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Determination of homocysteine and its related compounds by solid-phase microextraction–gas chromatography–mass spectrometry

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Abstract

The purpose of this study was to develop a simple and accurate analytical method to determine homocysteine (Hcy), cysteine (Cys), and methionine (Met) in aqueous samples. Until now, the most frequently used method for the assay of Hcy, Cys, and Met has been high-performance liquid chromatography with fluorescence detection after fluorescent tagging. The newly developed method involves the employment of the SPME (solid-phase microextraction) technique together with GC–MS. For application to a gas chromatographic system, alkyl formate derivatives were prepared in the form of *N*(*O*,*S*)-alkoxycarbonyl alkyl ester with the analytes in the aqueous samples. The optimum derivatizing regent for *N*(*O*,*S*)-alkoxycarbonyl alkyl ester was chosen by comparing the efficiency of the derivatized analytes in a GC through the SPME method and liquid–liquid extraction. The optimum conditions of the SPME system for the analytes derivatized with *N*(*O*,*S*)-ethoxycarbonyl propyl ester in the aqueous matrix were pH 3.0 and no salt, and 30 min equilibration time using an 85 μm PA (polyacrylate) fiber. The developed method is inexpensive, easy and rapid.

Keywords: Homocysteine; Cysteine; Methionine

1. Introduction

The sulfur-containing amino acid homocysteine (Hcy) is formed from dietary methionine (Met) during the *S*-adenosylmethionine (SAM)-dependent transmethylation reaction and can be converted to cysteine (Cys) via cystathionine [1]. A marked increase in the concentration of Hcy indicates reduced folates, Vitamins B_{12} and B_6, and sulfur amino acids. Even a mildly elevated plasma level of Hcy, referred to as hyperhomocysteinaemia, is considered to be an independent risk factor for premature atherosclerosis, resulting in coronary heart disease, strokes and peripheral vascular disease [2–5]. Furthermore, a moderate increase in plasma Hcy has been reported in renal failure, psoriasis, leukemia and solid tumors [6–9]. Thus, the Hcy, Met and Cys in blood can be used as biomarkers for the risk assessment of vascular disease, evaluation of nutritional status, monitoring of renal status and cancer

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prognosis. Most of the Hcy (~70%) in blood is protein-bound by disulfide bonds and another part exists as Hcy–Cys mixed disulfides. Therefore, it is necessary to cleave the disulfide bond in order to determine the total homocysteine.

Homocysteine in plasma is determined by ion-exchange chromatography using an amino acid analyzer, high-performance liquid chromatography (HPLC), and gas chromatography–mass spectrometry (GC–MS). However, an amino acid analyzer is not selective for sulfur amino acids and lacks sensitivity [10]. HPLC methods based on electrochemical detection [11,12] and on fluorescence detection [13–23] are commonly used to determine the total Hcy in plasma or urine. The HPLC fluorescence methods are highly sensitive, but are difficult for routine analysis due to the need for a gradient system, demanding complex derivatization, a clean-up procedure to remove the interference matrix for the selective extraction and column safety.

The assay method for Hcy using gas chromatography (GC) has rarely been reported, because of the incompatibility of Hcy with the GC system. In the structure of homocysteine there are many polar groups such as amine ($-NH_2$), carboxylic acid (–COOH) and thiol (–SH), thus homocysteine cannot be easily extracted from biological fluids using organic solvents. Furthermore, derivatization is needed to increase both the solubility in the organic solvent and the volatility for the chromatographic properties [24–26].

Recently, a novel technique, solid-phase microextraction (SPME) [27], has been developed, and is an inexpensive, rapid, simple and solvent-free technique for the extraction of the organic compounds from an aqueous sample matrix. This method combines the extraction and pre-concentration procedure, and extracted analytes are introduced into the gas chromatograph directly. Recently, the SPME method has been applied to the analysis of various groups of compounds from aqueous matrices, including phenols [28,29], polychlorobutadienes [30], phosphorous-containing insecticides [31], polycyclic aromatic hydrocarbons [32], tricyclic antidepressants [33] in plasma, organic acids in tobacco [34] and amphetamines in blood [35].

In this paper, an integrated method which combines direct derivatization in the plasma by the alkyl chloroformate reagent with simultaneous extraction and concentration by SPME is described. The sensitivity and/or reactivity of six alkyl chloroformates as derivatizing reagents are compared, and also the affinities of *N*,*O*-alkoxycarbonylpropyl ester forms for the SPME coating fiber are studied. The optimized conditions for direct derivatization and SPME analysis for Hcy, Met and Cys are described.

2. Experimental

2.1. Reagents and instrumentation

A HP 5988A mass spectrometer (Hewlett Packard, Palo Alto, CA, USA) coupled to an HP 5890A gas chromatograph (Hewlett Packard) controlled by a HP 59970C MS ChemStation was used. The mass spectrometer was operated with a filament current of 300 μA and an electron energy of 70 eV in the EI mode. A HP-1 (Hewlett Packard) capillary column (16 m×0.2 mm I.D. with a film thickness of 0.11 μm) was installed in the gas chromatograph and inserted directly into the ion source of the mass spectrometer. Helium was used as the carrier gas at a flow-rate of 0.9 ml/min. The oven temperature was held at 100°C for 0.5 min, increased to 300°C at 10°C/min and finally held at 300°C for 3 min. The splitting ratio was 1:10. The coated fiber was desorbed in the injection port of the GC at 240°C for 0.5 min. The mass range in the SCAN mode was *m*/*z* 40–400, and the selected masses in the SIM (selected ion monitoring) mode were: *m*/*z* 61, 129, 189 (methionine); *m*/*z* 74, 146, 220 (cysteine); *m*/*z* 56, 128, 189 (homocysteine). DL-Homocysteine, *S*-2-aminoethyl-L-cysteine hydrochloride (IS) and L-cysteine were obtained from Sigma (St. Louis, MO, USA), while the L-methionine was purchased from Fluka (Buchs, Switzerland). Ethyl, propyl, isopropyl, butyl, isobutyl and allyl chloroformate were purchased from Aldrich (Milwaukee, WI, USA). Deionized water was prepared with a Milli-Q water purification system (Millipore, Milford, MA, USA). The SPME holder for the manual sampling, 100 μm PDMS (polydimethylsiloxane), and 85 μm PA (poly-

acrylate) fiber were purchased from Supelco (Bellefonte, PA, USA).

2.2. Derivatization and liquid–liquid extraction

The derivatization technique for Hcy, Met and Cys with alkyl chloroformates was a slightly modified version of the method described by Husek [26], who derivatized the amino acids using methyl and ethyl chloroformate.

To obtain free homocysteine from disulfide bonds, 500 μl plasma was mixed with 500 μl deionized water, 100 μl dithiothreitol solution (12.5%, w/v) and incubated for 30 min at 40°C [24]. After incubation, proteins were precipitated by the addition of 100 μl trichloroacetic acid solution (72%, w/v) and Vortex-mixed. Supernatants were collected after centrifugation for 5 min.

A 400 μl mixture of propyl alcohol–pyridine (4:1, v/v) was added to 600 μl aqueous sample, with subsequent addition of 50 μl alkyl chloroformate. The mixture was Vortex-mixed for 3 min at room temperature and 1 ml chloroform containing 1% alkyl chloroformate was added. The derivatized sample mixture was centrifuged for 5 min at 730 *g* and 1 ml organic phase was transferred to a new test tube. Chloroform was evaporated using a nitrogen gas stream. After complete evaporation, the residue was taken up in 100 μl chloroform containing 1% alkyl chloroformate. The sample (2 μl) was injected into the GC.

2.3. SPME method

To 600 μl aqueous sample was added 400 μl propyl alcohol–pyridine (4:1) solution and 50 μl alkyl chloroformate, and the mixture Vortex-mixed for 3 min at room temperature. After addition of 2 ml deionized water, the SPME fiber was immersed in aqueous solution in a 4 ml vial containing a magnetic stirring bar. The vial was sealed with a pierced cap with stirring through the use of a magnetic stirring plate. After equilibration, the septum-piercing needle of the SPME device was injected into the gas chromatograph and desorbed for 0.5 min in the injection port.

3. Results and discussion

3.1. Comparison of the reactivity of various derivatization reagents

The reactivity of ethyl, propyl, isopropyl, butyl, isobutyl and allyl chloroformates towards Hcy, Met and Cys using the liquid–liquid extraction method was compared. The amine ($-NH_2$) and thiol (–SH) groups of Hcy, Met and Cys were converted to the alkoxycarbonyl form and carboxylic acid (–COOH) was converted into its ester form. Fig. 1 shows a comparison of the reactivity of several alkoxycarbonyl derivatives using liquid–liquid extraction. In the case of Hcy and Met, butyl and isobutyl derivatization was more reactive than that of ethyl and isopropyl chloroformates, and Hcy and Met were not effective with isopropyl derivatization.

3.2. Comparison of the efficiency of several derivatives by the SPME method

A comparison of the sensitivity of the ethyl, butyl, isobutyl and allyl derivatives for the 85 μm PA and 100 μm PDMS coating fiber was performed. In the experiments the pH and ionic strength were not adjusted and the equilibration time was 30 min. All experiments were carried out in triplicate and the average GC peak area counted for comparison. Since the coating fiber was degraded by the derivatizing reagent mixture, which was propyl chloroformate or isopropyl chloroformate, propyl and isopropyl derivatives, which were performed in the liquid–liquid extraction method experiment, were excluded. Fig. 2 shows a comparison of the efficiency of 85 μm PA fiber according to the derivative regents. Allyl and

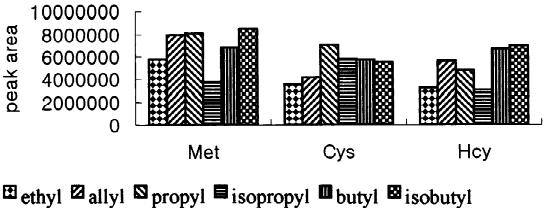

Fig. 1. Comparison of the reactivity of the alkoxycarbonyl propyl ester derivatives.

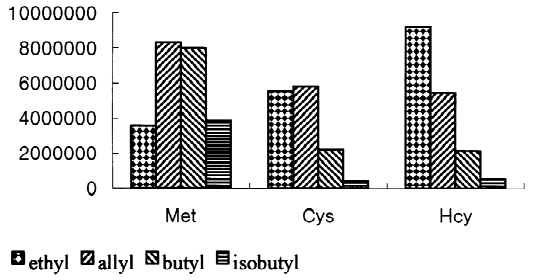

Fig. 2. Sensitivity of 85 μm PA for the various derivatives.

butyl derivatives for Met and ethyl and allyl derivative for Cys are more effective for 85 μm PA. In the case of Hcy, the ethyl derivative is predominantly effective for 85 μm PA. From a comparison of the efficiency of the 100 μm PA fiber with respect to several derivative reagents (Fig. 3) the isobutyl derivative is more effective than the other derivatives. However, during the isobutyl derivatization procedure there was a phase separation which resulted in poor reproducibility [RSD (n=3): Hcy, 28.4%; Cys, 20.1%; Met, 9.3%]. According to the above results, the ethyl derivatization reaction [RSD (n=3): Hcy, 3.5%; Cys, 5.4%; Met, 5.4%] and the 85 μm PA coating fiber were selected as the derivatization method and the species for the coating fiber of the SPME for the analysis of Hcy, Met and Cys.

3.3. Effect of pH and ionic strength

The effects of pH and salt, singularly and in combination, were investigated as a means of enhancing the amount extracted by the fiber. After adjusting the pH by addition of 5 *M* HCl and 5 *M* KOH, the effects of pH on the extraction efficiency were investigated. The pH of the spiked sample in the presence of distilled water was 5. The amount of standards extracted at several pH values was compared with that of control samples (pH 5, no salt). Fig. 4 shows the decreasing trend of extraction to the coating fiber with increasing pH from 1 to 14. In general, the lower the pK_a value, the greater the increment in the extracted amount. At neutral pH, the compounds are slightly in their ionic form. When the pH is decreased, their acid–base equilibrium shifts towards the neutral form, which has a greater affinity for the coating fiber, thereby more of the analyte was extracted to the fiber. In contrast, when the pH is increased, the affinity for the fiber is decreased. The addition of salt to the water solution generally causes a decrease in the solubility of the organic compounds in water, and this has been used to enhance the extraction of analytes on the fiber. Because the ionic strength of the aqueous solution increases, more ionized analytes are formed at the expense of the neutral molecules. Therefore, the effect of the different ionic strengths on extraction was investigated. Table 1 shows the effects of pH and salt, singularly and in combination. Because the analyte was already in the neutral form at pH 5, a change upon adding the salt was not observed. For analytes that have a considerable portion of their molecules in the ionized form, the salting-out effect is a negative factor.

The effects of both pH and salting out were investigated when used in combination. At pH 1.0 and no salt, the amount of analytes showed a small increment. At pH 5 and 10% added salt, Hcy and Cys showed a decrease by a factor of 0.3 and 0.6. At pH 1.0 and 10% salt concentration, the amount extracted for each analyte in the mixture solution was increased by a factor of 3–8 over that of the

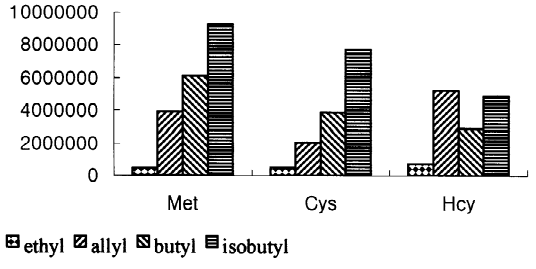

Fig. 3. Sensitivity of 100 μm PDMS for the various derivatives.

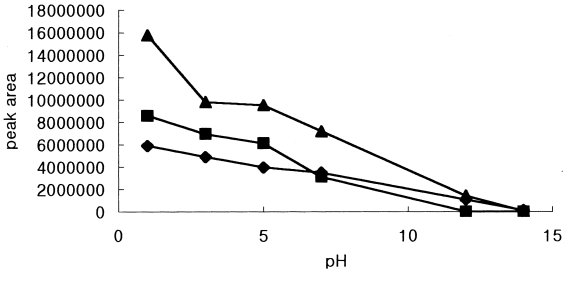

Fig. 4. Effects of pH on extraction to the 85 μm PA coating fiber: (♦) Met, (■) Cys, (▲) Hcy.

Table 1
Effect of salt concentration and pH

NaCl (%):	pH 5.0 (control)				pH 3.0				pH 1.0			
	0	10	20	30	0	10	20	30	0	10	20	30
Hcy	1	0.32	0.11	0.23	1.45	1.87	0.07	0.03	1.66	2.88	0.27	0.12
Met	1	1.79	1.09	0.86	1.23	1.24	0.13	0.01	1.49	8.26	1.58	0.75
Cys	1	0.64	0.18	0.29	1.13	1.12	0.11	0.02	1.40	3.85	0.36	0.17

control sample at pH 5.0 and with no salt added. Under these conditions, the analytes were slightly in their neutral form and salted out of aqueous solution and partitioned into the coated fiber. However, at lower pH and with added salt, degradation of the SPME fiber was found, and it was impossible to obtain good reproducibility and the fiber was removed from the connected holder. Therefore, although the amount of analytes extracted at low pH and with added salt showed a small increment, the optimum conditions of the aqueous matrix was pH 3.0 with no salt.

3.4. Equilibration time

The equilibration time between the coating fiber and aqueous solution was measured at pH 3.0 and no salt. Agitation by magnetic stirring bar was performed to achieve rapid mass transport. The time required to reach equilibrium was about 30 min (Fig. 5).

3.5. Calibration and limit of detection

In general, the plasma concentration is 7–21 μmol/l for Hcy, 13–45 μmol/l for Met and 170–380 μmol/l for Cys [13,18–20,25]. In this study, the limits of quantitation were <5 μmol/l for Hcy, Met and Cys in plasma. Therefore, this method can be applicable to Hcy, Met and Cys in human plasma. The calibration curve was linear over the range 5–50 μmol/l for Hcy and Cys and 40–400 μmol/l for Met, and the correlation coefficients of Hcy, Cys and Met were 0.991, 0.986 and 0.998, respectively. The method presented here is precise and sensitive for the detection of even low concentrations of Hcy, which is considered a pre-requisite for monitoring in blood. The method would be equally suitable for the determination of the total homocysteine.

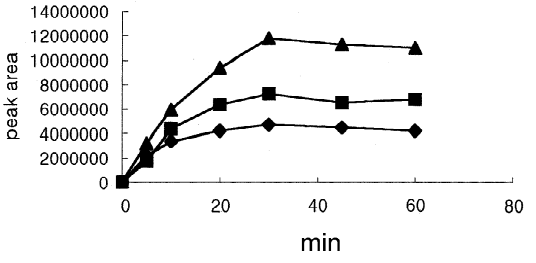

Fig. 5. Effects of immersion time on the extraction of Hcy, Cys and Met using the 85 μm PA coated SPME fiber: (♦) Met, (■) Cys, (▲) Hcy.

3.6. Applications

Fig. 6 shows the EI mass spectra of derivatized Hcy, Cys, and Met. The derivatized Hcy and Met show the molecular ions, which are at m/z 321 and 263, respectively. However, the molecular ion of derivatized Cys was not detected under these MS conditions.

To apply a human plasma sample, the sample treatment procedure was the same as described in Section 2.3. A GC–MS chromatogram (SIM) from the SPME method for a human plasma sample obtained from St. Paul's Hospital, Seoul, Korea, is shown in Fig. 7. The selected masses in the SIM (selected ion monitoring) mode were: m/z 61, 129, 189 (methionine); m/z 74, 146, 220 (cysteine); m/z 56, 128, 189 (homocysteine). Hcy, Cys, and Met were detected at a retention time of 6.27, 5.66, and 4.84, respectively. For the quantitation of homocysteine and its related compounds using SPME in human plasma samples, the equations for the curves were $y = 0.3995x - 0.4483$ for Hcy, $y = 0.1531x + 1.0139$ for Cys, and $y = 0.0912x + 0.0694$ for Met. The correlation coefficient (r^2) of each calibration

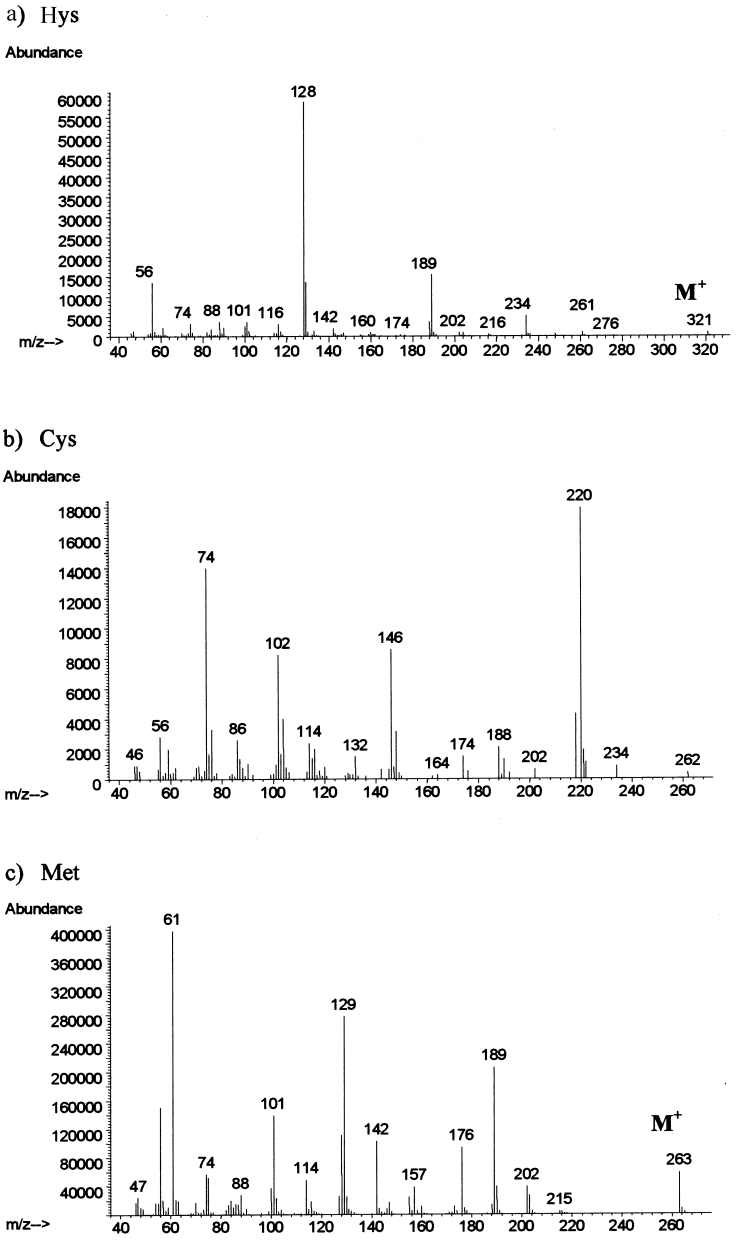

Fig. 6. Electron impact mass spectra of (a) homocysteine, (b) cysteine, and (c) methionine.

curve was 0.991 for Hcy, 0.998 for Met, and 0.986 for Cys. The measured amount of Hcy, Cys, and Met was 11.2±0.6 μmol/l (RSD 5.5%, $n=3$), 465.5±58.3 μmol/l (RSD 12.5%, $n=3$), and 29.5±3.7 μmol/l (RSD 12.4%, $n=3$), respectively.

4. Conclusions

To apply GC–MS for the determination of Hcy, Met and Cys after ethoxycarbonyl propyl ester derivatization in aqueous media directly, the SPME

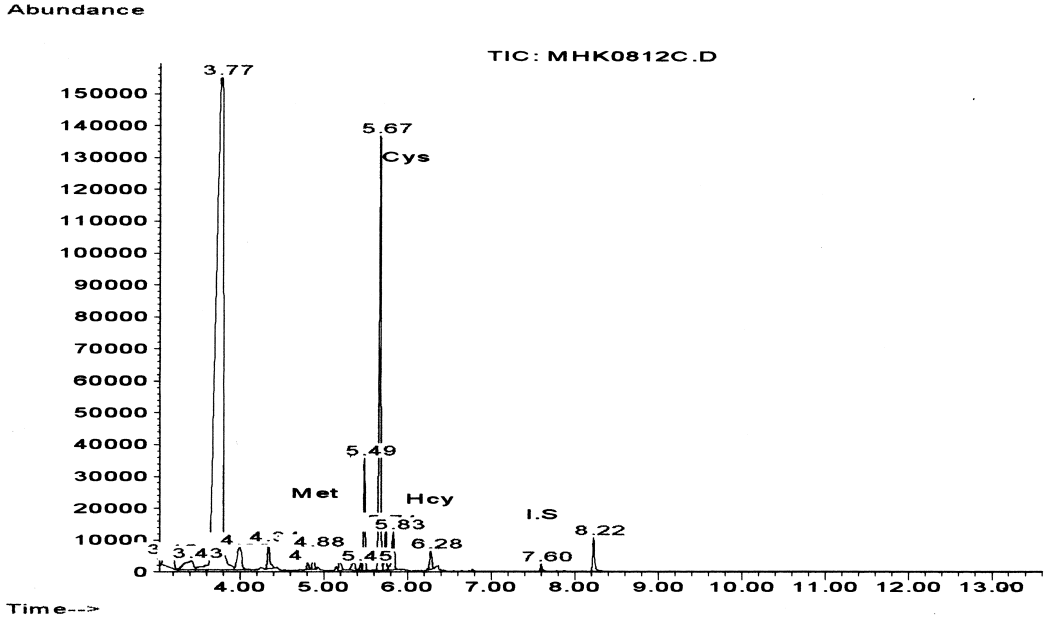

Fig. 7. GC–MS chromatograms of Hcy (6.28 min), Cys (5.67 min), Met (4.87 min) and internal standard (7.62 min) obtained from a human plasma sample.

method, in which extraction and concentration are integrated, was tested as a pre-treatment step. The developed method, which is simple and inexpensive, can be used to determine Hcy, Met and Cys in plasma and urine.

References

[1] D.B. Marks, A.D. Marks, C.M. Smith (Eds.), Basic Medical Biochemistry, Williams & Wilkins, Baltimore, MD, 1996, p. 601.
[2] L.M. Taylor Jr., R.D. DeFranf Jr., E.J. Harris, J.M. Porter, J. Vasc. Surg. 13 (1991) 128.
[3] L. Brattstorm, B. Israelsson, B. Norrving, D. Bergqvist, J. Thorne, B. Hultberg, A. Hamfelt, Atherosclerosis 81 (1990) 51.
[4] S.S. Kang, P.W.K. Wong, M.R. Malinow, Annu. Rev. Nutr. 12 (1992) 279.
[5] L.L. Wu, J. Wu, S.C. Hunt, B.C. James, G.M. Vincent, R.R. Williams, P.N. Hopkins, Clin. Chem. 114 (1994) 552.
[6] D.E.L. Wilcken, V.J. Gupta, A.K. Betts, Clin. Sci. 61 (1981) 743.
[7] C. Soria, B. Chadefaux, M. Coude, O. Gaillard, P. Kamoun, Clin. Chem. 36 (1990) 2137.
[8] H. Refsum, F. Wesenberg, P.M. Ueland, Cancer Res. 51 (1991) 828.
[9] H. Refsum, P.M. Ueland, S. Kvinnsland, Cancer Res. 46 (1986) 5385.
[10] D.E.L. Wilcken, V.J. Gupta, Clin. Sci. 57 (1979) 211.
[11] M.R. Malinow, S.S. Kang, L.M. Taylor, P.W.K. Wong, T. Inahara, D. Mukerjee, Circ. Res. 79 (1989) 1180.
[12] L.A. Smolin, J.A. Sneider, Anal. Biochem. 168 (1988) 374.
[13] A. Ariki, Y. Sako, J. Chromatogr. 422 (1987) 43.
[14] K. Imai, T. Toyo'oka, Y. Watanabe, Anal. Biochem. 128 (1983) 471.
[15] J.B. Ubbink, W.J.H. Vermaak, S. Bissbort, J. Chromatogr. 565 (1991) 441.
[16] B. Vester, K. Rasmussen, Eur. J. Clin. Chem. Clin. Biochem. 29 (1991) 549.
[17] P.E. Cornwell, S.L. Morgan, W.H. Vaughn, J. Chromatogr. 617 (1993) 136.
[18] T. Fiskerstrand, H. Refsum, G. Kvalheim, P.M. Ueland, Clin. Chem. 39 (1993) 263.
[19] H. Refsum, P.M. Ueland, A.M. Svardal, Clin. Chem. 35 (1989) 1921.
[20] M.A. Mansoor, A.M. Svardal, P.M. Ueland, Anal. Biochem. 200 (1992) 218.
[21] I. Fermo, C. Arcelloni, E. De Vecchi, S. Vigano, R. Paroni, J. Chromatogr. 593 (1992) 171.
[22] M.N. Reddy, C. Behnke, J. Liq. Chromatogr. Relat. Technol. 20 (1997) 1391.

[23] A. Andersson, A. Isaksson, L. Brattstrom, B. Hultberg, Clin. Chem. 39 (1993) 1590.
[24] J.O. Sass, W. Endres, J. Chromatogr. A 776 (1997) 342.
[25] H. Kataoka, K. Takagi, M. Makita, J. Chromatogr. B 664 (1995) 421.
[26] P. Husek, J. Chromatogr. 552 (1991) 289.
[27] D. Louch, S. Motlagh, J. Pawliszyn, Anal. Chem. 64 (1992) 1187.
[28] K.D. Buchholz, J. Pawliszyn, Anal. Chem. 66 (1994) 160.
[29] K.D. Buchholz, J. Pawliszyn, Environ. Sci. Technol. 27 (1993) 2844.
[30] E. Fattore, E. Benfenati, R. Fanelli, J. Chromatogr. A 737 (1996) 85.
[31] S. Magdic, A. Boyd-Boland, K. Jinno, J. Pawliszyn, J. Chromatogr. A 736 (1996) 219.
[32] D.W. Potter, J. Pawliszyn, Environ. Sci. Technol. 28 (1994) 298.
[33] X.-P. Lee, T. Kumazawa, K. Sato, O. Suzuki, J. Chromatogr. Sci. 35 (1997) 302.
[34] T.J. Clark, J.E. Bunch, J. Chromatogr. Sci. 35 (1997) 209.
[35] N.Y. Nagasawa, M. Ashiki, Y. Iwasaki, K. Hara, T. Kojiwa, Forensic Sci. Int. 78 (1996) 95.