Journal of Chromatography, 234 (1982) 225–230
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,294

Note

Rapid concentration and analysis of short chain carboxylic acids: variation on a theme

R. L. PATIENCE* and J. D. THOMAS
School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG (Great Britain)
(Received August 17th, 1981)

Short-chain carboxylic acids (C_1–C_5) are ubiquitous in natural systems, such as food and drink[1–5], living organisms[6–19] and water bodies and sediments[20–22], and are widely studied. However, problems can arise in analysis, since these are polar, comparatively volatile compounds, which, in biological systems, may make up only a small proportion of the total organic content. The most popular analytical technique is gas chromatography (GC) of the underivatized acids, and whilst most problems associated with GC of such polar compounds (reviewed by Ackman[23]) can be overcome with care, the exception is insensitivity to the flame-ionization detector (FID). Thus an appropriate derivative of an acid can be used to either increase GC sensitivity or facilitate use of high-performance liquid chromatography (HPLC). Formation of the *p*-bromophenacyl ester[30] was chosen from the methods available, *e.g.* refs. 24–37, as this reaction is easy to use, tolerant to water and the ester formed has a strong UV absorbance.

However, the literature provides few satisfactory ways for rapid preparation of aqueous samples ready for derivatization. Solvent extraction with diethyl ether has been used previously[38–43], and was adapted for this work to provide a concentrated, salt-free aqueous solution of short-chain carboxylic acids, which can be analysed directly by GC, or derivatized for GC or, here, HPLC. This technique is currently in use to study concentrations of carboxylic acids in the snail hosts of schistosomiasis, and in decaying plants on which they feed.

EXPERIMENTAL

Glassware

All glassware was scrubbed in detergent, then rinsed in hot and cold tapwater and finally in double distilled (DD) water (the second distillation from alkaline potassium permanganate solution).

Reagents

p-Bromophenacyl bromide (Aldrich) and 18-Crown-6 (Aldrich) were co-crystallized (10:1 ratio) after dissolution in HPLC-grade acetonitrile (Rathburn Chemicals) by addition of excess DD water, then filtered and dried *in vacuo*. The dried mixture was made up as a 10^{-2} *M* solution of the bromide/10^{-3} *M* of the crown

0021-9673/82/0000–0000/$02.75

ether in acetonitrile, and stored in the dark. Potassium hydrogen carbonate (AnalaR; BDH) was recrystallized after dissolution in DD water, by addition of excess methanol (AnalaR; BDH). The purified chemical was used either as the solid, or as a 0.1 *M* solution in DD water. Hydrochloric acid (1 *M*) was prepared by dilution of a concentrated solution (AnalaR; BDH) and was extracted with diethyl ether (May & Baker) before use. Carboxylic acid standards (acetic and propanoic acids: BDH; butanoic acid: Sigma) were made up, without purification, as concentrated solutions (0.1 *M*) in DD water and stored frozen.

Apparatus

The HPLC system comprised a Milton Roy Minipump (5000 p.s.i.g. maximum) with pulse dampening, a variable volume loop injector (typically 10 μl injected) coupled to a μBondapak C_{18} column (30 cm × 3.9 mm I.D.; Waters Assoc.). Column effluent passed through a 8-μl flow cell in a Carl Zeiss UV/Vis variable wavelength spectrophotometric detector set at 254 nm. The mobile phase was acetonitrile–water (50:50), flow-rate 1 ml/min.

Procedure

To an aqueous sample (1–2.5 ml, pH ≈ 7) in a centrifuge tube (*ca.* 10 ml) was added sufficient 1 *M* HCl (0.1–0.25 ml) to make the pH < 2. Diethyl ether (2–3 × aqueous volume) was added and the two layers mixed (30 sec) using a Whirlimixer (Fisons). If necessary, the solutions were centrifuged briefly (1500 *g*; 2 min) to separate the two layers. The diethyl ether layer was transferred, using a pasteur pipette, to a vial (⩾7 ml total volume). Extraction with ether was repeated once or twice more (see Table I for exact conditions). The ether was reduced, if necessary, to *ca.* 6 ml under a stream of nitrogen, and then placed in a deep-freeze (−20°C, 30 min)

TABLE I

RECOVERY OF BUTANOIC ACID (0.5 μMOLES) FROM (i) WATER (1 ML) CONTAINING KNOWN CONCENTRATIONS OF INORGANIC SALTS[44] AND (ii) NEUTRALIZED 2 *M* PERCHORIC ACID (2.5 ML)

	Experiment	*No.*	*Recovery of butanoic acid (μmoles)***	*Recovery (%)*	*Mean*	±	*Std. dev.*
(i)	Water + salts*	1	0.41	83			
		2	0.42	83			
		3	0.37	75	79		4
		4	0.38	76			
		5	0.39	77			
(ii)	Neutralized	1	0.31	62			
	perchloric acid**	2	0.29	57			
		3	0.22	43			
		4	0.16	32	46		13
		5	0.18	35			

* Extracted with 2 × 3 ml diethyl ether.

** Extracted with 3 × 6 ml diethyl ether.

*** Determined by HPLC of *p*-bromophenacyl ester and comparison with standard.

so that trace amounts of water (containing high concentrations of HCl and salts) crystallized out. The dry and inorganic salt-free ether remained liquid and was decanted or filtered in the deep-freeze into a small vial with gas-tight screw top [either a 7-ml McCartney vial with rubber liner, or preferably a 3.5-ml Reactivial (Pierce), with PTFE/silicone liner]. Aqueous $KHCO_3$ ($\leq$0.1 ml of a 0.1 *M* solution) was added to the ethereal solution; the vial was capped and Whirlimixed (1 min). At the end of this period, the pH of the water layer was tested by applying 0.2 μl (using a 1 μl SGE syringe) to pH paper (1–14 range). If the pH was 7–8, the aqueous layer was made up to 0.1 ml total with DD water. If pH $<$ 7, more aqueous $KHCO_3$ was added up to a total volume of 0.1 ml. The addition of $KHCO_3$ converts any carboxylic acid present into the potassium salt necessary for the derivatization. Finally, the ether layer was evaporated off under a stream of nitrogen.

The carboxylic acids (K^+ salts) in the aqueous layer (0.1 ml) were esterified by addition of excess of the *p*-bromophenacyl bromide/18-crown-6 solution in acetonitrile, made up to 1 ml total volume with acetonitrile. After heating (80°C, 20–30 min), the *p*-bromophenacyl esters were analyzed by HPLC. Structures were confirmed in two ways: (i) by co-injection and/or comparison of retention times of authentic standards on HPLC; (ii) by separation of the esters on thin-layer chromatography (TLC) (silica gel G; hexane–diethyl ether, 7:3, eluent). The following R_F values were obtained: *p*-bromophenacyl (*p*-bpa) bromide, 0.82; *p*-bpa butanoate, 0.68; *p*-bpa propanoate 0.56; *p*-bpa acetate, 0.38. Individual bands were removed, the ester recovered and re-analyzed by HPLC.

RESULTS AND DISCUSSION

The results of two solvent extraction experiments, chosen as typical of different sample origins, are presented in Table I. Sample i, an inorganic salt solution[44] spiked with butanoic acid (0.5 μmoles), represents a marine or freshwater source, and here contained calcium chloride (2 m*M*) as the major salt. Sample ii was perchloric acid (2 *M*), again spiked with butanoic acid (0.5 μmoles), which was neutralized with solid $KHCO_3$. Perchloric acid was chosen because it is commonly used for digesting organisms since it degrades proteins, thus preventing enzymatic action in tissues after death. Both experiments gave good recoveries of the small amount of the standard: *ca.* 79% for sample i and *ca.* 46% for sample ii (determined by HPLC: see Fig. 1 for example analysis of standard compounds). The lower recovery and high standard deviation in the latter case is not surprising because of the larger number of steps involved in sample preparation; in particular, loss through volatilization could occur when perchloric acid is neutralized with $KHCO_3$. It is worth noting that the acids are never reduced to dryness by this method (in contrast to typical solvent extraction, and also freeze drying, a common concentration technique for aqueous samples). In particular, adding aqueous $KHCO_3$ before removing the diethyl ether under nitrogen, added significantly to the efficiency of recovery.

The main departure, however, from standard solvent extraction is the removal by freezing of trace quantities of inorganic salt-containing water carried over with the diethyl ether. This step is important, particularly if the acids are to be derivatized. Even with direct analysis by GC (*i.e.*, acids not derivatized), high concentrations of inorganic ions can contaminate injectors and/or columns. In this case, the standard

was esterified to form the *p*-bromophenacyl ester[30]. This reaction has the advantage of tolerance to quite high proportions of water (>10%); but it was found here to be sensitive to chloride ion, whether as hydrochloric acid or potassium chloride. With chloride ion present, *p*-bpa bromide (starting material) "decays" to form a compound which co-elutes with propanoate ester on the reversed-phase column used here (Fig. 1). This "decay" product may be *p*-bpa chloride as (i) it is non-reactive for esterification purposes, (ii) it cannot be separated on silica gel G TLC (hexane–diethyl ether, 7:3, eluent) from the bromide ($R_F \approx 0.82$), although readily separable from the propanoate ester ($R_F \approx 0.56$) under these conditions. In fact, TLC was found to be an excellent method for purification of samples, or for structure confirmation, since the acids (as esters) can be separated into individual compounds (see Experimental for R_F values).

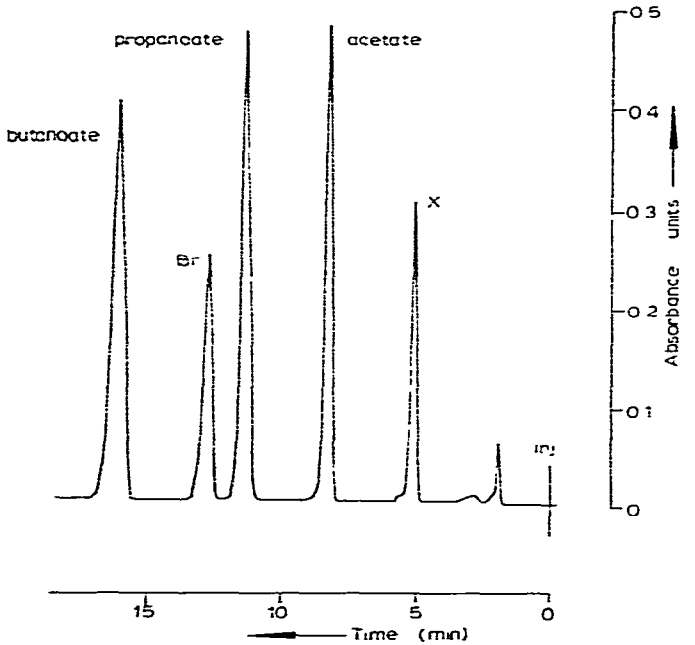

Fig. 1. HPLC separation of standard carboxylic acids (C_2–C_4) as *p*-bromophenacyl esters. Conditions: 10 μl (5 nmoles per component) loop injection on μBondapak C_{18} column; acetonitrile–water (50:50) elution; flow-rate 1 ml min; UV detection, 254 nm. Br = *p*-bromophenacyl bromide; X = reaction by-product from excess $KHCO_3$.

A number of different tests were undertaken to confirm the origin of the "decay" compound. It was formed under all the following experimental conditions, when chloride was present, and never when chloride was excluded: (i) μmole amounts of neutral KCl were added to a reaction mixture containing only the bromide and crown ether; (ii) samples containing chloride were heated in stirred and unstirred sealed vials or (iii) in an open system under reflux; (iv) samples were treated to preliminary ion exchange (K^+ form) only, without addition of HCl or solvent extraction; (v) an acidified aqueous medium was extracted/esterified in a two phase, single-step process using dichloromethane/phase transfer reagent $[(C_4H_9)_4NBr]$/*p*-bromophenacyl bromide (similar to procedure of L'Emeillat *et al.*[45]).

Because the presence of this "decay" product can be taken as evidence for propanoate on reversed-phase columns, exclusion of inorganic salts generally and chloride especially proved essential. Solvent extraction, then removal by freezing of traces of salt-containing water, is an efficient, reliable and speedy way of concentrating comparatively volatile carboxylic acids in good yield, free from both inorganic salts and ether-insoluble organic matter.

ACKNOWLEDGEMENTS

We thank UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases [grant no. T16/181/B2/2(A)] and SERC (grant no. GRA 44879 and GRB 51239) for financial support. We are also grateful to Professor S. Shall and Mr. C. Leech (School of Biology, University of Sussex) for use of HPLC equipment, Mr. A. Hamnett (ARC Unit of Invertebrate Chemistry and Physiology, University of Sussex) for assistance with HPLC, and Professor R. Andrew for providing laboratory facilities.

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