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Short Communication

High-performance liquid chromatography of a mixture of two dodecyltins by sensitive fluorescence tagging with morin

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Abstract

A high-performance liquid chromatographic (HPLC) method for the determination of a 6:4 mixture of tris(isooctyloxycarbonylmethylthio)monododecyltin (**I**) and bis(isooctyloxycarbonylmethylthio)didodecyltin (**II**) combined with fluorescence detection following postcolumn morin complexation is presented. The HPLC method is selective for the compounds investigated as no other peaks were observed. The instrumental detection limit was 14 ng for **I** and 9 ng for **II** per 5-μl injection (*i.e.* 2.8 μg/ml of **I** and 1.8 μg/ml of **II** in 2-propanol), the method detection limit was 10 μg/ml of 6:4 mixture in peanut oil and the limit of determination was 0.22 mg/ml. The linearity of the detector signals was verified within the range 50 ng–1.0 μg of 6:4 mixture per injection (*i.e.* 2.5–50 μg/ml in 2-propanol). Within the range 0.22–50 mg/ml, the accuracy was 90–102% of the expected values and the intra-assay precision (relative standard deviation) was <5%. The HPLC results and atomic absorption spectrometric data were concordant.

1. Introduction

Organotins are extensively used as PVC stabilizers, polymerization catalysts and biocides in industry and agriculture. To investigate their toxicological potential, the organotins are administered to test animals via suitable formulations. A mixture of monododecyltin and didodecyltin isooctylthioglycolates has been recommended as a stabilizer for rigid PVC in food packaging and is included in the recommendations of the Plastics Committee of the German Federal Health Office (BGA). As a part of the toxicological characterization of this mixture, studies on embryotoxicity in rats and rabbits were performed. Oral administration was performed using solutions in peanut oil. The preparations need to be checked for the correct concentration and stability of the active ingredients.

UV and fluorescence detectors are commonly applied in high-performance liquid chromatography (HPLC). However, owing to the lack of chromophores the direct determination of organotins by HPLC is difficult. Consequently, HPLC is often combined with element-specific detectors such as atomic-absorption spectrometers [1–5], flame photometers or inductively coupled plasma atomic emission spectrometers [6,7]. However, such equipment is highly sophisticated and expensive. Another approach is achieved by UV and fluorescence labelling with 8-quinolinol [8] and morin [9–15], respectively. The 8-quinolinol complexation was per-

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formed with an on-column procedure. Pre/on-column [9,10] or postcolumn techniques [11–15] were used to obtain highly fluorescent organotin–morin complexes. Ebdon and Garcia Alonso [15] and Kleiböhmer and Cammann [16] reported that the fluorescence of these complexes could be enhanced by using micellar systems. In this paper, a postcolumn technique with morin for organotin derivatization in a micellar system is presented. The procedure was applied to two novel PVC stabilizers.

2. Experimental

2.1. Materials

Tris(isooctyloxycarbonylmethylthio)monododecyltin (**I**) and bis(isooctyloxycarbonylmethylthio)didodecyltin (**II**) (Fig. 1) were synthesized in the laboratories of Schering (now Witco) (Bergkamen, Germany). A 6:4 (w/w) mixture of **I** and **II** was used for the experiments. Liquid chromatographic grade acetonitrile, 2-propanol and water were obtained from Merck (Darmstadt, Germany). Analytical-reagent grade morin, lithium chloride, Triton X-100 and acetic acid were also purchased from Merck.

2.2. Apparatus

The HPLC equipment consisted of a Model 510 pump (Waters, Eschborn, Germany), set at a flow-rate of 1.2 ml/min and equipped with a membrane pulse filter and a 25 cm × 4.6 mm I.D. stainless-steel column packed with 10-μm silicon carbide by VDS Optilab Chromatographie Technik (Berlin, Germany), an ISS-100 autosampler (Perkin-Elmer, Überlingen, Germany), a reagent-delivery module (Waters), set at a flow-rate of 1.0 ml/min, and a Perkin-Elmer LC-240 fluorescence detector. The detector was connected via an interface to a VAX 8810 mainframe computer (Digital Equipment, Munich, Germany) for data acquisition and evaluation. The chromatograms were evaluated with ACCESS · CHROM 1.6 (Perkin-Elmer).

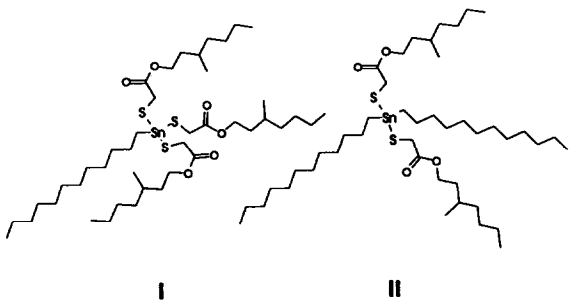

Fig. 1. Structural formulae of compounds **I** and **II**.

2.3. Chromatography and postcolumn derivatization

Chromatographic columns of dimensions 2 cm × 4.6 mm I.D. (analytical column) and 12.5 × 4.6 mm I.D. (precolumn) was packed with Hypersil MOS and SAS Hypersil, respectively, by M & W Chromatographie Technik (Berlin, Germany). The mobile phase was a mixture of 800 ml of acetonitrile, 140 ml of doubly distilled water, 60 ml of acetic acid and 6 g of lithium chloride. The postcolumn reagent proposed by Ebdon and Garcia Alonso [15] was modified and consisted of 15 mg of morin, 600 μl of acetic acid, 7 g of Triton X-100 and ad 500 ml of water containing 37% (v/v) 2-propanol. The mobile phase and postcolumn derivatization reagent were degassed under water-jet vacuum.

2.4. Sample work-up

The peanut oil solutions were diluted with 2-propanol by volume factors from 20 (samples of 0.5 mg/ml) to 2500 (samples of 150 mg/ml). Aliquots of 5–10 μl were injected into the HPLC system.

2.5. Calibration and evaluation

Six calibration samples for an analytical run were prepared by dissolving the 6:4 mixture in 2-propanol. The proportion of the peanut oil in

the calibration samples was the same (0.04–5%, v/v) as that in the real samples diluted for injection. For example, in the analysis of samples containing 5 mg/ml of 6:4 mixture in peanut oil, the portions of the analytes injected were in the range 50–600 ng of 6:4 mixture per 5 μl. Thus the calibration range was from 1.0 to 12.0 mg/ml. With respect to evaluation, peak heights were preferred because peak areas were difficult to determine owing to peak tailing. An unweighted linear regression (model: $y = a + bx$) was used to fit the data. The calculation of the found 6:4 mixture concentration was normally performed by evaluating peak height of **II**, which was converted to the 6:4 mixture concentration in the sample, and if necessary by use of the peak height of **I**. That can be done because the proportion of **I** to **II** is fixed and both for calibration and for the preparation of the samples the same substance batches were used.

2.6. Experimental characterization of the HPLC procedure

The instrumental detection limit was determined at a signal-to-noise ratio ≥2 and the linearity by use of 2-propanol standard solutions. The mass loss caused by traces of peanut oil in the injected solutions was investigated by the analysis of real samples combined with calibration standards that contained no peanut oil. The results are reported in terms of recovery. Accuracy and intra-assay precision data were obtained by the analysis of real samples on the basis of **I** and **II**. The inter-assay precision was calculated based on the standard deviation obtained in routine analyses. Finally, the acceptance criteria for the method's limit of determination were a relative standard deviation (R.S.D.) of ≤5% and a bias of ≤10% of expected concentration obtained following the analysis of a series of real samples with decreasing concentrations. Additionally, selected samples were analysed by atomic absorption spectrometry (AAS) as described below. The total tin concentrations found were compared with the HPLC data.

2.7. Comparative analysis by AAS

Some real samples were analysed by means of AAS as described by Tölg [17] and Welz [18] to confirm the results obtained by HPLC. The samples were dissolved and diluted with acetone–2-propanol (1:1, v/v). Each sample was processed in triplicate. For six-point calibration, an oily tin standard was dissolved and diluted with acetone–2-propanol (1:1, v/v). Within the analytical run the calibration standards were measured before and after the samples. Atomic absorption was measured at 286.3 nm. Linear regression was applied for the calculation of the calibration graph. Within each assay a quality control sample containing dibutyltin bis(2-ethylhexanoate) was analysed.

2.8. Stability tests

The stability of the dodecyltins in peanut oil was monitored over a period of 1 week. The expected concentrations were 0.5, 5.0 and 50 mg/ml of 6:4 mixture. Samples of 100 ml were prepared and stored at room temperature protected from light in a glass container. Immediately after preparation and 6 h, 3 days and 7 days afterwards 3–5 aliquots of 0.5 ml were withdrawn, diluted with 2-propanol and injected into the HPLC system.

3. Results and discussion

3.1. Separation and detection of the organotins

Because of its two lipophilic hydrocarbon chains, **II** is retained much more strongly than **I** on octadecyl reversed phases. An acceptable retention behaviour of the two compounds and a short run time of 10 min were achieved by use of the very small analytical columns filled with octyl reversed-phase material. Longer columns and octadecyl phases yielded flat and strongly retained peaks of **II** that could not be evaluated. Also, strong tailing of the peaks of **I** and **II** was observed and is probably due to the strong interaction of the organotin cations with the

residual hydroxy silanol groups of the stationary phase. These unwanted adsorption effects could be reduced significantly by addition of lithium chloride to the mobile phase as proposed by Lakata *et al.* [8]. However, the autosampler and pump have to be protected from corrosion by purging with water and 2-propanol after the runs. Finally, chromatograms of samples containing ≤0.5 mg/ml of 6:4 mixture often showed negative peaks interfering with the peak of **I**. Consequently, the evaluation of the disturbed peaks was difficult. It could be shown that negative peaks were caused by 2-propanol and disappeared when using small injection volumes and additionally the 12.5-cm precolumn, which improved the diffusion of injected 2-propanol in the mobile phase.

In order to achieve peaks of roughly the same height, the wavelengths were changed during the progress of each run. At the start of each chromatography, the fluorescence detector was set at an excitation wavelength of 400 nm and an emission wavelength of 560 nm. The emission wavelength was chosen to be above the emission maximum of **I** of 500 nm because **I** showed much stronger fluorescence than **II** and the proportion of **I** in the samples was 50% higher than that of **II**. As a result, the fluorescence of **I** was prevented from exceeding the dynamic range of the detector. Before the elution of **II** the detector was reset to the optimum sensitivity (emission maximum 460 nm). A representative chromatogram of the two compounds is shown in Fig. 2.

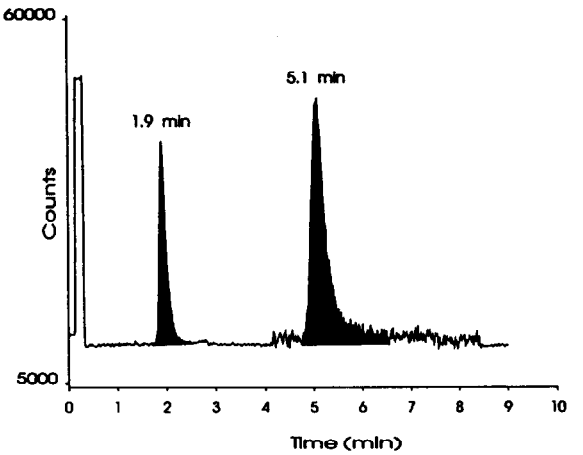

Fig. 2. Chromatogram of a peanut oil sample containing 5.0 mg/ml of 6:4 dodecyltin mixture. Compounds **I** and **II** were eluted at 1.9 and 5.1 min, respectively.

3.2. Characterization of the HPLC method

The system was very selective for the compounds investigated as no other peaks were observed. The instrumental detection limit was 25 ng of 6:4 mixture (corresponding to 14 ng of **I** and 9 ng of **II**) per injection. Including a dilution factor of 20 and an injection volume of 5 μl, the method detection limit was then 10 μg/ml of 6:4 mixture in peanut oil. The sensitivity for **I** could be enhanced further by a factor of at least ten if optimum detections conditions for **I** (emission maximum 500 nm) were applied. Additionally, flow-rates of the postcolumn reagents ≤1.0 ml/min led to higher sensitivity. The limit of detection of **II** could be lowered by increasing the injection volume and perhaps also by application of postcolumn UV irradiation as reported by Stäb *et al.* [13].

The linearity of the peak heights of the dodecyltins was tested within the range of 50 ng–1.0 μg of 6:4 mixture per injection (*i.e.* 2.5–50 μg/ml) and could be verified.

The recovery data are summarized in Table 1. The recovery of **I** was 80 and 75% and that of **II** was 66 and 62% at the levels of 0.22 and 0.51 mg/ml of 6:4 mixture, respectively. At the 5 and 51 mg/ml levels, the recovery of **I** was 92 and 103% and that of **II** was 86 and 97%, respectively. No mass loss of the two compounds was observed only with the sample of 51 mg/ml. The loss was presumably caused by the peanut oil matrix, which contaminated the stationary phases and adsorbed the dodecyltins on the column. To obtain optimum accurate analytical results in spite of this mass loss, the calibration standards therefore ought to be spiked with peanut oil according to the samples involved. In addition, a special sequence for the analyses of samples containing ≤1.0 mg/ml of 6:4 mixture should to be adopted. First, the calibration standards (low, medium and high levels) were chromatographed, then the blank peanut oil

Table 1
Recovery, accuracy and intra-assay precision data

Expected concentration (mg/ml of 6:4 mixture)	Recovery (% of expected values)		Accuracy and intra-assay precision			
			I		II	
	I	II	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)
0.22	80	66	98	4	95	5
0.51	75	62	92	3	90	3
4.99	92	86	98	2	98	2
50.66	103	97	102	1	100	2

The accuracy and intra-assay precision of the method are given as mean values and R.S.D.s of analyses performed with five replicates. The accuracy data are given as a percentage of the expected concentration of the prepared samples. Under the heading Recovery the results obtained without mass compensation are tabulated. The mean values of five replicates are given. The results were related to the expected concentrations. The data were calculated on basis of the peak heights of both I and II.

sample, subsequently a dodecyltin sample in triplicate, the blank peanut oil sample again, followed by the same calibration standards, blank sample, dodecyltin samples, and so on.

To determine the accuracy and intra-assay precision of the method, the four peanut oil solutions mentioned in Table 1 and another four samples containing 0.50–0.55 mg/ml of 6:4 mixture were analysed. At the 0.50–0.55 mg/ml level, the accuracy ranged from 87 to 93% of the expected values when the peak of I and from 90 to 105% when the peak of II was evaluated. For the other samples the expected concentrations were exactly met. Hence, the two steps proposed above diminished the influence of the mass loss so that sufficient accuracy was obtained.

The results of the total tin determination and the HPLC data are summarized in Table 2. The

Table 2
Comparison of the results obtained by analysis of real samples by HPLC and AAS (mean values of 2–5 determinations)

Expected concentration (mg/mol of 6:4 mixture)	Accuracy (% of expected concentration)		
	AAS (total tin)	HPLC	
		I evaluated	II evaluated
0.5	114	123	113
	118	137	113
	104	74	97
	98	84	98
	111	90	102
5.0	104	104	106
	102	102	104
50	104	99	115
	106	97	98

comparison showed that the HPLC data (evaluation of the peak of **II**) agreed very well with the AAS data. Only for one sample did the deviation of the result exceed 10%. Following evaluation of the peak of **I**, the difference between the AAS and HPLC data was up to 30%.

The intra-assay precision (R.S.D.) was in the range 1–4% and 2–5% when the peaks of **I** and **II** were used for evaluation, respectively. With respect to the inter-assay precision, the R.S.D.s obtained from different analytical runs were about 6% at the 0.5 mg/ml of 6:4 mixture level, about 2% at the 5.0 mg/ml level and about 4% at the 15–150 mg/ml level.

The recovery, accuracy, precision and AAS data show that the method always gave optimum results when the concentration of the 6:4 mixture was calculated on the basis of **II**. The lowest concentration tested, 0.22 mg/ml, was defined as limit of determination.

3.3. Application of the method

Within the framework of an analytical service for the validation of toxicological studies, the formulations used are monitored systematically for correct preparation. The stability of the active ingredients has to be tested before the studies begin. If the results are within the range of acceptance (90–110% of the expected concentration), the formulations are considered to be correctly prepared and the preparation process to be valid. The stability of the test substance is deemed to be proved for a defined time period when the decrease in concentration amounts to ≤10% with reference to the initial value. For the 6:4 mixture of dodecyltins the peanut oil formulations that had been routinely analysed so far conformed with the acceptance range and were considered to have been prepared correctly.

With respect to stability at the 5 and 50 mg/ml of 6:4 mixture levels, the analyses resulted in negligible changes in the concentrations of **I** and **II** during 1 week, as shown in Fig. 3. However, at the lowest concentration level of 0.5 mg/ml the concentration of **I** decreased from 84% to 14% within 1 week; the level of **II** remained

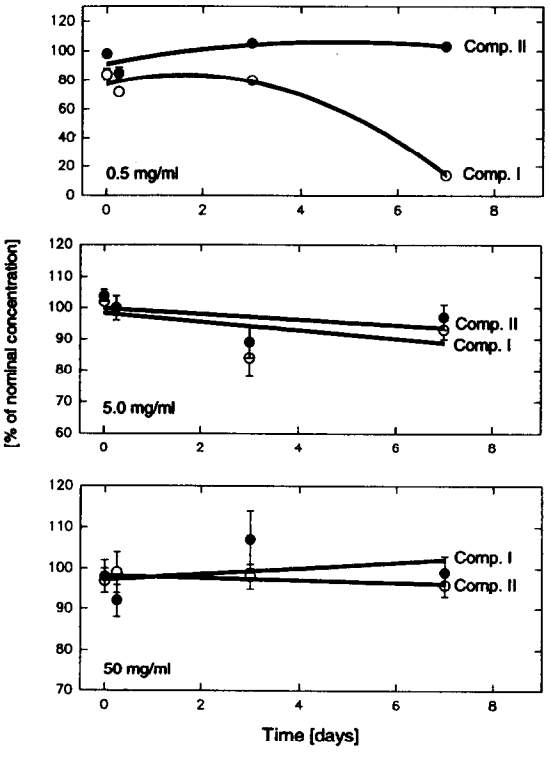

Fig. 3. Stability of the two docecyltins at different concentration levels. The graphs show the analytical results of the stability test. At the 5 and 50 mg/ml of 6:4 mixture levels, five aliquots per time point were processed for HPLC. The sample processing was performed in triplicate at the lowest concentration level (0.5 mg/ml of 6:4 mixture). In the top graph, the error bars disappear behind the symbols indicating the mean values because the standard deviation of the results is very low.

almost constant, 98% being found at the beginning and 103% at the end of the study. At the 0.5 mg/ml level, the 6:4 mixture of dodecyltins was considered to be stable for only a maximum of 3 days because of the decrease in **I** after that time. This means that formulations with concentrations of >5 mg/ml can be used for administration during 1 week and samples containing 0.5–5 mg/ml for a maximum of 3 days. Hence formulations have to be prepared twice per week for use in studies with daily administration.

Finally, the conclusion can be drawn that the described method is suitable for the analysis of peanut oil samples containing ≥0.22 mg/ml of

6:4 dodecyltin mixture. Good sensitivity, excellent selectivity and sufficient accuracy and precision are features of the procedure. The analytical results were reliable and both the correct preparation of formulations and the stability of the active ingredients could be monitored successfully.

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