *m/z* 220 as the base peak, hence this abundant and relatively high mass ion was selected for Cys SIM (hence minimizing interferences from low mass matrix and background ions). A 70 eV EI mass spectrum of ECF-Cys over the representative mass range of *m/z* 160–300 (a mass range that should display the main ionic species used for analyte characterization and quantitation) was also acquired using the DIMP-T&R-MIMS system and single quadrupole MS detection (Fig. 2). Whereas the *m/z* 207 ion is a known chemical noise from the silicone membrane,[23]



Scheme 1 Derivatization of Cys with ECF

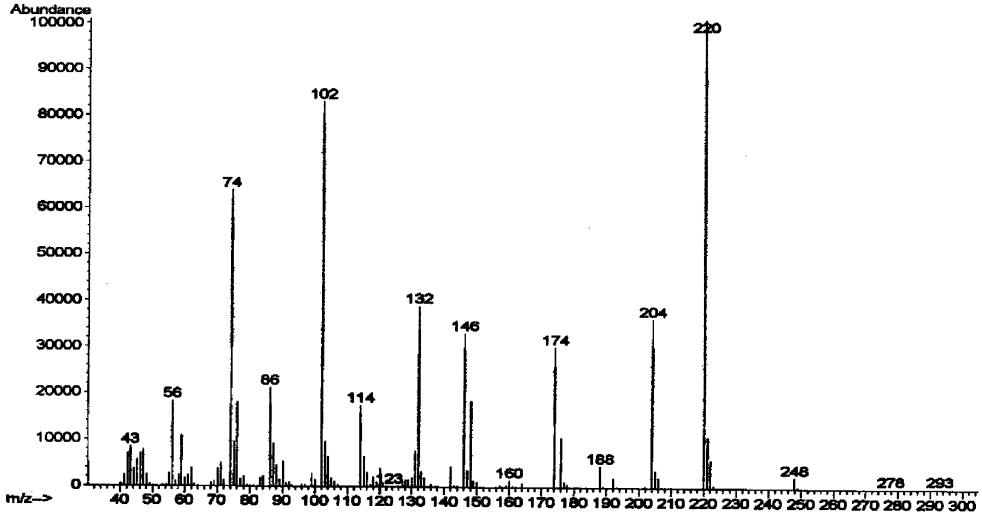


Fig. 1 70eV EI mass spectrum of Cys acquired after derivatization with ethyl chloroformate (ECF-Cys) and GC separation: The base peak, the ion of *m/z* 220, is the $[M - H]^+$ fragment.

characteristic fragment ions of ECF-Cys of *m/z* 220 and 204 are easily detected.

Trapping time

To determine the optimal trapping time for the best combination of sensitivity and speed, 100 μM of aqueous solution of Cys were treated as described above, divided into five aliquots, and then analyzed by the DIMP-T&R-MIMS technique using SIM of *m/z* 220 and increasing trapping times (Fig. 3). From 5 to 20 min of trapping the sensitivity increases almost linearly, but after 20 min of trapping the sensitivity levels off and even decreases after 30 min owing likely to ion source saturation. Therefore, to achieve the best compromise between speed and sensitivity, 15 min. was selected as the ideal trapping time.

Linearity

Several calibration curves were constructed to test the linearity of Cys quantitation by the present technique, using Cys concentrations ranging from 5 to 350 μM and SIM of the ion of *m/z* 220, and a representative collection of SIM peaks are shown in Fig. 4. The average linear range shows that the correlation coefficients vary from 0.994 to 0.998, thus demonstrating the quite good linearity for the method when applied to tCys quantitation. Linearity was also satisfactory (r = 0.99 at least) up to the limit of detection, that is, 2 μM.

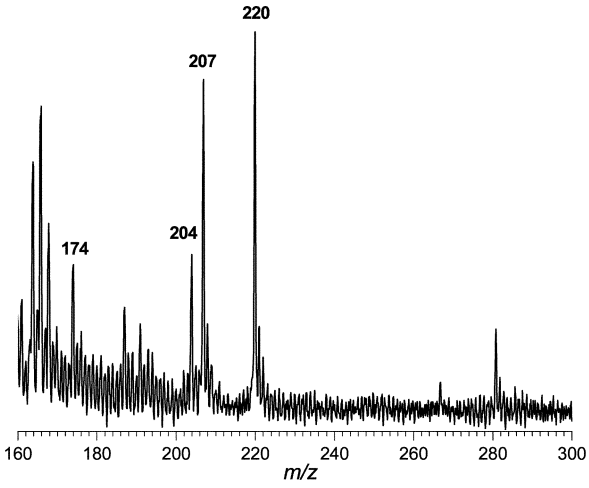


Fig. 2 DIMP-T&R-MIMS 70 eV EI mass spectrum of ECF-Cys acquired during the short thermal desorption period.

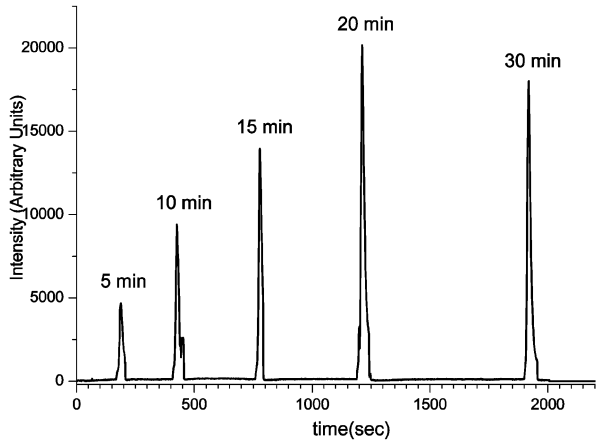


Fig. 3 SIM of the *m/z* 220 ion for ECF-Cys as a function of the trapping time.

Recovery

The test the method's recovery; hence, its susceptibility to interferences (specially from the complex, variable, multi-component serum (or plasma) matrix as well as from the chemicals used in the sample preparation and derivatization) standard solutions of Cys were added to plasma samples before treatment. High recoveries were observed, with an average of 95%, for concentrations ranging from 10 to 350 μM.

Reproducibility

Reproducibility was tested in several ways in a number of experiments performed over a time period of several months from low to high Cys concentration (10–300 μM) plasma samples. As an example, data from a serum sample to which a known (and relatively high) amount of Cys (200 μM) was added was taken. After treatment, division in five aliquots, and SIM using the DIMP-T&R-MIMS technique, a signal fluctuation of just 4% was observed; on average, within-day reproducibility ranged from 3 to 5% whereas between-day reproducibility ranged from 5 to 15%.

Limit of detection

Fig. 5 shows a collection of SIM peaks obtained while testing for the LOD for tCys by the present method. Aqueous Cys

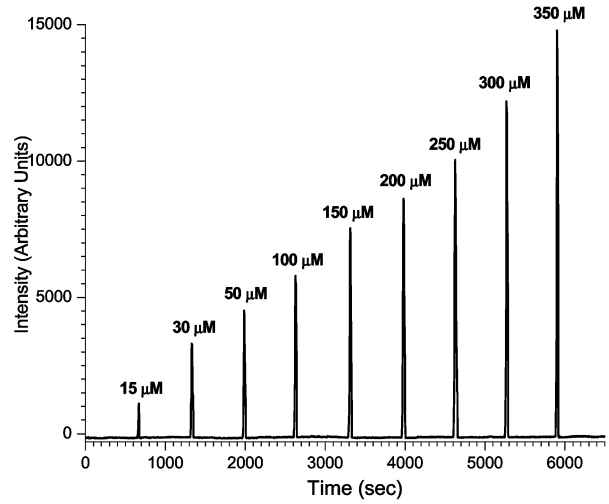


Fig. 4 SIM of the *m/z* 220 ion for ECF-Cys as a function of concentration.

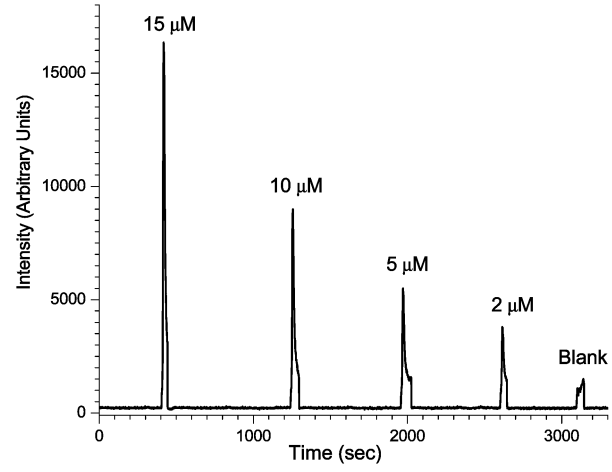


Fig. 5 SIM of the *m/z* 220 ion for ECF-Cys as a function of concentration showing the LOD of 2 μM.

solutions with concentrations ranging from 2 to 15 μM were treated as for a normal plasma or serum sample, and then analyzed using SIM of *m/z* 220. An LOD of 2 μM was achieved. Note that this LOD is well below the mean tCys concentration in human blood, see below.

tCys mean concentrations in human serum

The tCys concentration in serum or human plasma from a group of people from European countries has been reported to be near 250 μM,[28] whereas Jacobsen *et al*.[29] reported a tCys range for healthy men of 152.8–266.5 μM. The blood anticoagulant used in the sample preparation procedure may also affect the measured tCy.[25] With the aim of measuring the mean tCy concentration in human serum by the present method, samples from a group of 60 healthy brazilians were analysed. The mean tCys concentration found was 132 ± 45 μM. Our previous work[24] using the same methodology has determined a mean tHcy concentration in human plasma of 11.6 ± 1.5 μM.

Conclusion

A new method for combined total cysteine (tCys) and total homocysteine (tHcy) quantitation in plasma using trap and release membrane introduction mass spectrometry with a removable direct introduction membrane probe (DIMP-T&R-MIMS) has been described, and its analytical and performance parameters determined. The method is relatively simple and efficient and displays, for both tCy and tHcy quantitation (two increasingly important markers of cardiovascular diseases), good linearity and reproducibility, high recoveries, and detection limits (2 μM) sufficiently below the mean tCys and tHcy concentrations found in human plasma. From samples of a large group (60) of healthy people, the mean tCys concentration found in plasma was 132 ± 45 μM. Although several pretreatment steps are involved, there should be no major difficulties in implementing automation of the sample prepararation. With regard to speed, however, trapping is the time-limiting step. Although we have used 15 min of trapping to provide good sensitivity for both tHcy and tCys quantitation, 10 or even 5 min of trapping should provide enough sensitivity for a single tCys quantitation. Dual or even multiple arrays of DIMPs could eventually be used to increase sample throughput, and a dual array would be easily adapted to our MS system.[23]

References

1 B. J. Mills, M. M. Weiss, C. A. Lang, M. C. Lui and C. Ziegler, *J. Lab. Clin. Med.*, 2000, **135**, 396.
2 W. A. Gahl, N. Bashan, F. Tietze, I. Bernardini and J. D. Schulman, *Science*, 1982, **217**, 1263.
3 M. A. Mansoor, C. Bergmark, A. M. Svardal and P. M. Ueland, *Arterioscler. Thromb. Vasc. Biol.*, 1995, **15**, 232.
4 P. M. Ueland, M. A. Mansoor, A. B. Guttorsmen, H. Refsum, P. Aukrust and A. M. Svardal, *J. Nutr.*, 1996, **126**, 1281.
5 A. Meiste, *J. Biol. Chem.*, 1988, **263**, 17205.
6 J. S. Stamler and A. Slivka, *Nutr. Rev.*, 1996, **54**, 1.
7 L. Stryer, *Biochemistry*, W. H. Freeman, 3rd edn., 1988, p. 415.
8 (*a*) R. B. Parsons, R. H. Waring, D. B. Ramsden and A. C. Williams, *J. Neurol. Sci.*, 1997, **152**, 562; (*b*) R. Janaky, V. Varga, A. Hermann, P. Saransaari and S. S. Oja, *Neurochem. Res.*, 2000, **25**, 1397.
9 (*a*) K. S. McCully, *Am. J. Pathol.*, 1969, **56**, 111; (*b*) G. N. Welch and J. Loscalzo, *New Engl. J. Med.*, 1998, **338**, 1042; (*c*) P. Berger, *ACC Educ. Highlights*, 1998, **13**, 1; J. Townend, J. O'Sullivan and J. T. Wilde, *Blood Rev.*, 1998, **12**, 23.
10 N. Hogg, *Free Radical Biol. Med.*, 1999, **27**, 28.
11 (*a*) N. Jacob, E. Bruckert, P. Giral, M. J. Foglietti and G. Turpin, *Atherosclerosis*, 1999, **146**, 53; (*b*) H. Björn, A. Anders and I. Anders, *Clin. Chim. Acta*, 1997, **262**, 39.
12 (*a*) M. K. Gaitonde, *J. Biochem.*, 1967, **104**, 627; (*b*) M. H. Malloy, D. K. Rassin and G. E. Gaull, *Anal. Biochem.*, 1981, **113**, 407; (*c*) A. Besada, N. B. Tadros and Y. A. Gawargious, *Mikrochim. Acta*, 1989, **3**, 143; (*d*) V. Ogwu and G. Cohen, *Free Radical Biol. Med.*, 1998, **25**, 362.
13 H. Kubo and A. Toriba, *Anal. Chim. Acta*, 1997, **353**, 345.
14 H. Wang, W. S. Wang and H. S. Zhang, *Talanta*, 2001, **53**, 1015.
15 G. Chwatko and E. Bald, *Talanta*, 2000, **52**, 509.
16 E. Kaniowska, G. Chwatko, R. Glowacki, P. Kubalczyk and E. Bald, *J. Chromatogr., A*, 1998, **798**, 27.
17 P. Capitana, T. Malmezata, D. Breuilleb and C. Obleda, *J. Chromatogr., B*, 1999, **732**, 127.
18 (*a*) F. R. Lauritsen and T. Kotiaho, *Rev. Anal. Chem.*, 1996, **15**, 237; (*b*) T. Kotiaho, *J. Mass Spectrom.*, 1996, **31**, 1; (*c*) R. C. Johnson, R. G. Cooks, T. M. Allen, M. E. Cisper and P. H. Hemberger, *Mass Spectrom. Rev.*, 2000, **19**, 1; (*d*) R. A. Ketola, T. Kotiaho, M. E. Cisper and T. M. Allen, *J. Mass Spectrom.*, 2002, **37**, 457.
19 For recent examples see: (*a*) M. E. Cisper, A. W. Garrett, D. Cameron and P. H. Hemberger, *Anal. Chem.*, 1996, **68**, 2097; (*b*) M. A. Mendes, R. S. Pimpim, T. Kotiaho and M. N. Eberlin, *Anal. Chem.*, 1996, **68**, 3502; (*c*) C. S. Creaser, J. W. Stygall and D. J. R. Weston, *Anal. Commun.*, 1998, **35**, 9H; (*d*) R. Kostiainen, T. Kotiaho, I. Mattila, T. Mansikka, M. Ojala and R. A. Ketola, *Anal. Chem.*, 1998, **70**, 3028; (*e*) L. A. B. Moraes, M. N. Eberlin, J. R. Cagnon and L. H. Urbano, *Analyst*, 2000, **125**, 1529; (*f*) M. A. Mendes, R. Sparrapan and M. N. Eberlin, *Anal. Chem.*, 2000, **72**, 2166.
20 (*a*) F. R. Lauritsen, M. A. Mendes and T. Aggerholm, *Analyst*, 2000, **125**, 211; (*b*) G. Matz and F. Lennemann, *J. Chromatogr., A*, 1996, **750**, 141; (*c*) F. R. Lauritsen and J. Rose, *Analyst*, 2000, **125**, 1577; (*d*) L. S. Riter, Z. Takats and R. G. Cooks, *Analyst*, 2002, **126**, 1980.
21 (*a*) S. Woldring, *J. Assoc. Adv. Med. Instr.*, 1970, **4**, 43; (*b*) A. Wald and K. H. Ranseholf, *J. Assoc. Adv. Med. Instr.*, 1971, **5**, 325.
22 F. R. Lauritsen and R. A. Ketola, *Anal. Chem.*, 1997, **69**, 4917.
23 M. A. Mendes and M. N. Eberlin, *Analyst*, 1999, **125**, 21.
24 R. Haddad, M. A. Mendes, N. F. Höehr and M. N. Eberlin, *Analyst*, 2001, **126**, 1212.
25 F. Salazar, B. Herbeth, G. Siest and P. Leroy, *Clin. Chem.*, 1999, **45**, 2016.
26 Both serum and plasma samples were analyzed using the same procedures. tCys concentrations in plasma were found to be 10–20% higher than those found in serum samples.
27 (*a*) P. Husek, *J. Chromatogr.*, 1991, **552**, 289; (*b*) P. Husek, *J. Chromatogr., B*, 1998, **717**, 57.
28 L. E. Khairy, P. M. Ueland, H. Refsum, I. M. Graham and S. E. Vollset, *Clin. Invest. Rep.*, 2001, **103**, 2544.
29 J. D. W. Jacobsen, V. J. Gatautis, R. Green, K. Robinson, S. R. Savon, M. Secic, J. Ji, J. M. Otto and L. M. Taylor, Jr., *Clin. Chem.*, 1994, **40**, 873.