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Note

Gas–liquid chromatography of the heptafluorobutyryl-O-isobutyl esters of amino acids

PETER FELKER

Department of Plant Sciences, University of California, Riverside, Calif. 92521 (U.S.A.)

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Gas–liquid chromatography (GLC) studies of amino acids are constantly being refined by excellent studies of various workers, including MacKenzie and Tenaschuk[1,2] who introduced the more suitable isobutyl ester and faster esterification procedures; March[3] who introduced better acylation conditions, and Pearce[4] who introduced the use of a SCOT column to increase resolution. Nevertheless numerous minor difficulties continuously arise, for which solutions must be sought and it is the intent of this communication to report modifications and solutions to various minor problems. In particular the effects of evacuation and ethyl acetate peroxide contamination on acylation conditions, simple tests for reagent quality, and the use of inexpensive sample reaction vials are reported here.

MATERIALS AND METHODS

The S-carboxyethylcysteine was from Tridom/Fluka (New York, N.Y., U.S.A.) the ethyl acetate (pesticide quality) from Matheson, Coleman & Bell (E. Rutherford, N.J., U.S.A.), the 2,6-di-*tert*.-butyl-4-methylphenol (BHT) from Aldrich (Milwaukee, Wisc., U.S.A.), the heptafluorobutyric anhydride from Pierce (Rockford, Ill., U.S.A.) and the analytical-reagent grade isobutyl alcohol from Mallinckrodt (St. Louis, Mo., U.S.A.). The S-carboxyethylcysteine, BHT and heptafluorobutyric anhydride were used without further purification, but the heptafluorobutyric anhydride was dispensed and sealed in glass ampules (0.5 ml) under nitrogen. The ethyl acetate was suitable for acylation upon arrival, but as ethyl acetate accumulates peroxides it was stored in a dark bottle under nitrogen over Linde 4A molecular sieves. Isobutyl alcohol was purified as previously described[1]. The equal weight amino acid stocks (1 μg per 10 μl) were prepared in 0.1 *N* HCl and kept at 4° except for the amino acids methionine, tryptophan, S-carboxyethylcysteine and norleucine which were dissolved in glass distilled water (1 μg/μl) and stored at 4°.

The peroxide test in ethyl acetate used here involves dissolving several potassium iodide crystals in approximately 1 ml of water in a test tube, followed by addition and mixing of several ml of ethyl acetate. If peroxides are present colorless iodide will be oxidized to yellowish-orange iodine in several min. More sensitive and quantitative tests for peroxides are reported elsewhere[5].

The GLC peaks were integrated with a Spectra-Physics Autolab I Integrator

using the following parameters: PW, 5; SS, 85; BL, 2; Tl, 375; T4, 2100; MA, 750; and, PL 10; the 5.2 m × 2 mm I.D. glass column of 3% SP2100 on 100–120 mesh Supelcoport was operated at 20 ml/min (35 p.s.i. at 90°) on a 6°/min program from 90° to 270° with a 2-min hold. The S-carboxyethylcysteine and tryptophan occurred as a doublet with a retention time approximately 2.3 min later than arginine with the S-carboxyethylcysteine eluting first.

The derivatization procedure employed here used the 20 min/120° esterification[2] with isobutanol–acetyl chloride prepared as previously described[6] and a 10 min/150° acylation[3] with 50 μl of ethyl acetate containing 0.03% BHT and 50 μl of heptafluorobutyric anhydride. Both the esterification and acylation were evacuated as previously reported[6]. Prior to esterification, but after evacuation, the samples were placed in a sonic oscillator bath for approximately 15–30 sec.

RESULTS AND DISCUSSION

In a comparison of previous work on esterification and acylation conditions necessary for GLC of amino acids as their perfluorobutyryl-isobutyl esters, Pearce[4] found that preparation of acetyl chloride–isobutanol according to Felker and Bandurski[6] followed by the 20 min/120° esterification proposed by MacKenzie and Tenaschuk[2] yielded satisfactory results. Pearce[4] did have difficulty in repeating earlier work[2,3,6] on the acylation of these amino acids. Sudden unexpected failures in this laboratory to successfully chromatograph methionine led to the testing of the ethyl acetate for peroxides with the potassium iodide test, and to the conclusion that the ethyl acetate had become contaminated with peroxides. It has been previously reported that alcohols, esters, aldehydes and ethers may accumulate peroxides[5]. Upon substituting peroxide-free ethyl acetate (from the original seldom opened amber bottle) for peroxide-contaminated ethyl acetate it was found, as shown in Table I, that the methionine relative-weight-response (RWR) coefficient went from 0.18 to 0.81. In work for a previous paper[6] it was observed that flushing with nitrogen and evacuating the acylation vessel markedly improved methionine, S-carboxyethylcysteine, arginine and tryptophan recoveries. At that time peroxide contamination in ethyl acetate was not suspected or tested for. In Table I it can be seen that evacuation of peroxide-contaminated ethyl acetate increased recoveries of methionine, S-carboxyethylcysteine and tryptophan remarkably. From Table I it appears that evacuation of the acylation mixture containing peroxide-free ethyl acetate does not greatly affect methionine, S-carboxyethylcysteine, tryptophan, and arginine RWR coefficients. If the optimum relative-molar-response (RMR) values obtained by Pearce[4] are recalculated as RWR coeficients one obtains 0.79 and 0.72 for methionine and arginine respectively. The values reported here in which the acylation is carried out at 150° are 0.77 and 0.83 for methionine and arginine when peroxide-free ethyl acetate is used as solvent and when the acylation mixture is evacuated as previously described[6]. It is possible that reduction of peroxide levels below that of more sensitive peroxide tests[5] may further increase tryptophan and S-carboxyethylcysteine RWR coefficients.

As previously stressed[6], the RWR coefficients have more meaning in a GLC context than do RMR coefficients due to the equal-per-carbon-atom response of flame ionization detectors (FIDs)[7]. For example, one would expect octane to have nearly double the response of an equimolar amount of butane. When the GLC amino

TABLE I

EFFECTS OF PEROXIDE-CONTAMINATED ETHYL ACETATE AND EVACUATION PRIOR TO ACYLATION ON AMINO ACID RWR COEFFICIENTS

Amino acids	*Evacuate for acylation*		*Peroxide-contaminated*		*Peroxide-free*	
	Peroxide-free	*Peroxide-contaminated*	*Evacuation for acylation*	*No evacuation for acylation*	*Evacuation for acylation*	*No evacuation for acylation*
Ala	0.84	0.85	0.89	0.88	0.93	0.94
Gly	0.92	0.88	0.92	0.98	0.96	1.00
Val	0.78	0.78	0.83	0.80	0.92	0.93
Thr	1.01	1.02	1.05	1.04	0.99	1.02
Ser	1.04	1.12	1.06	1.05	0.96	1.04
Leu	0.94	0.97	0.99	0.96	0.98	1.05
Ile	0.90	0.95	0.98	0.87	0.96	1.00
Nle	1.00	1.00	1.00	1.00	1.00	1.00
Pro	1.06	1.08	1.03	1.11	1.02	1.03
HyPro	1.03	1.09	1.04	1.03	1.00	1.02
Met	0.81	0.18	0.60	0.00	0.77	0.74
Asp	1.15	1.17	1.14	1.12	1.08	1.08
Phe	1.09	1.11	1.04	1.10	1.08	1.07
Glu	1.12	1.11	1.10	1.14	1.08	1.09
Lys	0.89	0.91	0.82	0.97	0.75	0.70
Tyr	1.08	1.08	1.14	1.03	1.15	1.22
Arg	0.73	0.67	0.58	0.47	0.83	0.81
CysR	0.79	0.55	0.67	0.32	0.62	0.47
Trp	0.57	0.33	0.50	0.38	0.79	0.71

acid responses are expressed as RWR values it becomes possible to assess qualitatively the degree to which amino acid derivatizations go to completion since all RWR's should be approximately equal to one. The fact that the value for methionine is low can be partially attributed to the sulfur present, which is not detected by the GLC–FID. The fact that aspartic and glutamic acid have RWR's greater than one, can be attributed to an additional esterified alcohol.

It will not be possible to assess quantitatively the degree to which amino acid derivatizations go to completion with the use of RWR coefficients until the "effective carbon numbers[7]" of the side chains are determined. Nevertheless, unlike the RMR coefficients, RWR coefficients provide a theoretical basis for comparison of amino acid derivatization.

It is this author's experience that the tryptophan acylation procedure of March[3], which employs a mixture of perfluorobutyric anhydride–ethyl acetate (50:50) (with 0.03% BHT) at 150° for 12 min, yields only one tryptophan peak whereas earlier procedures[2,6] yielded 2 peaks consisting of mono- and diacylated tryptophan[6]. March's[3] highest tryptophan RMR of 0.94 corresponds to an RWR of 0.60, which compares to a value of 0.71 to 0.79 reported here for the peroxide-free evacuated acylation mixture. The 0.71–0.79 tryptophan RWR coefficients reported here were atypically high in this particular experiment and were more typically around 0.60. Because of the low percent nitrogen in tryptophan, and the aromatic ring, this author would expect a tryptophan RWR coefficient greater than one, as is the case with phenylalanine and tyrosine. The fact that the RWR is approximately $^1/_2$ leads one to suspect that considerable degradation is taking place. As it would be unreasonable to expect a degradation coefficient to remain constant with varying degrees of amino acid purification prior to derivatization, the utility of a tryptophan RWR

coefficient derived from standards is dubious. An attempt to determine tryptophan in lysozyme using the dithioethane–tin protectant[5], without the isotope dilution method[8], yielded approximately 40% of the theoretical value. Difficulties may also arise in tryptophan determination by traditional amino acid analysis on divinylbenzene-sulfonic acid resins, since these resins catalyze destruction of the indole-containing plant hormone indole-3-acetic acid when present at quantities less than 20 nmoles[9].

Not only has the ethyl acetate been found to be contaminated, but a lot of 1-ml ampules of perfluorobutyric anhydride were found to be unsatisfactory by causing an unknown peak to occur between glycine and valine, and by reducing the threonine and tyrosine peaks to 10% of their normal value. The unusual behavior was correlated with a yellow discoloration obtained upon heating only the ethyl acetate and perfluorobutyric anhydride for 5 min at 150°. A replacement shipment of perfluorobutyric anhydride eliminated the unknown GLC peaks, yielded good threonine and tyrosine peaks, and did not discolor upon heating at 150° with ethyl acetate.

Increasingly poor resolution of lysine–tyrosine as well as severe tailing of glycine sometimes has been found to occur after as few as eight injections, as Pearce[4] also reported. Repacking the top several millimeters of column-packing and replacing the glass wool will restore the lysine–tyrosine resolution and severely reduce glycine tailing. Even better resolution could probably be achieved if column-packings were sieved prior to column packing since over 25% of 3% SP2100, 100–120 Supelcoport Lot E9979 packing passed a 120-mesh sieve. Occasionally it is necessary to bake even the most expensive septums (HT-9 septums, Applied Science Labs., Lot 7) to avoid unwanted GLC peaks despite claims by the manufacturers to the contrary.

Somewhat fortuitously it has been found that the Varian GLC-auto sampler 2-ml screw-cap vials with PFTE-faced, silicone-backed septa (96-000099-00) are quite inexpensive and admirably derivatize 100-μl acylation samples under vacuum at 150° for 10 min. A vacuum is most conveniently admitted into the auto sampler vials with the previously described vacuum apparatus, but with the use of non-coring hypo needles (Pierce) which become plugged less frequently.

GLC of amino acids other than cysteine, histidine and tryptophan can be performed straightforward. The only reported GLC method for determination of cysteine, histidine and tryptophan in proteins[8] involves a complicated isotope dilution method and this places GLC of amino acids at a serious disadvantage to new high-pressure amino acid techniques. To this investigator the hydrolysate preparation of Inglis *et al.*[10], followed by ion-exchange amino acid analysis with equipment available for $ 10,000 (Durrum, Sunnyvale, Calif., U.S.A.), capable of determining all amino acids in 90 min, looks especially attractive.

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