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Comparison of silylation and esterification/acylation procedures in GC-MS analysis of amino acids

Three derivatization agents used in GC analysis of amino acids were compared: *N,O*-bis(trimethylsilyl)trifluoroacetamide, (BSTFA), *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), and isobutyl chloroformate (iBuCF). It was shown that the analytical characteristics achieved in the case of silylation with MTBSTFA are comparable to those obtained for esterification/acylation. However, since the former approach requires laborious sample preparation to isolate the compounds in question prior to derivatization, determination of amino acids as *N(O,S)*-alkoxycarbonyl alkyl esters seems to be preferable in many cases. Application of the esterification/acylation procedure to analysis of lyophilized *E. coli* microbial culture was demonstrated.

Key Words: Amino acids; Derivatization; Silylation; Alkylation; GC-MS

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1 Introduction

Identification and quantitation of amino acids in various matrices is a common task, since amino acids are fundamental units of any biological object ranging from bacteria to humans. Foods, beverages, and fruits are also characterized by their specific sets of amino acids. To detect these compounds, various techniques are used including gas chromatography and high-resolution liquid chromatography. Although gas chromatographic methods have some advantages over HPLC, derivatization of amino acids needs to be performed before analysis to produce volatile compounds. Usually, a silylation is carried out. Formerly, BSTFA [1] was the silylation method of choice; however, anhydrous conditions and continuous heating were required. Moreover, the silylation conditions reported by Gehrke and coworkers were sometimes contradictory. Then a new reagent, MTBSTFA, was proposed for silylation, whose derivatives were found to be less moisture-sensitive and required milder conditions to complete the derivatization. Numerous publications have appeared on the application of MTBSTFA to gas chromatographic amino acid analysis [2–6]. At the same time, when the determination of amino acids in aqueous media is necessary, alkoxycarbonyl alkyl esters [7–10] seem to be more attractive because of simple sample preparation and good analytical characteristics. Unlike silylation, in the case of alkoxycarbonyl alkyl esters it should be possible to derivatize amino acids while keeping sugars and some related compounds unaffected, thereby simplifying the resulting chromatogram. This is important when working with biological samples that contain large amounts of such compounds.

Among *N(O,S)*-alkoxycarbonyl alkyl esters of amino acids, isobutoxycarbonyl isobutyl esters can be considered as an optimal solution, regarding both the sensitivity achieved and the price of reagents required. In a recent paper [11], we reported the EI mass spectra of these amino acid derivatives. Since the NIST mass spectral library has no entries for these compounds, this information could be helpful for proper identification and quantitation of *N(O,S)*-isobutoxycarbonyl isobutyl esters of amino acids.

Consequently, this paper aims to compare some of the most widely used derivatization agents, BSTFA, and MTBSTFA, with iBuCF. The esterification/acylation procedure was applied to analysis of lyophilized *Escherichia coli* microbial culture for amino acids to demonstrate the benefits of this approach.

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2 Experimental

2.1 Chemicals

All the amino acids used in the study were obtained from Sigma (St. Louis, MO, USA). Isobutyl chloroformate (iBuCF) and pyridine (Py) were from Aldrich (Milwaukee, WI, USA). 2-Methyl-1-propanol, chloroform, and acetonitrile (ACN) were purchased from Lecbiopharm (Moscow, Russia). *N,O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) was supplied by Pierce (Rockford, IL 61105, USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Regis Technologies Inc. (Morton Grove, IL 60053, USA).

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2.2 Amino acid solutions and derivatization procedures

The derivatization of the amino acids using BSTFA and MTBSTFA was carried out as follows. Standard solutions of the amino acids used for silylation were prepared in 10 mL of 0.1- and 0.01 M HCl at concentration 10^{-7} g/μL each. 100 μL of each solution was evaporated to dryness in a gentle nitrogen stream at room temperature. The residual water was removed with methylene chloride. Derivatization of the dry residue was carried out using either BSTFA or MTBSTFA. In the first case, 100 μL of acetonitrile and 100 μL of BSTFA were added to the residue, and the solution obtained was heated for 4 h at 135°C on an oil bath (vials were immersed in the oil only to the level of the liquid inside the vial). In the second case, 100 μL of acetonitrile and 100 μL of MTBSTFA were added to the residue. After 30 s of sonication the mixture was heated at 70°C for 30 min. 1 μL of the reaction mixture was injected into the GC-MS in both cases.

A standard solution of the amino acids used for preparation of isobutoxycarbonyl derivatives was prepared in bidistilled water at a concentration of 10^{-7} g/μL each. 10 μL of the solution was mixed with 80 μL of bidistilled water, 30 μL of isobutanol, and 10 μL of Py, and 30 μL of iBuCF was added to the mixture; the mixture was sonicated for 30 s. After that the derivatives formed were extracted with 500 μL of chloroform by vigorous shaking followed by centrifugation of the solution. 1 μL of chloroform layer was injected into the GC-MS.

2.3 Sample preparation and derivatization of *Escherichia* lyophilizate

The lyophilized culture of *E. coli* was treated as follows: 500 μL of bidistilled water was added to 100 mg of the lyophilizate; the mixture obtained was sonicated for 5 min and then centrifuged at 7000 rpm. 500 μL of yellowish transparent solution above the undissolved residue was transferred to a vial containing 150 μL of isobutanol, 50 μL of pyridine, and 150 μL of isobutyl chloroformate was added, and then the mixture was sonicated for 30 s. After that the derivatives formed were extracted with 500 μL of chloroform by vigorous shaking followed by centrifugation of the solution. 1 μL of chloroform layer was injected into the GC-MS.

2.4 GC-MS

GC-MS analysis was carried out on a "TRACE 2000" gas chromatograph (ThermoQuest, CE Instruments, Italy) connected to an "Automass Multi" quadrupole mass spectrometer (ThermoQuest, Finnigan, France).

The trimethylsilyl/*tert*-butyldimethylsilyl derivatives (TMS/TBDMS) of the amino acids were separated on a RTX-5MS fused-silica capillary column (30 m × 0.32 mm × 0.25 μm), while the isobutoxycarbonyl ones were separated on Chrompack CP-Sil 24CB (30 m × 0.32 mm × 0.50 μm). Helium was used as a carrier gas at a flow rate of 1.5 mL/min. Split/splitless injection was used (splitless time 30 s, then split 20:1). The injector temperature was 280°C. The column temperature was programmed for the isobutoxycarbonyl derivatives separation as follows: isothermal 50°C for 7 min, then heating up to 300°C at rate of 10 K/min, and hold at final temperature 300°C until the elution of the last component; for TMS/TBDMS derivatives separation: isothermal 50°C for 7 min, then heating up to 280°C at rate of 5 K/min, and hold at the final temperature 280°C until the elution of the last component.

EI mass spectra were scanned in the range of 90–750 amu in case of TMS/TBDMS derivatives of the amino acids and 90–400 amu in case of the isobutoxycarbonyl derivatives. Ion source temperature was 200°C, the transfer line temperature 280°C.

Upon obtaining the chromatograms, the amino acids derivatives were identified using the NIST library search whenever possible.

3 Results and discussion

The literature contains ambiguous data about the conditions of trimethylsilylation of amino acids (135 or 150°C, derivatization time of 15 min, 2.5 h, or 4 h) using BSTFA. In his early paper Gehrke, one of the pioneers in gas chromatographic analysis of amino acids, recommended that the derivatization be conducted at 135°C and sample removal performed after 15 min, since most of the amino acids have already been converted. GC analysis of a second sample of the reaction mixture after 4 h of heating was then recommended to obtain the derivatives of arginine, lysine, histidine, tryptophan, and cystine as the derivatization of these amino acids was the most difficult. In subsequent papers derivatization at 150°C for 2.5 h was considered optimal. Thus, it is clear that such variation in the data means the absence of certain, strict conditions necessary for the reliable derivatization. We used heating at 135°C for 4 h and succeeded in obtaining the derivatives of 14 amino acids out of 17 (exceptions were arginine, asparagine, and histidine). **Table 1** lists the derivatized amino acids and their retention times. They were prepared by derivatization of a solution of the amino acids in 0.1 M HCl after evaporation.

The detection limits estimated for the TMS derivatives in the total ion current mode were ca. 10^{-9} g/μL.

It is known that the trimethylsilyl derivatives of amino acids are very sensitive to moisture, and great care should be taken of to remove water traces at every step of sample preparation. *tert*-Butyldimethylsilyl derivatives are known to be more stable. Having carried out the respective deri-

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