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Ultrasonic-assisted derivatization reaction of amino acids prior to their determination in urine by using single-drop microextraction in conjunction with gas chromatography

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Abstract

A derivatization–extraction method that avoids tedious preconcentration steps is established in order to determine amino acids accurately at nanogram levels. The method involves conversion of the analytes of concern to *N*(*O*,*S*)-ethoxycarbonyl amino acid ethyl esters and subsequent extraction by single-drop microextraction (SDME) followed by GC analysis. The reaction proceeds smoothly and rapidly under ultrasonication which removes the bubbles from the bulk solution. Precision is acceptable and 12 non-hydrolyzed amino acids can be determined in urine in this manner. As long as the extraction conditions are consistently applied, quantitative analysis can be performed accurately. The limits of detection were satisfactory in the range 0.010–0.025 μg/ml for GC–FID and 0.26–68 ng/ml for GC–MS(SIM) with 1 ml sample volume.

Keywords: Amino acids; Ethylchloroformate; Derivatization; Urine; Single-drop microextraction; GC

1. Introduction

Amino acids are fundamental units of any biological object ranging from bacteria to humans [1]. The analysis of amino acids in such samples has become an important issue of scientific interest. In protein chemistry, amino acid analysis is an integral tool used to obtain information on both the absolute amount and amino acid composition of the sample. To detect these compounds, various techniques have been used. Flow injection and sequential injection-based analyzers have been proved capable of monitoring amino acids but these approaches were not intended for complete profiling. Only total amino acid analysis [2,3] or the assay of a limited number of individual amino acids is possible [4–6].

Due to the complex matrices that are often encountered, analytical methods for amino acids rely heavily on separations using gas chromatography [7–9], liquid chromatography [10–12] and capillary electrophoresis [13–15].

Gas chromatographic methods have some advantages over HPLC, however, derivatization of amino acids needs to be performed before analysis to produce volatile compounds. Usually, silylation is carried out using BSTFA [16–18] and MTBSTFA [19–21] under anhydrous conditions and heating or esterification/acylation in the two successive steps [22,23]. When the derivatization of amino acids in aqueous media is inevitable alkoxycarbonyl alkyl esters seem to be more attractive because of simple sample preparation and highly acceptable analytical features [24–27]. Moreover, unlike silylation, the latter method is possible to derivatize amino acids while keeping sugars and related compounds uninfluenced. This is important, in particular when working with biological samples.

Despite the remarkable achievements in separation and detection of amino acids, sample preparation before analysis remains an integral and often time consuming part of methodology. In trace analysis, this step should result in an extract compatible with the measurement analytical

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technique. Current methods for the extraction and concentration of amino acids in biological samples are liquid–liquid extraction [27,28] and solid phase extraction [29,30].

Single-drop microextraction (SDME) is a relatively new method of sample preparation which provides extraction of analytes into a small volume of organic solvent and is performed quickly with simple equipment found in most analytical laboratories. Liu and Dasgupta [31] and Jeannot and Cantwell [32] first introduced the concept of using a small drop of water-immiscible solvent for sample preparation, combining analyte extraction and preconcentration prior to analysis. Psillakis and Kalogerakis [33] recently provided a detailed report on the developments, modes and applications of SDME.

Herein, we describe a methodological development of amino acids analysis based on gas chromatography after the rapid ultrasonic-assisted formation of *N*(*O*,*S*)-ethoxycarbonyl amino acid ethyl esters and the SDME of the resulting derivatives. The experimental conditions to obtain high efficiency in the derivatization and extraction step were established and the method was directly applied to the analysis of amino acids in urine. The method is sensitive, simple and accurate, capable of determining amino acids at nanogram levels in urine samples.

2. Experimental

2.1. Materials

The amino acids: alanine, glycine, valine, leucine, isoleucine, proline, asparagine, methionine, phenylalanine, cysteine, lysine, glutamine, serine, threonine, arginine and tyrosine were obtained from Fluka Chemie (Switzerland). Standard solutions of each amino acid (2 mg/ml, except for tyrosine, 0.4 mg/ml) were prepared by weight weekly in double distilled water (DDW) and stored refrigerated. *n*-Pentadecane (internal standard) was obtained from Sigma, chloroform, toluene, hexane, *iso*-octane and pyridine were obtained from Sigma–Aldrich (Hellas) and dichloromethane from Merck (Darmstadt, Germany).

The derivatization reagent ethylchloroformate, was obtained from Fluka at high purity (98%). All chemicals and solvents were of analytical-reagent grade.

Early morning urine samples from two healthy volunteers were collected and processed immediately using the proposed method.

Screw-capped vials of 10 ml sealed with PTFE-lined silicon septa were used for storing the standard solutions. The vials were cleaned with AP-13 Extran alkaline soap (Merck) for 24 h rinsed consecutively with deionized water and AR acetone and baked at 110 °C overnight. Volumetric flasks were washed as described above but were air-dried. Extraction and injection were performed using an angled-cut needle tip (0.6 mm glass barrel i.d.; 0.11 mm needle i.d., HP part no. 9301-0511), a magnetic stir bar (1.0 cm × 0.4 cm), reaction vials of 5 ml, a magnetic stir plate and a syringe stand.

The ultrasonic cleaner was a Transsonic 420 (Elma-Singen/HtW) model.

2.2. Derivatization and extraction

Amino acids in 1 ml of aqueous or urine sample were derivatized with 400 μl of ethanol–pyridine (4:1, v/v) and 100 μl of ethylchloroformate. The reaction vial was ultrasonicated for 10 min following addition of 50 mg of sodium chloride and vigorous stirring for 2 min, until all the suspended air and CO_2 produced by the reaction is removed. For the single-drop microextraction a 10 μl Hamilton syringe containing 1.5 μl (plus 0.7 μl of the needle volume), of chloroform–toluene 3:1 was immersed in the stirred solution and fixed 0.4 cm below the meniscus of solution. The plunger was depressed to cause the solvent containing a fixed amount of internal standard to form a drop suspended at the edge of the microsyringe needle. After sampling-extracting for 5 min from a solution stirred at 200 rpm, the drop is drawn back into the syringe and immediately transferred into the GC injection port for analysis.

2.3. Gas chromatography–mass spectrometry

The GC–MS analyses were performed on a Shimadzu GC-17A gas chromatograph interfaced with a Shimadzu QP 5000 mass spectrometer. Samples were injected in the splitless mode. Chromatography was conducted on a fused silica capillary column (Supelco SPB-5, 30 m × 0.25 mm i.d. film thickness 0.25 μm). Helium was used as the carrier gas at a flow rate of 1 ml/min. The GC conditions were as follows: injector temperature, 250 °C; transfer line, 280 °C; initial oven temperature, 60 °C; then increased to 270 °C at 8 °C/min, with a final time of 8.75 min at 270 °C. The total time programme was 35 min. The mass-selective detector was operated in the electron impact (EI) mode at 70 eV and electron multiplier voltage of 1.25 kV. To confirm the mass fragments of the derivatives, data were obtained in the full scan mode in the scan range from *m*/*z* 50 to 400. Groups of selected ions, characteristic of each derivatized amino acid, were thereafter monitored at appropriate time intervals. Data were collected and integrated with a personal computer using the CLASS-5000 Version 1.24 Chromatography Software (Shimadzu Chem. Lab. Analysis System and Software).

The GC–FID system used was a Shimadzu GC-17A gas chromatograph equipped with a flame ionisation detector (FID) and a 30 m × 0.25 mm i.d. fused-silica capillary column (OV-5, film thickness 0.25 μm). Analyses were performed using the same set of chromatographic conditions, as above.

2.4. Method validation

To determine calibration curves, amino acid aqueous samples at various concentrations depending on the detection

system were prepared and analyzed accordingly using the above procedure. The calibration curves were obtained by plotting the peak height ratio between the derivatives of amino acids and that of *n*-pentadecane (I.S.). For measuring limits of quantification and detection, standards were serially diluted and processed according to the procedure detailed previously. Reproducibility was evaluated by analysing fortified urine samples containing two different concentrations of amino acids on the same day in five replicates (intra-day reproducibility) and for 5 consecutive days in duplicate (inter-day reproducibility). Two urine sub-samples were fortified with the individual amino acids at concentrations three and five times the limits of quantitation of the respective amino acids.

3. Results and discussion

All the amino acids selected except for arginine were successfully derivatized. The mass spectra of the derivatized amino acids are featured by a limited number of ion fragments in high abundance. The prominent ions of the spectra are attributed to the cleavage of bonds towards the detachment of ethyl-, ethoxy- and ethyl ester-group. The most significant ions along with the ions selected for quantitation are gathered in Table 1. Molecular or quasi-molecular ion peaks are not present in all spectrum derivatives. Once present, they are in low abundance. Details on mass spectrometric fragmentation and interpretation of this family of derivatives is reported by Huang et al. [34].

3.1. Selection of the reaction conditions

The literature contains a host of data about the derivatization conditions of amino acids with alkylchloroformates. In his early paper, Hušek, one of the pioneers in GC analysis of *N*-alkoxycarbonyl alkyl esters of amino acids, recommended

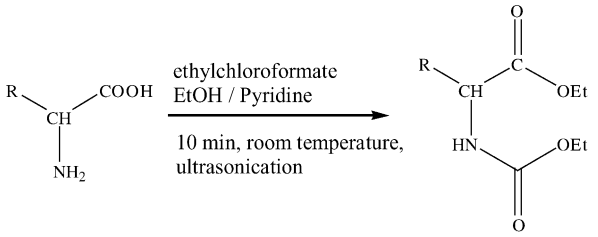

Fig. 1. Derivatization reaction for analyzing *N*(*O*,*S*)-alkoxycarbonyl alkyl esters of amino acids.

that derivatization be conducted at ambient temperature under the catalytic influence of pyridine [24]. In this work, an ultrasonic step is inserted to drive the reaction to completion at a considerably shorter period of time so as to increase the sensitivity of SDME method while improving, at the same time, the precision.

Ethylchloroformate with ethanol were examined for the derivatization of amino acids using Hušek's method in order to optimize the chromatographic conditions and acquire the fragmentation pattern of the modified amino acids. The reaction scheme of these reagents with amino acids is shown in Fig. 1. The derivatization comprises an exothermic reaction with the production of CO_2 and is considered to be rapid. Following the release of CO_2, one may falsely arrive at the conclusion that when air bubbles subside the reaction is complete. The reaction was performed by studying the relative recovery of the 12 amino acids using stirring at room temperature, at 70 °C and under mere ultrasonication. The visualized results in Fig. 2 reveal that the reaction at room temperature by stirring takes long time to complete and barely levels off after 80 min. In contrast, heating and ultrasonication exhibits a remarkable acceleration of the reaction with the latter being unequivocally better choice once the rapid completion of the reaction is aimed. Ultrasonication is able to reveal subtle interactions and particular effects of entropic and enthalpic origin. The cavitation and collapse of cavita-

Table 1
Mass fragment ions in EI spectra of *N*(*O*,*S*)-ethoxycarbonyl amino acid ethyl esters

Amino acid	Retention time (min)	Important ions (*m*/*z*)
Ala	10.54	**189**, 116(100), 88(5), 70(20), 174, 144, 102
Gly	10.68	**175**, 102(100), 74(30), 56(30), 175, 147, 130, 118, 84
Val	12.72	**217**, 144(100), 55(70), 174, 129, 116, 98
Leu	13.92	**231**, 158(100), 102(30), 231, 185, 174, 142, 129,112
Ser	13.95	**60** (100) 132(60), 74(40), 175, 86, 129, 101
Ile	14.20	**231**, 158(100), 102(30), 74(30), 231, 174, 129, 112
Thr	14.20	**219**, 129(100), 101(100), 74(95), 175, 146, 101, 83
Pro	14.57	**215**, 70(100), 142(70), 215, 170, 126, 114, 98, 82, 56
Asn	14.77	69 (100), 141(40), 214, 174, 113, 95, 80, 56
Met	18.00	**249**, 61(100), 175(30), 249, 157, 142, 129, 114, 101
Phe	19.78	**265**, 176(100), 91(80), 74(90), 220, 192, 148, 120
Cys	20.22	74(100), 220(80), 102(85), 204, 174, 146, 132, 114
Gln	22.41	84 (100), 173 (40), 200, 156, 128
Lys	24.15	156(100), 226(30), 272, 199, 171, 144, 128, 115, 102
Tyr	26.25	107(100), 192(30), 280, 264, 220, 207, 179, 120, 102

The ions in bold indicate the molecular ion peaks in the spectra. The underlined ions were used as target ions in SIM analyses. Values in parentheses represent the relative abundance.

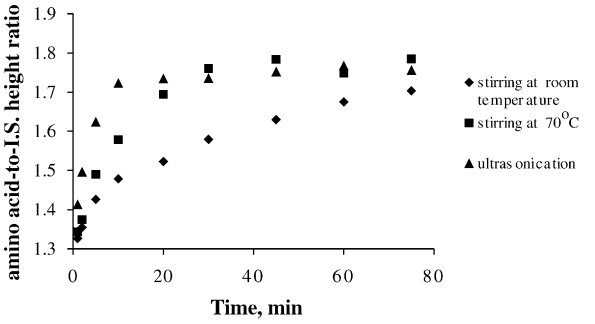

Fig. 2. Progress of the derivatization reaction under stirring, heating and ultrasonication. *Y*-axis refers to the sum of the peak heights for the 12 amino acids.

tion bubble, where transient hot spots with locally extremely harsh conditions occur, are generally accepted as the origin of the chemical effects [35,36]. Another beneficial effect of ultrasonication compared to the corresponding silent reaction is the efficient removal of bubbles from the bulk solution. The presence of bubbles is detrimental to the SDME process as they can attach to the drop, thus minimizing the surface available to extraction and facilitating its dislodgement.

In view of the above results, a 10 min ultrasonication was adopted prior to the 2 min of vigorous stirring. These conditions deliver the corresponding derivatives in increased yields up to 20–35%, depending on the amino acid itself.

Arginine is reportedly difficult to derivatize due to the guanidine-group in its molecule. Our derivatization method did not lead to any improvement on this matter. As for serine and threonine, both these hydroxyl amino acids were derivatized and extracted in the single drop. Because of the poor resolution of the pairs leucine–serine and isoleucine–threonine (see Table 1) in the non-polar phases (i.e. OV-5 and SPB-5) we decided to exclude from the subsequent optimization process the amino acids serine and threonine. It has been reported that the more polar phases are of higher resolving power for all them [24]. Glutamine is derivatized–extracted at low yield under ultrasonication, although in higher degree than silent conditions. As compared to the rest of amino acids tested, glutamine exhibited depressed chromatographic peak, so it was not involved in the proposed study.

3.2. Optimisation of the single-drop microextraction step

Method development was examined from a univariate optimization approach considering the equilibrium reaction conditions.

Previous studies on SDME showed serious advantage in adding the internal standard to the extraction drop rather than the aqueous solution [37]. Thus, *n*-pentadecane was added to the organic solvent mixture prior to extraction simplifying sample preparation. The optimization was studied with the extraction of all 12 amino acids and in all cases the peak heights and ratios of analyte height with the I.S. were calculated.

To characterize the performance of this SDME method, several experimental parameters were studied. Initially, the experiments for the selection of the extraction solvent were carried out. The organic solvent should compromise high extraction efficiency, low solubility in water and high surface tension so as the drop to be held easily at the tip of the microsyringe. When extraction time and other conditions are fixed, the recovery achieved by the solvents used showed the following order: chloroform (solubility in water: 0.795 g/100 ml) ≥ dichloromethane (1.32 g/100 ml) ≥ toluene (0.052 g/100 ml) ≥ ethyl acetate (8.0 g/100 ml) > *i*-butanol (9.5 g/100 ml) > *n*-hexane (0.000947 g/100 ml). Mixture of chloroform–toluene were also used to compensate for a high extraction efficiency and low solubility. Best recoveries for each of the investigated derivative substances were acquired by the use of chloroform–toluene at a ratio 3:1.

Further experiments were carried out to determine the optimum drop size of the organic solvent. The volume of the solvent drop and time of extraction are crucial for the optimum recoveries of the modified amino acids. Different drop volumes (0.5–3 μl) were studied in duplicate for an extraction period of 5 min. The amounts of analytes extracted by the organic drop have been found to be linearly proportional to the drop size at equilibrium [38]. Actually, proportional increase was obtained with the size of drop in the studied range, however, 1.5 μl forms even drops among the experiments and was used to study the performance of the method. Higher volumes cause difficulties to hold the drop on the tip of microsyringe during SDME. This effect is more pronounced in urine matrix where the solvent drop was proved more prone to detachment from the microsyringe.

The effect of extraction-sampling time was investigated by monitoring the variation of the total chromatographic peak area with time. The analytical signal increases up to 10 min; prolonged sampling brings about drop dissolution and non-satisfactory results. An extraction time of 5 min is long enough for improved precision, as extraction rate has considerably slowed after the passage of this period, at equilibrium reaction conditions.

Sample volume cannot increase indefinitely due to increase in drop solubility. On the other hand, sample volume should be compatible with the requirements of a limited availability of biological fluids. Therefore, a volume of 1 ml was adopted for the experiments.

The contribution of stirring to the extraction performance was studied by checking the amino acids recoveries in 1.5 μl drop of chloroform–toluene in the range 50–300 rpm. Stirring speed of 200 rpm ensured sufficient extraction yield with low risk of drop dislodgement. At 250 rpm, the drop is scarcely held at the tip of the syringe.

In many extraction methods, a high content of inorganic ions brings about various extraction efficiencies. Most frequently, the addition of salt can decrease the solubility of analytes in the aqueous sample and enhance the partitioning in the organic solvent (salting out effect). The derivatization reaction was performed under the established conditions

with the addition of sodium chloride up to 350 mg/ml. Salting out effect at 350 mg/ml which corresponds to the saturated quantity, increased the extraction of the derivatized amino acids even by 30%, depending on the amino acid derivative itself. However, the drop stability decreases during stirring, by virtue of the higher viscosity of the sample and the presence of granules of sodium chloride. A 20% improvement in peak height was feasible even with as low as 50 mg sodium chloride/ml without sacrificing sensitivity.

Finally, all the SDME experiments were performed at ambient temperature since high temperatures can cause fast dissolution of the organic drop.

3.3. Performance characteristics—real samples

The linearity of the chromatographic responses versus concentrations was studied. Linear responses were observed over the concentration range of 0.028–11.6 μg/ml for the amino acids with GC–FID (Table 2) and 0.70–2420 ng/ml with GC–MS(SIM) analyses (Table 3). The correlation coefficients for the calibration curves of the tested amino acids ranged from 0.9963 to 0.9991. The limits of detection ranged from 0.010 to 0.025 μg/ml for GC–FID and from 0.26 to 56.0 ng/ml for GC–MS(SIM). The feasibility of using this method for amino acid analysis in urine was then tested. The optimized extraction protocol was applied to the urine samples and their concentrations were calculated from aqueous extraction calibration lines. Accuracy was studied using spiked preparations at two different concentration levels; the first one three times the limits of quantitation and the other one five times these values for the respective amino acids. As SDME is a non-exhaustive extraction procedure, the recovery was determined as the ratio of the concentrations found in urine and distilled water samples under the optimum operating conditions. The recoveries obtained were within the range 92–101%. The intra-day and inter-day relative standard deviations (R.S.D.s) for the two afore-mentioned concentration levels in urine were 6.7–8.9% (five replicates) and 5.2–6.9% (five days), respectively. Compared with the R.S.D. of other methods published in the literature, the derived values from this method were found to be acceptable provided that the experimental conditions are consistently applied and the drop is formed evenly among the experiments.

Table 2
Analytical figures of merit of the method for the GC–FID analysis

Analyte	Calibration curve			
	R^a	DLR[b] (μg/ml)	LOD[c] (μg/ml)	LOQ[d] (μg/ml)
Ala	0.9984	0.041–11.6	0.014	0.041
Gly	0.9979	0.049–13.8	0.016	0.049
Val	0.9985	0.038–6.4	0.013	0.038
Leu	0.9968	0.055–6.4	0.018	0.055
Ile	0.9989	0.029–6.6	0.010	0.029
Pro	0.9991	0.048–5.2	0.016	0.048
Asn	0.9965	0.063–9.0	0.021	0.063
Met	0.9972	0.055–6.2	0.018	0.055
Phe	0.9988	0.028–7.4	0.010	0.028
Cys	0.9984	0.086–6.4	0.035	0.076
Lys	0.9986	0.053–19.3	0.018	0.053
Tyr	0.9966	0.051–6.8	0.017	0.051

[a] Correlation coefficient. Calculated from three replicates for each concentration level.
[b] DLR: dynamic linear range.
[c] Limit of detection: signal-to-noise ratio: 3.
[d] Limit of quantitation: signal-to-noise ratio: 10.

Table 3
Analytical figures of merit of the method for the GC–MS(SIM) analysis

Analyte	Calibration curve			
	R^a	DLR[b] (ng/ml)	LOD[c] (ng/ml)	LOQ[d] (ng/ml)
Ala	0.9991	0.80–111.6	0.26	0.80
Gly	0.9987	0.85–103.8	0.27	0.82
Val	0.9981	0.90–68.4	0.30	0.90
Leu	0.9978	0.90–62.4	0.30	0.90
Ile	0.9975	1.0–63.6	0.33	1.0
Pro	0.9982	0.90–55.2	0.30	0.90
Asn	0.9989	67–900	22.3	67
Met	0.9969	10.5–242	3.6	10.5
Phe	0.9993	0.70–74.4	0.23	0.70
Cys	0.9963	56–754	56.0	156
Lys	0.9976	55–1290	18.0	55
Tyr	0.9971	78–948	26.0	78.0

[a] Correlation coefficient. Calculated from three replicates for each concentration level.
[b] DLR: dynamic linear range.
[c] Limit of detection: signal-to-noise ratio: 3.
[d] Limit of quantitation: signal-to-noise ratio: 10.

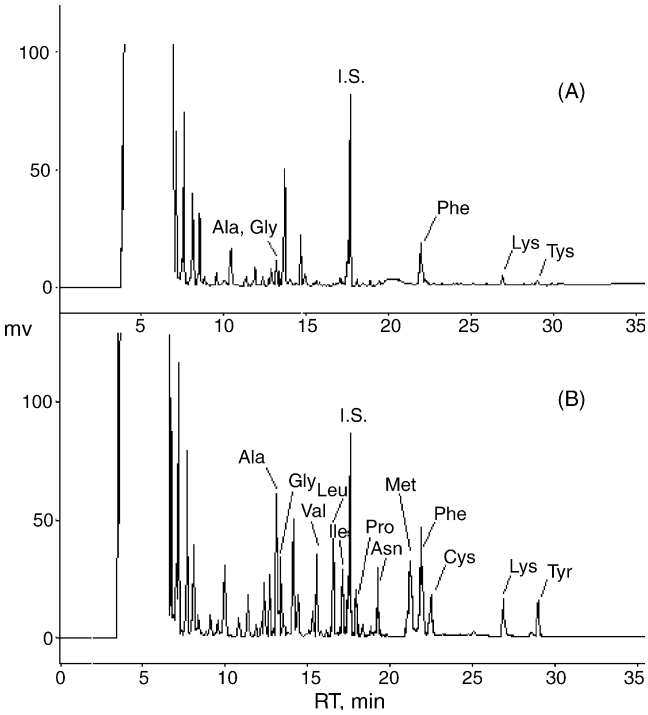

Fig. 3. GC–FID traces of pure urine (A) and fortified urine sample (B) from a volunteer after derivatization and extraction according to the proposed method.

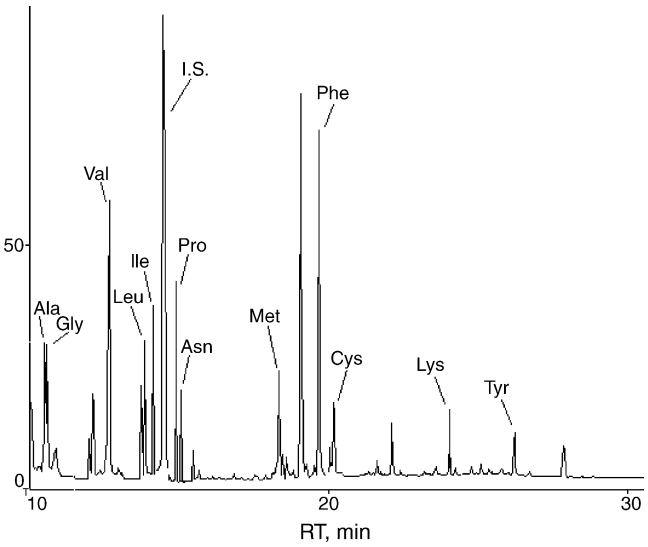

Fig. 4. GC–MS(SIM) trace of a fortified urine sample after derivatization and extraction according to the proposed method.

Typical chromatograms extracted from pure and spiked urine are shown in Figs. 3 and 4 and illustrate the ability of SDME to detect amino acids in conjunction with gas chromatography. Several extraneous peaks occurred in the chromatograms of urine extractions which could correspond to esters of fatty, dicarboxylic or short-chain hydroxycarboxylic acids. Although many of these peaks are large, no interferences to this study were observed since no peaks occurred at the retention times of interest.

4. Conclusion

A derivatization–extraction method which avoids tedious preconcentration steps is established in order to determine amino acids accurately at nanogram levels. The method proceeds through the conversion of the analytes of concern to *N*(*O*,*S*)-ethoxycarbonyl amino acid ethyl esters via an ultrasonic-assisted step and extraction by SDME before GC analysis. Precision is acceptable and 12 non-hydrolyzed amino acids can be determined in urine in this manner. Since the entire microdrop can be injected into the GC, any concentration steps required when using large amounts of organic solvent are eliminated. SDME is inexpensive as it requires common laboratory equipment, 1.5 μL of toxic organic solvent and it does not suffer from carryover between extractions. As long as the extraction conditions are consistently applied, quantitative analysis can be performed accurately. Other advantages of SDME include simplicity, speed and potential for ease automation.

References

[1] I. Matsumoto, S. Sakamoto, T. Kuhara, M. Sudo, M. Yoshino (Eds.), GC–MS Practical Chemical Diagnosis—Inherited Metabolic Disorders, Soft Science Publications, Tokyo, 1995.
[2] J.F. van Staden, T. McCormack, Anal. Chim. Acta 369 (1998) 163.
[3] H. Silvaieh, M.G. Schmid, O. Hofstetter, V. Schurig, G. Gübitz, J. Biochem. Biophys. Methods 53 (2002) 1.
[4] C.D. Stalikas, M.I. Karayannis, S. Tzouwara-Karayanni, Talanta 41 (1994) 1561.
[5] N. Kiba, M. Tachibana, K. Tani, T. Miwa, Anal. Chim. Acta 375 (1998) 65.
[6] D. Janasek, U. Spohn, Sens. Actuators B: Chem. 74 (2001) 163.
[7] I. Revelsky, Y. Yashin, T. Sobolevsky, A. Revelsky, B. Miller, V. Oriedo, Eur. J. Mass Spectrom. 9 (2003) 497.
[8] C. Rodier, R. Sternberg, F. Raulin, C. Vidal-Madjar, J. Chromatogr. A 915 (2001) 199.
[9] P. Dallakian, H. Budzikiewicz, J. Chromatogr. A 787 (1997) 195.
[10] I. Molnár-Perl, J. Chromatogr. A 987 (2003) 291.
[11] D. Fekkes, J. Chromatogr. B: Biomed. Appl. 682 (1996) 3.
[12] D.H. Spackman, W.H. Steine, S. Moore, Anal. Chem. 30 (1958) 1190.
[13] S. Oguri, K. Yokoi, Y. Motohase, J. Chromatogr. A 787 (1997) 253.
[14] Z. Shen, Z. Sun, L. Wu, K. Wu, S. Sun, Z. Huang, J. Chromatogr. A 979 (2002) 227.
[15] J.T. Smith, Electrophoresis 20 (1999) 3078.
[16] C.W. Gehrke, H. Nakamoto, R.W. Zumwalt, J. Chromatogr. 45 (1969) 24.
[17] K.-L. Woo, D.-S. Lee, J. Chromatogr. B 665 (1995) 15.
[18] M.W. Duncan, A. Poljak, Anal. Chem. 70 (1998) 890.
[19] H.J. Chaves das Neves, A.M.P. Vasconcelos, J. Chromatogr. 392 (1987) 249.
[20] P. Simek, A. Heydova, A. Jegorov, J. High Res. Chromatogr. 17 (1994) 145.
[21] I. Starke, E. Kleinpeter, B. Kamm, Fresenius' J. Anal. Chem. 371 (2001) 380.
[22] R. Zumwalt, D. Roach, C.W. Gehrke, J. Chromatogr. 53 (1970) 171.
[23] Z. Hell, A. Cwik, Z. Finta, Z. Horváth, J. Mol. Catal. A: Chem. 184 (2002) 191.
[24] P. Hušek, J. Chromatogr. 552 (1991) 289.
[25] P. Hušek, J. Chromatogr. B 717 (1998) 57.
[26] H. Kataoka, S. Matsumura, M. Makita, J. Pharm. Biomed. Anal. 14 (1997) 1271.
[27] A. Namera, M. Yashiki, M. Nishida, T. Kojima, J. Chromatogr. B 776 (2002) 49.
[28] H. Kataoka, S. Matsumura, H. Koizumi, M. Makita, J. Chromatogr. A 758 (1997) 167.
[29] C. Cheng, S.-C. Wu, J. Chromatogr. A 896 (2000) 299.
[30] P. Hušek, P. Šimek, LC–GC 19 (2) (2001) 986.
[31] H. Liu, P.K. Dasgupta, Anal. Chem. 68 (1996) 1817.
[32] M.A. Jeannot, F.F. Cantwell, Anal. Chem. 68 (1996) 2236.
[33] E. Psillakis, N. Kalogerakis, Trends Anal. Chem. 21 (2002) 54.
[34] Z.-H. Huang, J. Wang, D.A. Gage, J.T. Watson, C.C. Sweeley, P. Hušek, J. Chromatogr. 635 (1993) 271.
[35] A. Tuulmets, S. Salmar, H. Hagu, J. Phys. Chem. B. 107 (2003) 12891.
[36] M.A. Beckett, I. Hua, J. Phys. Chem. A 105 (2001) 3796.
[37] L.S. de Jager, A.R.J. Andrews, Analyst 125 (2000) 1943.
[38] L. Hou, H.-K. Lee, J. Chromatogr. A 976 (2002) 377.