Journal of Chromatography, 467 (1989) 321–326
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

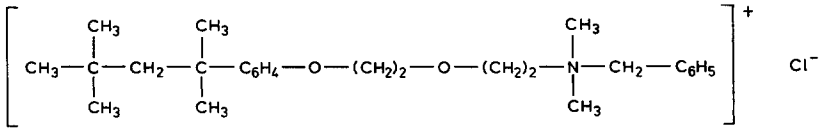
CHROM. 21 250

Note

Rapid high-performance liquid chromatographic method for the determination of bencetonium chloride residues in fish products; confirmation by thin-layer chromatography

Th. B. A. REUVERS*
Dpto. of Bromatologia, Centro Nacional de Alimentación y Nutrición, Majadahonda, Madrid (Spain)
G. ORTÍZ
University of Bogotá, Bogotá (Colombia)
and
M. RAMOS and M. MARTÍN DE POZUELO
Dpto. of Bromatologia, Centro Nacional de Alimentación y Nutrición, Majadahonda, Madrid (Spain)
(First received February 18th, 1988; revised manuscript received January 4th, 1989)

Bencetonium chloride is a surface-active agent and exhibits antiseptic properties.



Benzyl(diisobutylphenoxyethoxyethyl)dimethylammonium chloride

It is used frequently as an algicide, bactericide and fungicide in dairies and food industries in many countries. The need for the detection and identification of its residues in food is of great importance as contamination of food with this quaternary ammonium compound (QAC) is forbidden.

Various techniques have been applied to the quantitative analysis of QACs; a common method used for the determination of QAC residues in milk[1–4] involves ion pairing of the material with an acid dye and subsequent extraction into an organic phase such as chloroform or dichloromethane, followed by spectrophotometric measurement. However, this technique does not provide any information about the QAC found and it is not very useful for residue analysis in food because of the formation of emulsions which inhibit extraction and lower recoveries.

The introduction of chromatographic techniques increased the specificity and sensitivity in the analysis of QAC, which are poor in the colorimetric method. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been applied in distribution studies of various QACs[5–9].

Larson and Pfeiffer[10] proposed a liquid chromatographic method with indirect photometric detection using benzyltrimethylammonium salt in the mobile phase as an

0021-9673/89/$03.50

UV-absorbing counter ion. Kawase *et al.*[11] present an interesting technique based on the separation of dialkyl-type cationic surfactants by HPLC and on-line ion-pair extraction of the surfactant with bromophenol blue into an immiscible organic phase. However, these methods cannot be applied to the determination of residues of QACs in a complex matrix.

Almost no relevant methods for the detection and quantitation of QACs are available and only a few authors have reported work in this field. Pohlmann and Cohan[12] describe the detection of QACs in biological fluids by GC, and Wee and Kennedy[13] developed a liquid chromatographic method with conductometric detection for the determination of trace levels of QACs in river-water.

The determination of QACs in meat and meat products has been described by Harke *et al.*[14]: the samples were extracted with organic solvents and the residues of QACs detected by thin-layer chromatography (TLC). Rojas *et al.*[15] proposed a colorimetric method for a cationic surfactant in fish, which resulted in a determination of the total QACs, without their individual identification. These two methods show some difficulties in the extraction and isolation of the QACs from the complex matrices. Therefore, Reuvers *et al.*[16] presented an extraction method in which 1-hexanesulphonic acid was used as the counter ion during the extraction of benzalkonium chloride from fish products. TLC based on the method as described by Harke *et al.*[14] was used for the detection of the surfactant.

HPLC has not yet been employed in the detection of bencetonium chloride residues in foods. Here, we propose a method based on such a technique, using a counter ion in the extraction and chromatographic procedure.

EXPERIMENTAL

Reagents and instrumentation

All solvents were reagent grade and used as received. The counter ion 1-hexanesulphonic acid was supplied by Scharlau (F.E.R.O.S.A., Barcelona, Spain) and hexachloroplatinic(IV) acid by BDH Chemicals (U.K.).

The HPLC equipment consisted of a Waters pump, an injector (U6K) and UV absorption was monitored at 254 nm with a fixed-wavelength detector (Waters R 441 UV) at a range setting of 0.02 a.u.f.s. The HPLC column (30 cm × 3.9 mm) used throughout this work was packed with μBondapak-CN (Waters); it was used at room temperature. The eluent consisted of acetonitrile–0.1 *M* ammonium acetate (75:25) with the addition of 15 ml PIC B6 (Waters) per litre of the mobile phase. Flow-rate: 1 ml/min.

Extraction and clean up of bencetonium chloride from fish samples

A 10-g sample of minced hake, European squid or shortfin squid was mixed thoroughly with 50 ml of 96% ethanol, 0.05 ml 1-hexanesulphonic acid and 0.5 ml 2 *M* HCl. After 1 h the suspension was filtered into a 250-ml round-bottom flask. The residue was extracted with a second portion of 50 ml 96% ethanol and the ethanolic supernatant filtered into the same flask. The filter cake was pressed and washed with 25 ml of ethanol and the pooled extracts were dried in a rotatory evaporator under vacuum at $< 70°C$. The dry residue was taken up in 0.5 ml 2 *M* HCl, 0.5 ml methanol, 0.05 ml 1-hexanesulphonic acid and 5 ml light petroleum (b.p. 30–40°C). The

suspension was agitated for 2 min and the mixture transferred to an extraction tube (25 ml, 3 cm I.D.). The round-bottom flask was rinsed subsequently with the same mixture and with 5 ml light petroleum; both rinses were transferred to the extraction tube.

The extracts were mixed and heated in a warm water-bath (70°C) until the organic layer had disappeared. A 2-ml volume of dichoromethane–light petroleum (1:1) was added to the retained aqueous phase followed by mixing for 1 min to achieve complete extraction of the bencetonium chloride–counter ion complex. The organic phase was used for analysis.

Detection and identification by HPLC

Different amounts of standard BC (bencetonium chloride) were injected to check the linear response of the detector. Recovery experiments were carried out on spiked samples of different fish products by injection of 5, 10 or 20 μl of the organic layer (2 ml) obtained under the conditions of extraction, purification and detection, as described above.

Confirmation by TLC

A 10-μl volume of the sample extracts was applied on Kieselgel 60 plates (20 cm × 20 cm) and eluted by *n*-butanol–acetic acid–water (4:1:1). The plates were air-dried and sprayed with the following reagent: 3 ml of 10% aqueous hexachloroplatinic(IV) acid mixed with 97 ml of water and 100 ml 6% aqueous KI. The detergent appeared inmediately as dark blue spots on a rose background.

RESULTS AND DISCUSSION

Different amounts of a 0.1% solution of bencetonium chloride in water were injected into the chromatographic system (0.02 and 0.05 a.u.f.s. × range setting) and peak heights were measured. The calibration graph was calculated using the method of least squares and can be expressed as $y = 25.8x + 15.8$, where y = BC peak height in mm at 254 nm (0.02 a.u.f.s.) and x = the amount of BC injected (on different days). The linearity was satisfactory with a correlation factor, $r = 0.983$ and was linear up to 10 μg injected. The standard deviation (S.D.) was 1.7 ng/mm at 0.02 a.u.f.s. with a coefficient of variation (C.V.) of 6.5%.

The retention time (about 6 min) of BC under these chromatographic conditions is long enough to assure the separation of the front peak in the fish samples. Fig. 1 shows a chromatogram of 2 μg of BC injected under the conditions described.

To check the validity of the proposed extraction and clean up, various commercial samples of fish products were homogenized and spiked with different amounts (up to 100 ppm) of BC and the extracts analyzed. Typical chromatograms of spiked (50 ppm) and unspiked hake extracts are shown in Fig. 2. It is seen that the separation of the BC peak from the front peak is sufficient to analyze BC in fish. Recoveries for hake are shown in Table I, based on the calibration graph.

The same procedures were carried out with commercial squid samples. The results are shown in Fig. 3 and Table II.

The "unusual" extraction and clean-up procedures are necessary to desorb and suspend all the sediments present on the walls of the flask. In our earlier extraction experiments[16], carried out without light petroleum in the extraction mixture,

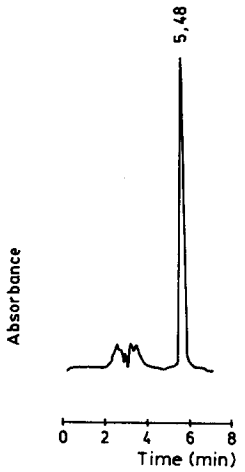


Fig. 1. Chromatogram of 2 μg bencetonium chloride.

sediments remained on the wall with BC adsorbed, resulting in very low recoveries. The presence of the counter ion in an acid medium facilitates the extraction of BC into the organic layer, which, however, does not contain all the BC present. That is the reason for the evaporation of the organic layer in the presence of the aqueous layer and

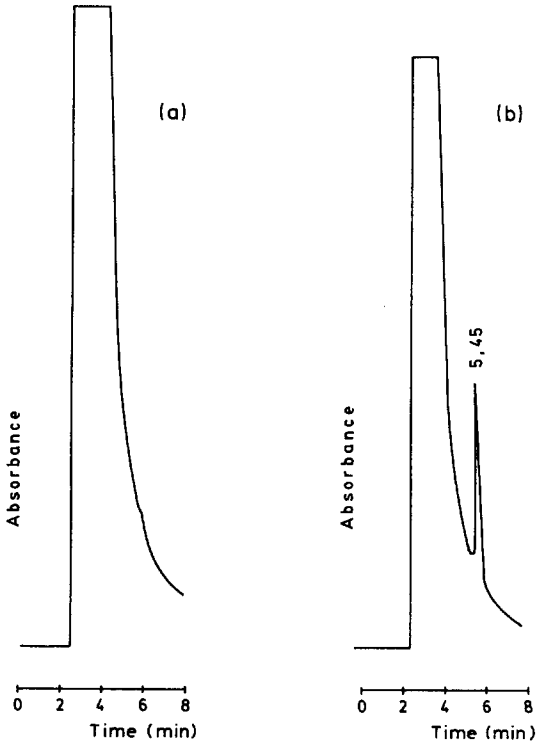


Fig. 2. Typical chromatograms of (a) unspiked and (b) spiked (50 ppm) hake extracts.

TABLE I
RECOVERY OF BENCETONIUM CHLORIDE IN HAKE

Hake	*Found mean value (ppm) (n=4)*	*Recovery (%)*	*C.V. (%)*
10 g	–	–	
10 g + 10 ppm	8.6	86.3	6.3
10 g + 25 ppm	23.0	92	13.4
10 g + 100 ppm	87.4	87.4	19.0

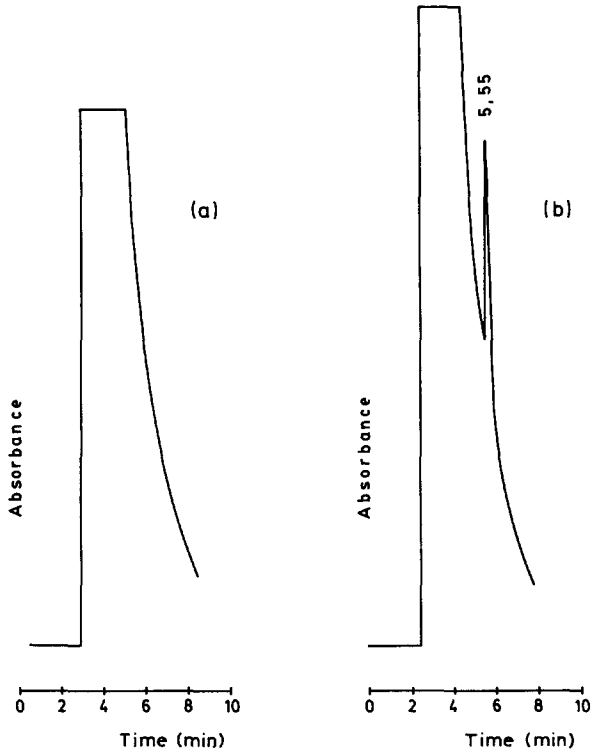


Fig. 3. Typical chromatograms of (a) unspiked and (b) spiked (50 ppm) squid extracts.

TABLE II
RECOVERY OF BENCETONIUM CHLORIDE IN SQUID

Squid	*Found mean value (ppm) (n=4)*	*Recovery (%)*	*C.V. (%)*
10 g	–		
10 g + 10 ppm	9.1	91.0	21
10 g + 25 ppm	22.1	88.4	19
10 g + 50 ppm	41.9	83.7	19
10 g + 100 ppm	88.1	88.1	9.2

its subsequent extraction with dichoromethane–light petroleum (1:1), which results in the complete transfer of the BC–counter ion complex into the organic layer. The presence of light petroleum in this mixture is necessary to assure that the organic layer is always the upper layer. The procedure described is suitable for fatty and non-fatty fish. Recoveries were satisfactory as shown in Tables I and II. The method was linear between 0 and 500 ppm, in which range BC occurs in fish products, and the detection limit is approximately 5–10 ppm.

However, the presence of BC should be confirmed. TLC may be used for the identification of BC. Under the conditions described, aliquots of 10 μl of the fish extracts and different amounts of the standard (1, 2, 5 μg) are added to the Kieselgel 60 (Merck) plate and eluted. Immediately after the development with the spray reagent, the spots are observed and quantitated by visual comparison of their intensities with these of standard spots. The detection limit is about 10 ppm and the TLC results are well in agreement with the values obtained using the HPLC method proposed. Other QACs such as benzalkonium chloride are visible, but they do not interfere because of their higher R_F values.

ACKNOWLEDGEMENTS

The authors thank N. Mendez and C. Díaz for their analytical assistance and A. Gómez for technical assistance in the preparation of the manuscript.

REFERENCES

1 G. Wildbrett and F. Einreiner, *Z. Lebensm.-Unters.-Forsch.*, 165 (1977) 34.
2 F. Munaò and O. C. Grillo, *Il Latte*, 5 (1980) 673.
3 S. G. Faber, H. G. Gasthuis, G. van der Haar and G. Veenkamp, *De Ware(n) Chemicus*, 8 (1978) 138.
4 *Methods of analysis of AOAC*, Association of Official Analytical Chemists, Washington, DC, 13th ed., 1980, method No. 20.077-20.089, pp. 333–336.
5 L.-K. Ng, M. Hupé and A. G. Harris, *J. Chromatogr.*, 351 (1986) 554.
6 S. L. Abidi, *J. Chromatogr.*, 200 (1980) 216.
7 A. Nakae, K. Kunihiro and G. Muto, *J. Chromatogr.*, 134 (1977) 459.
8 J. Kawase, Y. Takao and K. Tsuji, *J. Chromatogr.*, 262 (1983) 48.
9 R. C. Meyer, *J. Pharm. Sci.*, 69 (1980) 1148.
10 J. R. Larson and C. D. Pfeiffer, *Anal. Chem.*, 55 (1983) 393.
11 J. Kawase, Y. Takao and K. Tsuji, *J. Chromatogr.*, 262 (1983) 293.
12 J. L. W. Pohlmann and S. L. Cohan, *J. Chromatogr.*, 131 (1977) 297.
13 V. T. Wee and J. M. Kennedy, *Anal. Chem.*, 54 (1982) 1631.
14 H. P. Harke, G. Bestmann and M. Linke, *Gordian*, 1 (1978) 7.
15 E. Rojas, P. Lasa, J. J. Rivera and E. Muro, *Quim. Ind. (Madrid)*, 28 (1982) 205.
16 Th. Reuvers, G. Ortíz, M. Martín de Pozuelo and M. Ramos, *II World Congress of Food Technology, Barcelona, March 3–6, 1987.*