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CAPILLARY GAS CHROMATOGRAPHY OF AMINO ACIDS, INCLUDING ASPARAGINE AND GLUTAMINE: SENSITIVE GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC AND SELECTED ION MONITORING GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETECTION OF THE N,O(S)-*tert.*-BUTYLDIMETHYLSILYL DERIVATIVES

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SUMMARY

Amino acids and the amino acid amides glutamine and asparagine can be simultaneously derivatized to the corresponding N,O(S)-*tert.*-butyldimethylsilyl derivatives in a one-step reaction with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide in acetonitrile. The solution is used directly for gas chromatography (GC). Losses due to evaporation steps are avoided. Except for the more basic amino acids, derivatization occurs at room temperature. Lysine, arginine and histidine require additional heating at 150°C for 2.5 h in order to complete derivatization. The derivatization has high reproducibility. The response factors relative to norvaline or cycloleucine lie between 0.40 and 1.30. Arginine is the most difficult amino acid to derivatize. The size of the *tert.*-butyldimethylsilyl (TBDMS) group prevents multiple silylation of the nitrogen atoms. Only a single peak is observed for each compound. Twenty-seven amino acid (and glutamine and asparagine) derivatives were simultaneously chromatographed and well separated in a single run on a 25 m × 0.20 mm I.D. glass capillary column coated with OV-1. The TBDMS derivatives possess very characteristic EI mass spectra at 70 eV, with intense diagnostic ions. This makes them very appropriate for GC–mass spectrometric (MS) work and selected ion monitoring GC–MS at the picomole level. The detection limit for arginine as the TBDMS derivative is less than 0.3 ng. The usefulness of the method is illustrated by the detection of amino acids in a peptide hydrolysate obtained from 1 μg of bovin insulin B-chain.

INTRODUCTION

Amino acids are fundamental constituents of living matter, involved in multiple metabolic functions, mainly as building blocks of proteins. Their free forms also

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participate in cell functions, as nutrients, neurotransmitters and biosynthetic percursors. The simultaneous analysis of free amino acids and dicarboxylic amino acid amides is of paramount importance in clinical chemistry and neurochemistry. The zwitterionic structure of amino acids prevents the application of direct gas chromatography (GC), and they must first be converted into volatile derivatives.

Derivatization of free amino acids for GC has received considerable attention. The most popular methods are based on multi-step procedures, involving esterification of the carboxyl group under strongly acidic conditions, followed by acylation of the remaining functional groups[1]. These methods have two major inconveniences that prevent the general application of GC to amino acid analysis, although the accuracy, speed of analysis, good resolution and sensitivity of modern capillary GC would offer substantial advantages over any other method routinely used. Some amino acids are difficult to derivatize reproducibly; histidine, for example, often requires additional acylation in order to complete derivatization[2,3]. On the other hand, the simultaneous analysis of amino acids and dicarboxylic amino acid amides, such as glutamine and asparagine, is not possible with the above-mentioned methods. Under the strongly acidic conditions used in the common esterification process, glutamine and asparagine are rapidly deaminated and converted into the same derivatives as the corresponding amino acids. Their independent determination still relies on ion-exchange procedures[4,5]. In order to overcome these difficulties, alternative methods have been proposed, based on mild esterification conditions[6], on the formation of 1,3-oxazolidinones under weakly basic conditions[7–9] or on the analytical control of the formation of the intermediate pyrrolidone carboxylic acid esters by enantiomer labelling[10].

The most attractive approach is offered by direct sylilation[11–19], but a serious drawback is the formation of multiple derivatives, owing to excessive silylation of the nitrogen atoms. It is clear that, if excessive silylation could be avoided, *e.g.* by the use of bulkier silyl groups, in order to remove the difficulty caused by multiple peak formation with some amino acids, the simplicity of the method, and also its suitability for the simultaneous determination of amino acids, glutamine, and asparagine, would be restored. Successful GC analysis of asparagine and glutamine and other proteic amino acids as the corresponding N(O)-dimethyl-*tert.*-butylsilyl derivatives has been reported[20]. However, the dimethyl-*tert.*-butylsilylation of protein amino acids gave multiple peaks in some cases[21]. Biermann *et al.*[22] could not resolve glutamate and arginine.

In this paper we report the use of N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), as a donor of the *tert.*-butyldimethylsilyl group (TBDMS), in a one-step derivatization procedure, and the simultaneous assay of amino acids, including asparagine and glutamine, by capillary GC. In addition, the TBDMS derivatives have very characteristic EI mass spectra, which make them very useful in sensitive GC–mass spectrometric (MS) work and for selected ion monitoring (SIM) GC–MS detection at the sub-nanomole level.

EXPERIMENTAL

Materials

Amino acids, glutamine and asparagine were obtained from Supelco (Bella-

fonte, PA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.) as chromatographic standards. Bovin insulin B-chain was a gift from Prof. E. Bayer. MTBSTFA was purchased from Pierce (Rockford, IL, U.S.A.). Acetonitrile (chromatographic grade) was obtained from Tokyo Kasei (Tokyo, Japan). The derivatization reactions were carried out in 1-ml Reacti-Vials (Pierce).

Instruments

GC was performed with a Carlo Erba Model HRGC 5300 instrument Mega Series, equipped with a splitter, a flame ionization detector and a 25 m × 0.20 mm I.D. laboratory-made borosilicate glass capillary, coated with OV-1. Hydrogen was used as the carried gas at 50 kPa. The injector temperature was 320°C and the detector temperature 350°C. The oven temperature was linearly programmed from 120 to 290°C at 3°C/min.

GC–MS and SIM GC–MS measurements were made with a Shimadzu Model QP-1000 instrument under the following conditions: ion source temperature, 200°C; interface temperature, 320°C; and multiplier, 2450 V. EI mass spectra (70 eV) were taken by continuously scanning the mass range m/z 50–700 every 1.5 s. The glass capillary was coupled directly to the ion source through a piece of 0.20 mm I.D. fused-silica tubing, connected to the capillary by means of a dead-volume butt connector (Supelco).

Quantitative measurements were made with a Shimadzu Model CR3-A computing integrator. Response factors were calculated relative to norvaline or cycloleucine.

Derivatization

A standard solution was prepared by dissolving approximately 5 mg of each amino acid, glutamine and asparagine in 25 ml of 0.1 *M* hydrochloric acid in a volumetric flask. Aliquots of 5–250 μl of the standard solution were transferred into 1-ml PTFE-lined screw-capped derivatization vials. The solvent was evaporated under a gentle stream of dry nitrogen and the residue was kept overnight over phosphorus pentoxide *in vacuo*. To the dry residue were added 50 μl of MTBSTFA, 50 μl of acetonitrile and 5 μl of ethanethiol. The mixture was sonicated for 30 min at room temperature, then heated at 150°C for 150 min. After cooling to room temperature, aliquots of the solution containing the derivatives were used directly for GC.

Peptide hydrolysis

Bovin insulin B-chain (100 μg) was weighed into a 1-ml derivatization vial and 500 μl of 6 *M* hydrochloric acid were added. The closed vial was heated at 110°C for 24 h and, after cooling to room temperature, the solvent was evaporated under a light stream of nitrogen. The residue was dried over phosphorus pentoxide *in vacuo* and the dry residue was derivatized as described by addition of 50 μl of MTBSTFA, 50 μl of acetonitrile and 5 μl of ethanethiol. After derivatization, 0.1–1-μl aliquots of the solution were used for SIM GC–MS.

RESULTS AND DISCUSSION

The use of pertrimethylsilylated amino acids in GC with packed columns has led to the successful separation of complex amino acid mixtures[23]. Trimethylsilylamines and imidazoles, being themselves efficient trimethylsilylating agents, easily transfer TMS groups to hydroxy groups present in the chromatographic system, including the silanol groups on glass surfaces. Owing to the much higher phase ratio of capillary columns, desilylation of this type of derivative may become more significant with insufficiently deactivated capillaries; therefore, it is not surprising that, in spite of its easy preparation, the TMS derivatives of amino acids have not been used extensively in capillary GC. The introduction of highly deactivated fused-silica columns, and the great progress achieved in modern column technology in producing highly inert capillaries[24], are important factors that justify a reconsideration of the importance of silylation in preparing amino acid derivatives for GC. The inconvenience of multiple derivative formation observed with some amino acids can be circumvented by the use of a bulkier silylating group.

In synthetic chemistry, the TBDMS group is finding increasing utility in the protection of hydroxylic groups[25]. The transformation of alcohols to the corresponding TBDMS ethers is easy. The stability of the ethers to solvolysis is approximately 10^4 times greater than that of the TMS analogues. MTBSTFA provides the same TBDMS group as the initial formulation of Corey and Venkateswarlu[25], but it is more effective in derivatizing alcohols, thiols and primary and secondary amines. The reactions are rapid and afford good yields of the derivatives[26].

Except for the more basic lysine, arginine and histidine, and also glutamine and asparagine, the amino acids are rapidly converted into their TBDMS derivatives when treated with MTBSTFA in acetonitrile (1:1) at room temperature. Complete derivatization of these compounds requires additional heating. Arginine is the most difficult amino acid to derivatize completely; therefore, it was used as a model compound for the determination of the optimal conditions for complete derivatization of all the amino acids. Samples containing cycloleucine as an internal standard were derivatized by heating at 150°C with MTBSTFA–acetonitrile (1:1). Samples were taken at 30-min intervals and the relative response factors were calculated. The maximal value of 0.40 for the arginine derivative was reached after 150 min. At 120°C, derivatization was still incomplete after 3 h. The addition of ethanethiol as an antioxidant to the derivatization mixture significantly improved the yield of the cysteine derivative.

Twenty-seven amino acid, glutamine and asparagine derivatives were well separated on a 25 m × 0.20 mm I.D. glass capillary coated with OV-1 (Fig. 1). Only a single peak is observable for each compound and extraneous peaks are negligible. The response factors relative to norvaline or cycloleucine are in the range from 1.30 for TBDMS-alanine to 0.40 for the arginine derivative, with good reproducibility (S.D. % = 1.5–2.0%). After 3 days at room temperature in a closed vial, no significant alterations were found.

The TBDMS derivatives of amino acids, asparagine and glutamine, have very typical EI mass spectra at 70 eV, dominated by diagnostic ions. All the spectra have a base peak m/z 73. A weak molecular ion or, more often, a weak $(M - 15)^+$ ion is present in most instances. In the mass spectra of the arginine and citruline deriv-

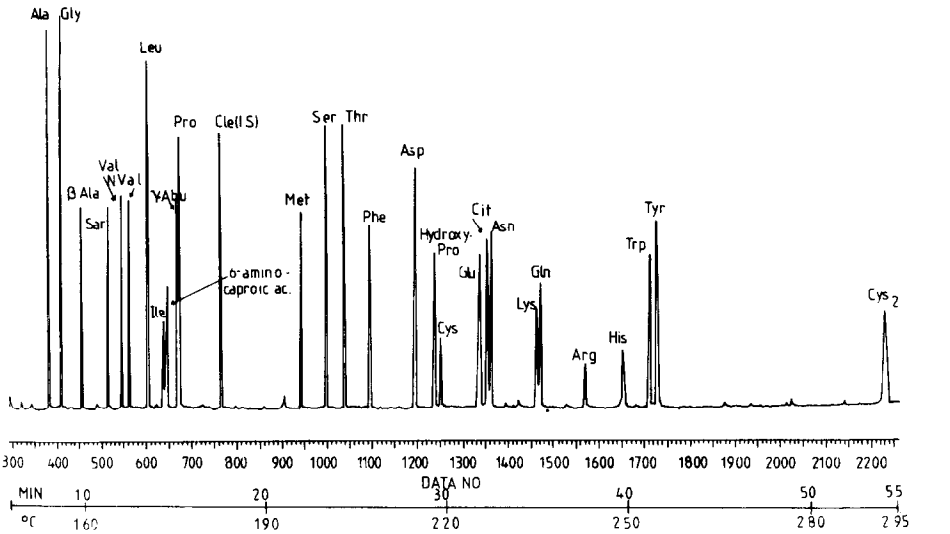

Fig. 1. TIC record obtained with a mixture of 50 ng each of standard amino acids as the corresponding TBDMS derivatives, with split injection (1:20). Conditions as in Experimental.

atives, $(M + 1)^+$ is sometimes observable, accompanied by the corresponding fragmentation ions. The most important characteristic of the spectra, however, is the presence of intense $(M - 57)^+$ and $(M - 159)^+$ ions. These ions originate directly from the molecular ion by loss of a *tert.*-butyl group and by cleavage at the α-carbon atom, respectively. The ion at *m/z* 159, corresponding to $(- CO_2TBDMS)^+$ is always present. Except in the case of special fragility towards electron impact, as with the citruline, arginine and aspartic and glutamic acid derivatives, for example, a fragment ion corresponding to the side-chain is present (Table I). Common to all the spectra are an ion at *m/z* 302, corresponding to $(M - \text{side-chain})^+$, and the ions resulting from this by loss of 57, 115, 131, 132, and 159 mass units (*m/z* 245, 187, 171, 172 and 143). The ions characteristic of the TBDMS group, *i.e.*, *m/z* 131 (TBDMSO), 132 (TBDMSOH) and 147 (CH_3O-TBDMS,H^+) are accompanied by other ions with a difference of 57 mass units. The ion at *m/z* 147 is generally very intense and seems to arise from rearrangement. Other diagnostic ions are represented by $(M - 43)^+$ and $(M - 85)^+$. The latter is frequently present in higher relative abundance than $(M - 57)^+$. Although we are not able, at present, to determine with certainty the origin of these ions, which are present in almost all the spectra, it seems reasonable to suggest their formation from $(M - 15)^+$ and $(M - 57)^+$ by rearrangement with loss of CO. Cystine is an exception to the regular fragmentation pattern of the TBDMS derivatives. In this instance, preferential cleavage takes place, as expected, at the S–S bond, giving an abundant *m/z* 348 ion. This is a very useful ion for the SIM of cystine. Although the arginine and citrulline derivatives give mass spectra, in which some of the usual fragments are present, the most characteristic fragmentation seems to be the loss of the TBDMS-guanido and TBDMS-ureido groups, via a McLafferty rearrangement, to afford an ion of *m/z* 343. This ion then loses the typical fragments. In the spectra of both derivatives, an ion of *m/z* 286

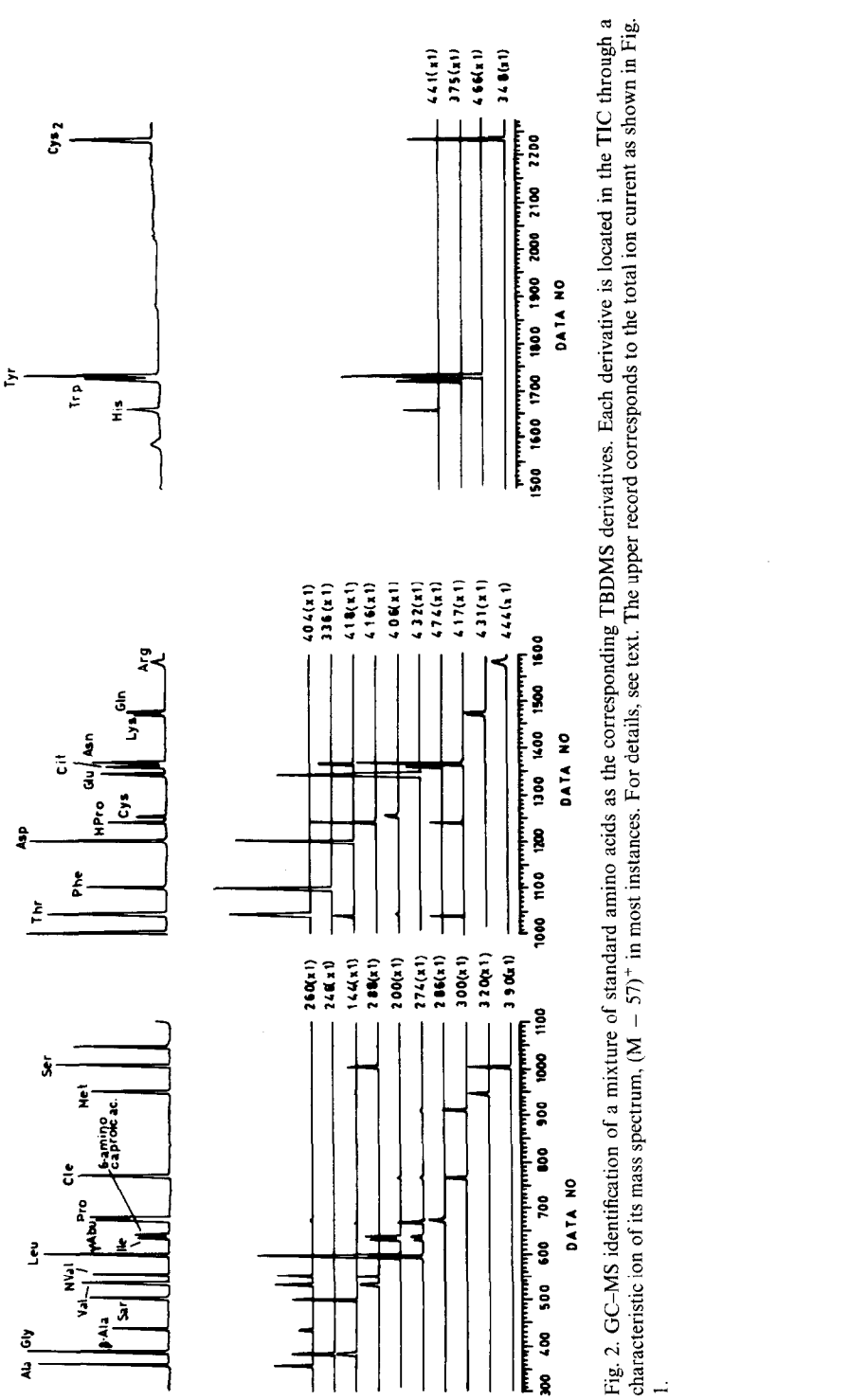

Fig. 2. GC–MS identification of a mixture of standard amino acids as the corresponding TBDMS derivatives. Each derivative is located in the TIC through a characteristic ion of its mass spectrum, $(M - 57)^+$ in most instances. For details, see text. The upper record corresponds to the total ion current as shown in Fig. 1.

TABLE I

DIAGNOSTIC IONS PRESENT IN THE EI MASS SPECTRA (70 eV) OF THE TBDMS DERIVATIVES OF AMINO ACIDS, GLUTAMINE AND ASPARAGINE, AS OBTAINED IN GC–MS EXPERIMENTS

Scan rate, *m/z* 50–700 in 1.5 s.

Compound	*m/z (relative intensity)**						
	M^+	$(M - 15)^+$	$(M - 43)^+$	$(M - 57)^+$	$(M - 85)^+$	$(M - 159)^+$	*Side-chain*
Ala	–	302 (2.0)	274 (1.7)	260 (25.7)	232 (45.0)	158 (68.5)	
Gly	–	288 (0.6)	260 (1.1)	246 (21.8)	218 (34.7)	144 (8.9)	
α-Ala	–	302 (1.4)	274 (0.7)	260 (15.8)	232 (32.2)	158 (32.6)	
Sar	–	302 (1.0)	–	260 (16.8)	–	158 (1.4)	
NVal	–	330 (0.4)	302 (5.8)	288 (10.8)	260 (19.9)	186 (57.6)	
Val	–	330 (2.1)	302 (1.2)	288 (14.5)	260 (35.8)	186 (73.1)	
Leu	–	344 (2.1)	–	302 (24.6)	274 (31.4)	200 (93.7)	57 (51.1)
Ile	–	–	316 (8.1)	302 (33.1)	274 (52.9)	200 (86.5)	57 (25.3)
6-Aminocaproic	–	–	–	302 (16.6)	–	200 (55.8)	57 (19.9)
GABA	–	316 (1.6)	–	274 (32.4)	–	–	
Pro	–	328 (0.4)	300 (2.3)	286 (15.7)	258 (29.6)	184 (100)	184
Cle	–	342 (0.3)	314 (0.4)	300 (10.0)	272 (18.3)	198 (55.6)	
Met	377 (0.4)	362 (0.7)	–	320 (17.2)	292 (33.0)	218 (33.2)	190 (2.4)
Ser	–	432 (1.5)	404 (0.8)	390 (10.4)	362 (15.0)	288 (24.3)	145 (1.0)
Thr	–	446 (7.0)	418 (10.8)	404 (22.0)	376 (10.0)	302 (27.0)	159 (12.6)
Phe	–	378 (4.7)	–	336 (23.3)	308 (35.0)	234 (32.4)	91 (26.2)
Asp	–	460 (8.8)	–	418 (21.3)	390 (13.0)	316 (11.3)	173 (1.0)
HPro	–	–	458 (2.2)	416 (37.1)	388 (42.0)	314 (85.4)	171 (1.6)
Cys	–	448 (2.6)	420 (1.5)	406 (40.4)	378 (42.0)	304 (43.5)	161 (0.2)
Glu	–	474 (1.5)	–	432 (41.2)	–	330 (23.6)	
Cit	–	–	474 (3.3)	460 (0.3)**	–	–	
Asn	–	459 (1.2)	–	417 (27.5)	–	315 (4.1)	172 (1.4)
Lys	488 (14.0)	–	–	431 (30.0)	–	329 (8.0)	186 (9.3)
Gln	488 (1.6)	473 (1.3)	–	431 (32.8)	–	329 (6.2)	186 (0.7)
Arg	–	501 (12.6)	–	460 (5.9)**	–	–	
His	–	483 (3.3)	–	441 (50)	–	339 (10.0)	196 (51.4)
Trp_2	–	–	–	375 (21.1)	347 (3.7)	273 (9.1)	130 (58.3)
Tyr	–	508 (2.3)	–	466 (25.4)	438 (8.5)	364 (4.3)	221 (6.3)
Cys_2***	–	–	–	639 (4.0)	611 (2.0)	537 (1.5)	

* Relative to *m/z* 73 = 100%.

** The principal fragmentation involves the loss of the ureido or guanido group via a McLafferty rearrangement to give *m/z* 343, from which the usual fragmentation pattern follows; *m/z* 286 (57.0%) is characteristic of these amino acids.

*** The most characteristic ion is, however, *m/z* 348 (see text).

(343 – 57) is present in high relative abundance (57.0%) and is, therefore, very characteristic.

The regularity of the pattern of the mass spectra of the TBDMS derivatives of amino acids is very useful in their GC–MS assay. Direct identification becomes easy in mass chromatography of the appropriate ions $(M - 57)^+$ (Fig. 2). Of course, other particularly significant ions may be chosen in order to confirm their identification, *e.g.*, *m/z* 444 (TBDMS-arginine). The presence of an ion of *m/z* 375 in the mass spectrum of tryptophan indicates that derivatization of the indolic nitrogen in

this amino acid has not taken place under the conditions used. The tryptophan derivative therefore elutes before the tyrosine derivative as a well formed peak.

When run in the SIM mode, the mass spectrometer represents the most versatile and selective detector and the sensitivity is much enhanced. These characteristics, together with the good mass spectrometric properties of the TBDMS derivatives, can be explored for their sensitive SIM GC–MS detection. The amino acids possessing the lowest response factors on derivatization, arginine, lysine and histidine, were used as model compounds for the SIM GC–MS measurements. SIM of M^+, $(M - 15)^+$, $(M - 57)^+$ and $(M - 159)^+$ was used for the specific detection of these amino acids as their TBDMS derivatives at the picomole level (Fig. 3). For arginine, *m/z* 444 ($M^+ - 15 - 57$) was introduced instead of $(M - 57)^+$, because its relative ion abundance is less subject to the influence of the amount of sample injected; however, it maintains all its diagnostic value. Amounts of as little as 2 ng of lysine, 1 ng of arginine and 4 ng of histidine can be reliably detected with a very high signal-to-noise ratio, meaning that the detection limit is considerably lower. In fact, the derivative corresponding to 0.3 ng of arginine can still be specifically detected (Fig. 3B).

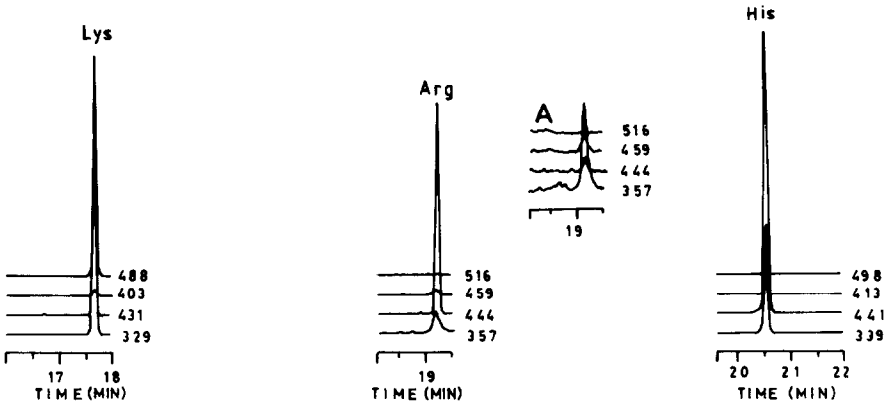

Fig. 3. SIM GS–MS records corresponding to 2 ng of lysine (*m/z* 488, 403, 431 and 329), 2 ng of arginine (*m/z* 516, 459, 444 and 357) and 4 ng of histidine (*m/z* 498, 413, 441 and 339), as the corresponding TBDMS derivatives. (A) SIM GC–MS corresponding to 0.3 ng of arginine injected.

SIM of the TBDMS-amino acids, allied to its suitability for quantitative work, is a very useful technique for the study of the amino acid composition of hydrolysates from peptides available only in minor amounts. As an example, the amino acid composition of a peptide hydrolysate corresponding to 1 μg of the B-chain of bovin insulin could be studied, after derivatization, by SIM GC–MS, under split injection conditions, using the 4×4 channels available in our instrument. The signal-to-noise ratio obtained is well above the detection limits (Fig. 4). If these aspects are considered, the inicial amount of peptide can still be significantly lowered.

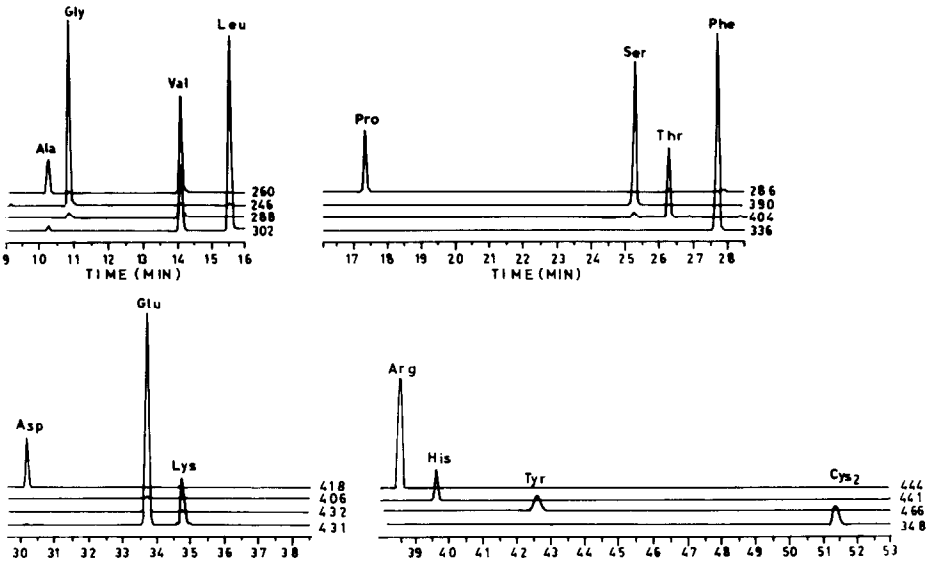

Fig. 4. SIM of the $(M - 57)^+$ ions of the TBDMS amino acids of an acid hydrolysate (6 *M* HCl, 24 h) of insulin B-chain, after derivatization. The amount injected corresponds to 1 μg of parent peptide.

CONCLUSIONS

The application of MTBSTFA to the derivatization of amino acids and dicarboxylic amino acid amides yields the corresponding TBDMS derivatives in good yields. With only one chromatographic peak obtained for each compound, a mixture of 27 amino acids, including asparagine and glutamine, could be separated as the corresponding TBDMS derivatives in a single chromatographic run; hence the simultaneous analysis of amino acids, glutamine and asparagine becomes possible. The characteristic mass fragmentation pattern of the derivatives makes them very useful for GC–MS work. Direct identification is easy. SIM of diagnostic ions is very sensitive and allows detection at the sub-nanomole level, even for the most difficult amino acids. Amounts of as little as 0.3 ng of arginine can be detected. The detection limit can be significantly lowered for less functionalized amino acids with a 4-fold relative response factor on derivatization.

The trace analysis of amino acids, glutamine and asparagine has great clinical and neurochemical significance. In natural product chemistry and biochemistry, the small amounts required for derivatization and detection make the procedure very useful in the amino acid assay of peptide hydrolysates that are available only in minor amounts.

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