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DETERMINATION OF ACETYL AND FORMYL GROUPS AS PENTAFLUOROBENZYL ESTERS BY MEANS OF GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION*

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SUMMARY

The determination of formate, acetate and propionate as their pentafluorobenzyl esters by glass capillary gas–liquid chromatography has been studied. The separation of pentafluorobenzyl esters of acids is obtained with columns coated with PPSeb stationary phase. Quantitative results are given with samples containing *ca.* 5 nmol of acetyl and formyl groups.

INTRODUCTION

Interest in methods for identification and quantitative determination of acetyl groups in proteins and peptides has grown rapidly since Narita[1] isolated and characterised the N^{α}-acetyl peptide of tobacco mosaic virus protein. Since then many proteins have been found to be N^{α}-acetylated, and a compilation of the sequences of these proteins has recently been published[2]. The presence of N^{α}-formyl groups has also been well established, for instance, N^{α}-formyl methionine in protein chain initiation on the ribosome, N^{α}-formyl valine in Gramicidin A (ref. 3) and N^{α}-formyl glycine in melittin[4].

Acetate has been determined by variety of methods as previously discussed[5,6]. In the previous report, bound acetate and formate were determined as phenacyl esters; the technique required a minimum of 20 nmol for successful derivatisation due to the limited sensitity of the flame ionisation detector (FID)[6]. The availability of the electron-capture detector enabled higher sensitivity to be achieved. Pentafluorobenzyl (PFB) esters of carboxylic acids are known to exhibit high sensitivity to the electron-capture detector and several methods have been proposed to convert carboxylic acids into their respective PFB derivatives[7–16].

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A sensitive method was desirable because of the small amount of formyl or acetyl group relative to the weight of the protein. Many proteins being studied are only available in small amounts in a purified form. A method is reported here for the direct determination of acetyl and formyl groups attached to amino acids and proteins. After alkaline hydrolysis of the sample, the sodium salts of acetate and formate are converted into PFB esters prepared by means of crown ether catalysis and determined by gas–liquid chromatography (GLC) with electron-capture detection (ECD).

EXPERIMENTAL

Materials

α-Bromo-2,3,4,5,6-pentafluorotoluene was from Aldrich (Milwaukee, WI, U.S.A.), 15-crown-5 from Fluka (Buchs, Switzerland), acetonitrile (redistilled from P_2O_5), toluene, sodium formate, sodium acetate, propionic acid, *n*-butyric acid, isovaleric acid, nitrobenzene, sodium hydroxide and sodium bicarbonate from BDH (Poole, Great Britain). Other chemicals were obtained as previously reported[6].

Gas chromatography

Gas chromatography (GC) was carried out with the following gas chromatographs: a modified D6 gas chromatograph with gas density balance (GDB) detector as previously reported[17]; Pye series 104, Model 24, fitted with dual flame ionisation detector and Pye Unicam GCD fitted with a 10 mCi ^{63}Ni electron-capture detector. Integration of the peak areas was carried out with Kent Chromalog 2 and Vidar Autolab 6300 digital integrators. The glass capillary column (30 m × 0.27 mm I.D.) was coated with 5% Chromosorb R and 5% PPSeb by a single-step coating method[18]. Direct injection onto the capillary column with sample volumes up to 2.0 μl were made without inlet heater[19].

Hydrolysis of samples and preparation of esters

About 5 nmol of N-acetylated compounds were hydrolysed with 20 μl of 1 *M* sodium hydroxide in 0.5 ml polypropylene microcapped centrifuge tubes in an autoclave at 15 p.s.i. for 3 h. After the hydrolysis, the known amount of internal standards (*n*-butyric acid and isovaleric acid) were added followed by 20 μl of 1 *M* HBr and 2 μl of 1 *M* $NaHCO_3$. Aliquots (5 μl) were transferred to silanised glass tubes (5 cm × 0.3 mm I.D.) and taken to dryness. PFB bromide (50 nmol) and 15-crown-5 (50 nmol) in acetonitrile solution (10 μl) were added. The tube was sealed and incubated at 80°C for 2 h with occasional shaking. A 1-μl aliquot was added to 2 ml of toluene and 1 μl was injected onto the GLC column.

RESULTS AND DISCUSSION

It was necessary to use a mixed stationary phase for the separation of phenacyl esters[6], and mixed stationary phases are always difficult to reproduce. Quantitation of phenacyl formate necessitates a perfectly silylated column. The technique required a minimum of 20–25 nmol for successful derivatisation due to the limited sensitivity of the flame ionisation detector. The availability of ECD enabled higher sensitivity to be achieved, and therefore PFB esters were prepared.

The separation of PFB esters was achieved with PPSeb stationary phase (4%, w/w) coated on 80–100 mesh Chromosorb W-HP, at 110°C and was comparable with that obtained with the phenacyl esters. Fig. 1a–c shows the separation of PFB esters with GDB detection, FID and ECD. In order to determine the yields of esters, standard solutions of PFB formate, acetate and propionate were prepared and run with the internal standards nitrobenzene and *n*-dodecane (C_{12}) on a 4% PPSeb column with a GDB detector. This enabled quantitative yields to be determined in absolute amounts (micrograms). The yields of PFB esters prepared from sodium salts of acids and determined by the GDB detector are given in Table I. Quantitative recoveries for three PFB esters were obtained. The recovery of nitrobenzene against C_{12} as determined by the GDB detector was 98 ± 3.2% (n = 8). The same solution (as for GDB) was diluted and gas chromatographed using the other detectors, from which their relative molar response (RMR) values with FID and ECD were determined (Table II). The separation of PFB esters on PPSeb SCOT column is shown in Fig. 2. A 10-fold increase in amount injected gave the same peak height compared with a packed column, coupled with shorter run time and improved resolution.

PFB esters were formed by using the potassium salts and dicyclohexyl 18-crown-6. Expected recoveries were obtained with a minimum of 25 nmol of starting materials. However, much higher values for formate and acetate were obtained with smaller amounts (in the region of 5 nmol). These higher values were attributed to putative impurities present in KOH. With sodium salts of acids reasonably good recoveries were obtained by using $NaHCO_3$ as a base and 15-crown-5 catalyst (the use of sodium hydroxide and Na_2CO_3 was not satisfactory). Davis[15] derivatised carboxylic acids and phenols with PFB bromide and crown ether catalysis, and has shown that the stronger bases K_2CO_3 and KOH gave good yields of both acids and phenols, whereas the weaker bases $KHCO_3$, CH_3COOK and KCN were suitable for the derivatisation of carboxylic acids, but not phenols. Chan[16] prepared phenacyl valproate by using the sodium salts of valproic acid and dicyclohexyl 18-crown-6. In this investigation, when sodium salts of acids and dicyclohexyl 18-crown-6 were used, no PFB esters were formed. For the sodium salts it was necessary to use 15-crown-5.

It was previously shown for the phenacyl esters[6] that the best results were obtained with phenacyl bromide and dicyclohexyl 18-crown-6 (10:1; mol/mol) in benzene solvent at 80°C for 30 min, but with small amounts much higher concentrations of 15-crown-5 were required. Similarly, for the derivatisation of carboxylic acids (less than 1 μg/ml) higher concentrations of PFB bromide and crown ether were

TABLE I

RECOVERY OF PENTAFLUOROBENZYL ESTERS DETERMINED BY GAS DENSITY BALANCE DETECTOR

Preparation of sample and GLC conditions as in Fig. 1.

Compound	*% Recovery ± S.D. (n = 12)*
PFB Formate	97.0 ± 3.68
PFB Acetate	99.6 ± 1.96
PFB Propionate	98.8 ± 1.50

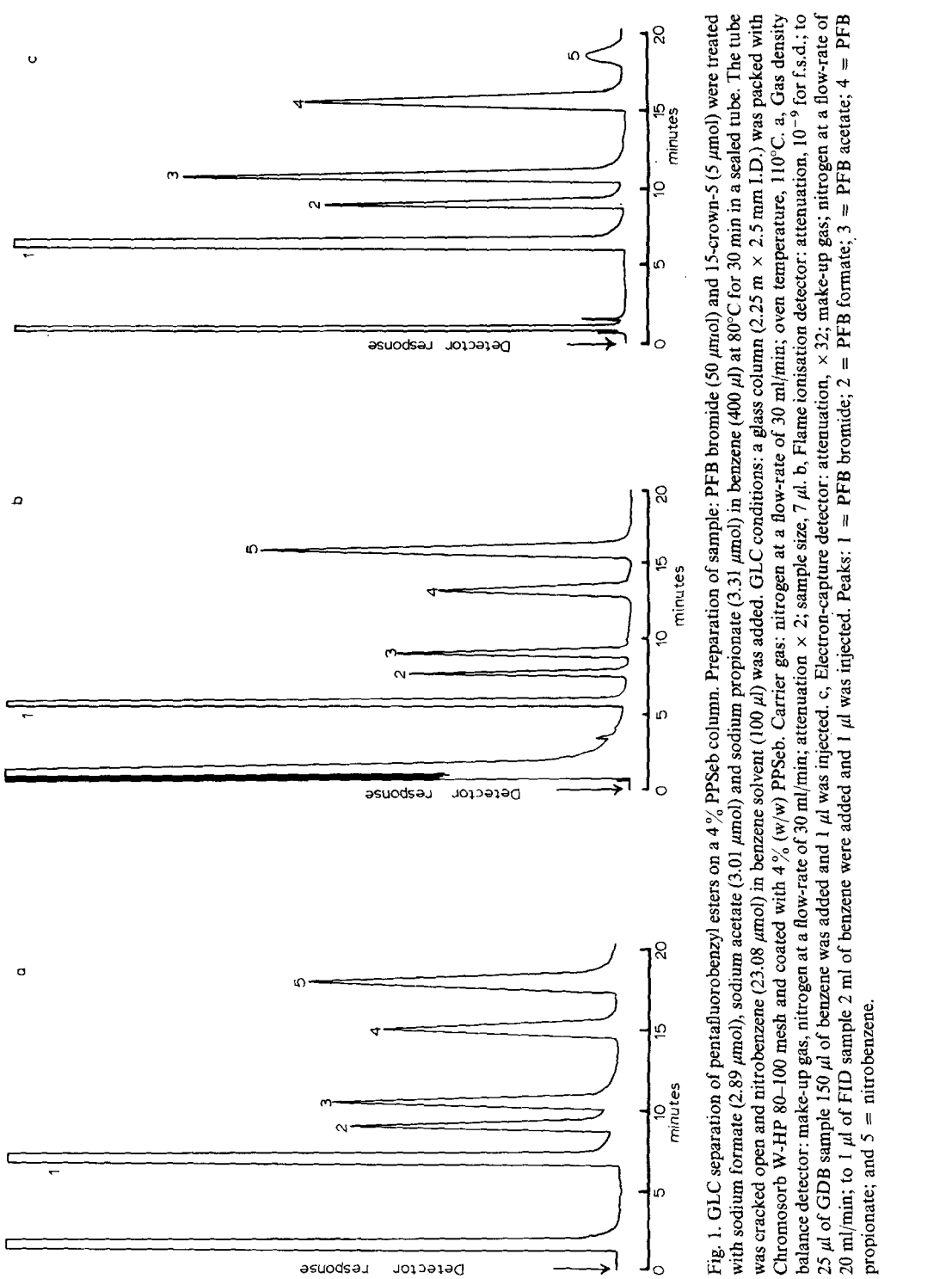

Fig. 1. GLC separation of pentafluorobenzyl esters on a 4% PPSeb column. Preparation of sample: PFB bromide (50 μmol) and 15-crown-5 (5 μmol) were treated with sodium formate (2.89 μmol), sodium acetate (3.01 μmol) and sodium propionate (3.31 μmol) in benzene (400 μl) at 80°C for 30 min in a sealed tube. The tube was cracked open and nitrobenzene (23.08 μmol) in benzene solvent (100 μl) was added. GLC conditions: a glass column (2.25 m × 2.5 mm I.D.) was packed with Chromosorb W-HP 80–100 mesh and coated with 4% (w/w) PPSeb. Carrier gas: nitrogen at a flow-rate of 30 ml/min; oven temperature, 110°C. a, Gas density balance detector: make-up gas, nitrogen at a flow-rate of 30 ml/min; attenuation × 2; sample size, 7 μl. b, Flame ionisation detector: attenuation, 10^{-9} for f.s.d.; to 25 μl of GDB sample 150 μl of benzene was added and 1 μl was injected. c, Electron-capture detector: attenuation, × 32; make-up gas; nitrogen at a flow-rate of 20 ml/min; to 1 μl of FID sample 2 ml of benzene were added and 1 μl was injected. Peaks: 1 = PFB bromide; 2 = PFB formate; 3 = PFB acetate; 4 = PFB propionate; and 5 = nitrobenzene.

TABLE II

RELATIVE MOLAR RESPONSE VALUES OF PENTAFLUOROBENZYL ESTERS DETERMINED AGAINST NITROBENZENE (= 1)

Preparation of sample and GLC conditions as in Fig. 1.

Detection	*Relative molar response ± S.D. (n = 12)*		
	PFB Formate	*PFB Acetate*	*PFB Propionate*
FID	1.18 ± 0.06	1.33 ± 0.04	1.59 ± 0.02
ECD	13.81 ± 0.59	22.62 ± 0.75	21.33 ± 0.76

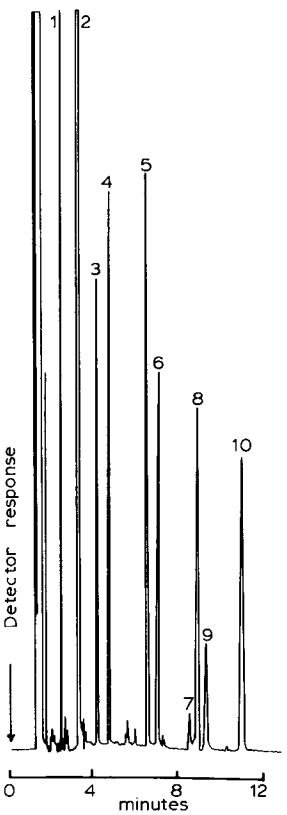

Fig. 2. GLC separation of PFB esters on PPSeb SCOT column. Preparation of sample: a solution containing sodium formate (5.1 nmol), sodium acetate (4.5 nmol), sodium propionate (5.6 nmol), sodium butyrate (5.0 nmol), sodium isovalerate (4.8 nmol) and sodium bicarbonate (250 nmol) was placed in a silanised glass test tube (5 cm × 0.3 mm I.D.) and taken to dryness. PFB bromide (75 nmol) and 15-crown-5 (75 nmol) in acetonitrile solution (20 μl) were added. The tube was sealed and incubated at 80°C for 30 min. A 1-μl aliquot was dissolved in 4 ml of toluene and 1 μl was injected onto the GLC column. GLC conditions: 30 m × 0.27 mm I.D. glass column coated with 5% Chromosorb R and 5% PPSeb; carrier gas, hydrogen at a flow-rate of 1.7 ml/min; make-up gas, nitrogen at a flow-rate of 50 ml/min; detector temperature, 250°C; column temperature, 100°C; attenuation, × 32. Peaks: 1 = PFB chloride; 2 = PFB bromide; 3 = PFB formate; 4 = PFB acetate; 5 = PFB propionate; 6 = nitrobenzene; 7 = artifact; 8 = PFB *n*-butyrate; 9 = artifact; and 10 = PFB isovalerate.

employed (0.3%)[15]. The presence of moisture effects derivatisation, and when samples were not completely dry lower RMR values and larger artifact peaks (Fig. 2, peaks 7 and 9) were obtained. These artifact peaks were attributed to the polymerisation of PFB bromide[15,20]. It was found that pure sodium salts of acids (0.5–5 nmol) were completely derivatised with PFB bromide and 15-crown-5 (1:1; mol/mol) at 80°C in 30 min, but in the presence of 10 μmol NaBr (excess NaBr was always present in the samples due to alkaline hydrolysis conditions) 2 h at 80°C were required.

Fig. 3 shows the response concentration curves for different PFB esters (femtomoles) against the peak area response. The linear dynamic range was *ca.* 100 for each compound under the conditions quoted. The RMR values on a SCOT column are given in Table III; these agree well with packed column RMR values (Table II).

The conditions for the release of bound acetate with alkaline hydrolysis were previously reported[6]. The results for the hydrolysis of N-acetyl and N-formyl amino acids with 1 *M* NaOH are given in Table IV. The recoveries for acetate and formate were close to the those expected.

Fig. 4 shows the GLC trace obtained with chicken egg albumin. Four different proteins were hydrolysed, and all gave peaks which showed the presence of small amounts of formate. Control values were obtained by preparing the PFB esters without previous alkaline hydrolysis. The quantitative results are given in Table V. The controls yielded values of 0.45 and 0.36 mole of acetate per mole of protein, and if these were deducted from the values after alkaline hydrolysis, the results agreed with the expected molar ratios of 4 and 1 for albumin and carbonic anhydrase, respectively.

The unknown protein was isolated from axoplasm of the marine worm (*Myxicola infundibulum*) and was shown to contain two polypeptides with mol.wt. 172,000 and 155,000 (ref. 21). Both polypeptides were blocked with acetate (Table V).

S_{18} (ribosomal protein from *Escherichia coli*) had a high control value of 9.23 moles of acetate per mole of protein, and if this was deducted from the value after alkaline hydrolysis, the molar ratio of 1.46 was obtained (Table V). Owing to the limited solubility of S_{18} in 1 *M* NaOH, the control value was lower then expected and therefore the determined value higher. It is essential with this method that protein should not be grossly contaminated with acetate and formate. However, owing to the limited amount available (S_{18}), it was not possible to dialyse the sample as simple

TABLE III

RELATIVE MOLAR RESPONSE VALUES OF PENTAFLUOROBENZYL ESTERS DETERMINED AGAINST NITROBENZENE (=1) ON A PPSeb SCOT COLUMN

Preparation of sample and GLC conditions as in Fig. 2.

Compound	*Relative molar response ± S.D. (n = 8)*
PFB Formate	13.53 ± 0.75
PFB acetate	22.15 ± 0.87
PFB Propionate	20.47 ± 0.73
PFB *n*-Butyrate	19.45 ± 0.92
PFB Isovalerate	20.83 ± 0.81

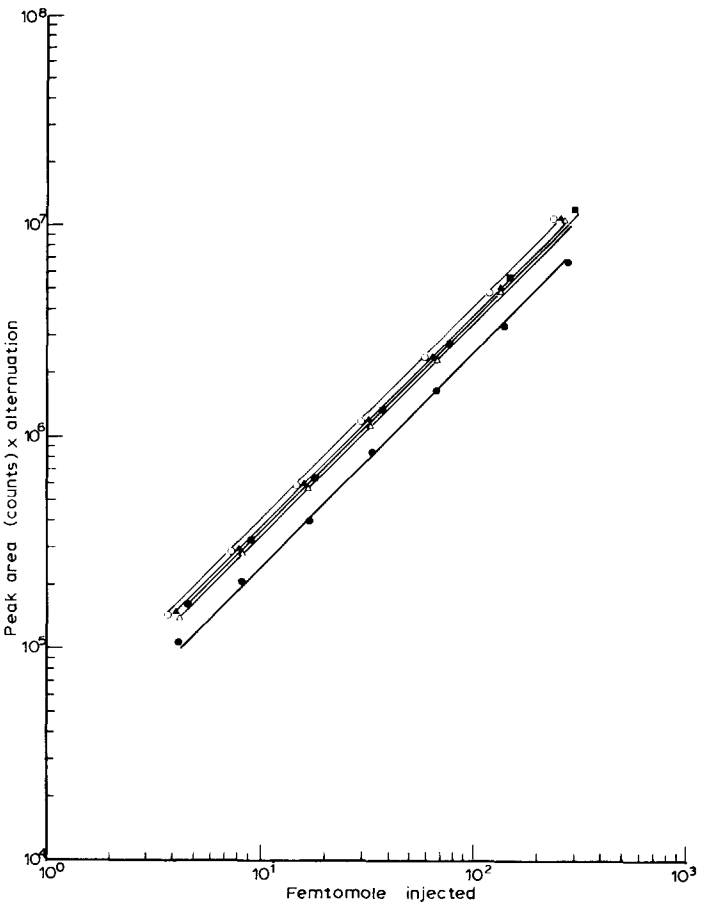

Fig. 3. Response–concentration curves for PFB esters. Preparation of sample and GLC conditions as in Fig. 2. ● = PFB Formate; ○ = PFB acetate; ■ = PFB propionate; △ = PFB *n*-butyrate; and ▲ = PFB isovalerate.

TABLE IV

RECOVERY OF ACETATE AND FORMATE AS PFB ESTERS AFTER ALKALINE HYDROLYSIS OF N-ACETYL AND N-FORMYL AMINO ACIDS

Preparation of sample: see Experimental; GLC conditions as in Fig. 2.

Compound	*Calculated (nmol)*	*% Recovery ± S.D. (n = 4)*
N-Acetyl alanine	4.6	94.2 ± 5.8
N-Acetyl glutamic acid	4.2	96.7 ± 4.3
N-Acetyl phenylalanine	5.6	98.6 ± 6.1
N-Formyl leucine	3.7	95.2 ± 4.6
N-Formyl valine	6.2	97.5 ± 3.8

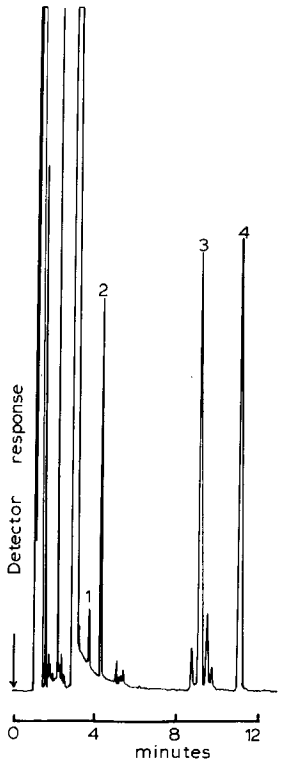

Fig. 4. GLC of PFB acetate and formate obtained from albumin after alkaline hydrolysis. Preparation of sample as in Experimental. GLC conditions as in Fig. 2. Peaks: 1 = PFB formate; 2 = PFB acetate; 3 = PFB *n*-butyrate (internal standard); and 4 = PFB isovalerate (internal standard).

dialysis removes most of the free acetate and formate present. Yaguchi[22] determined the primary structure of S_{18} and showed it to be N-acetylated.

Most of methods reported for the determination of bound acetyl groups either require complicated procedures or large amounts of starting materials. The enzymic methods make use of highly purified enzymes and require 100–900 nmol[23], 17–250 nmol[24], 80 nmol with an average recovery of 90%[25], and 3–12 nmol[26] of acetate.

TABLE V

DETERMINATION OF ACETATE IN PROTEINS

Preparation of sample: see Experimental; GLC conditions as in Fig. 2; (n = 4).

Protein	*Acetate–protein ratio (mol/mol)*		
	Without hydrolysis	*With hydrolysis*	*Difference*
Albumin	0.45 ± 0.05	4.57 ± 0.16	4.12
Carbonic anhydrase	0.36 ± 0.03	1.42 ± 0.09	1.06
Unknown protein	0.62 ± 0.10	2.73 ± 0.13	2.11
S_{18}	9.23 ± 0.75	10.69 ± 1.09	1.46

Protein samples containing 8–17 μmol[27], 0.45–1.13 μmol[28], and 20–100 nmol[6] were analysed by GLC. The method described here allows the determination of *ca.* 5 nmol of covalently bound acetate (also formate and propionate). The limiting factor on this assay's sensitivity is not the detection (ECD can detect 3 fmol of PFB esters) but mainly inherent constraints dependent upon the chemistry of the derivatisation procedure. This method can also be used for the determination of short chain fatty acids in physiological samples (plasma and urine).

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