Chapter VI

Chemical Determination of Drug Residues in Seafoods Using High Performance Liquid Chromatography (HPLC)

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A. INTRODUCTION

The classical methods for determining antibiotics are inhibition bioassays. In most instances, these bioassays are primary procedures for residue screening and quantitative analysis.

One of the advantages of microbial inhibition assays is that they detect almost all members of several antibiotic classes simultaneously at a relatively low cost. However, drawbacks may occur because some analytes have a reduced detection sensitivity to test organisms or an inhibition by possible coextractant such as lipids from animal tissues or lysozyme.

The use of chemical methods for antibiotic determination is limited, particularly for older antibiotics, because microbial inhibition tests are commonly used for regulatory purposes. In many instances chemical methods are only used for chemical identifications.

In past two decades numerous chemical methods (gas chromatography, GC; thin-layer chromatography, TLC; high-performance liquid chromatography, PHLC) have been developed (1-3). These physicochemical methods tend to be more specific, more precise, and have lower limits of detection (i.e. they are more sensitive compared to microbiological methods).

Looking to the future, we can expect to see a continued evolution of test methods for antibiotics in food animals in both field tests and confirmatory tests (chemical) applied in laboratories. With the signing of the new General Agreement on Tarrifs and Trade (GATT) agreement, increased recognition of validated laboratory methods can be expected. The GATT will focus on the Codex Alimentarius Commission and its subsidiaries for scientific support in the resolution of disputes. Other bodies such as AOAC INTERNATIONAL and the International Union of Pure and Applied Chemistry

(IUPAC) will play important role in reviewing validated methods involving both microbiological bioassays and chemical analysis.

The past decade has brought a shift from a total reliance on microbiological assays for the detection and identification of antibiotics to an increased use of chemical separation techniques. New techniques, such as supercritical fluid extraction, capillary zone electrophoresis, and the various combinations of separation techniques with mass spectrometry (MS) and tandem mass spectrometry, will bring further evolution to the regulatory program tests in all countries. Other developments in immunochemical and biochemical analysis will offer opportunities for fast, sensitive, and selective screening tests. There will be more collaboration amongst regulatory laboratories (agencies) around the world. The evolution of new methodologies will take into consideration the international implications and national and international requirements will have to be fully respected.

B. SIMULTANEOUS DETERMINATION OF SULFADIAZINE, SULFADIMETHOXINE TRIMETHOPRIM AND ORMETOPRIM IN SHRIMPS

Scope:

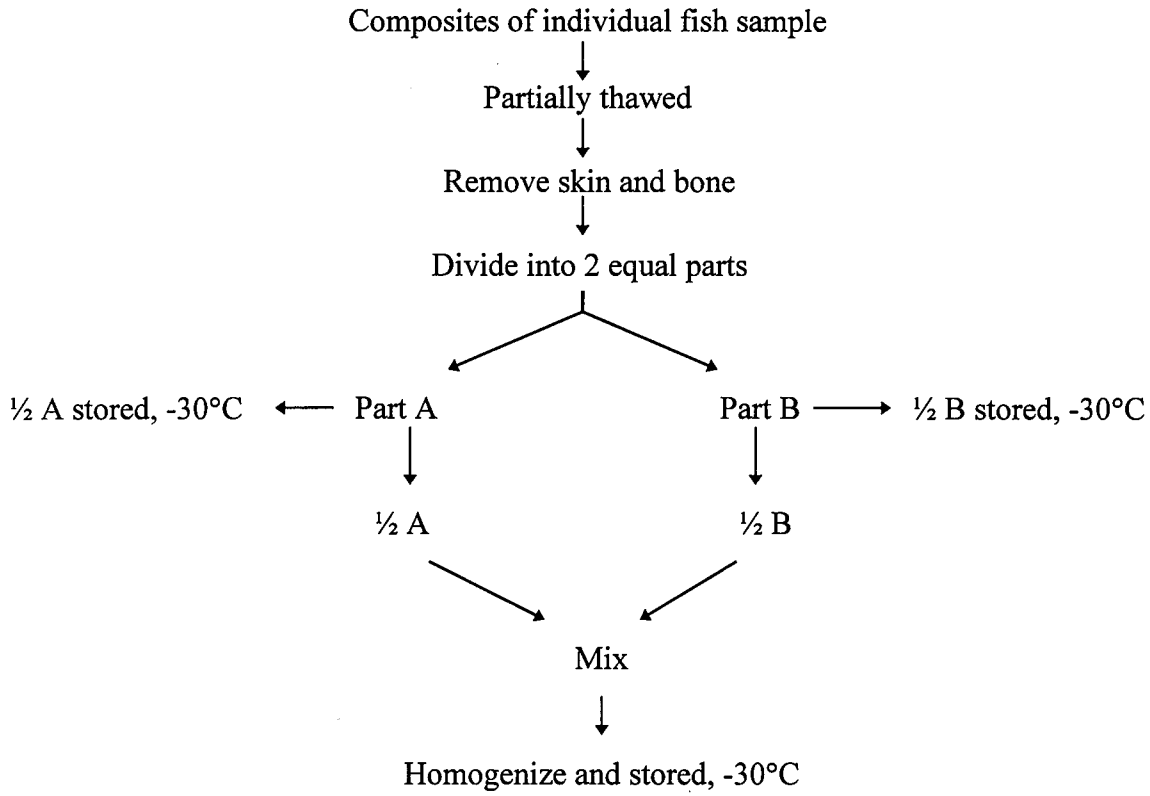
Romet 30 and Tribrissen are frequently used in the treatment of diseases in aquacultured fish. Romet 30 contains sulfadimethoxine (SDM), and ormetoprim (OMP), a potentiator while Tribrissen carries sulfadiazine (SDZ) and trimetoprim (TMP).

Chemical methods for the analysis of these residues have been reported and include TLC[1-4], GC[5], and HPLC[6-9]. The method described here involves simultaneous detection and determination of four sulfa residues.

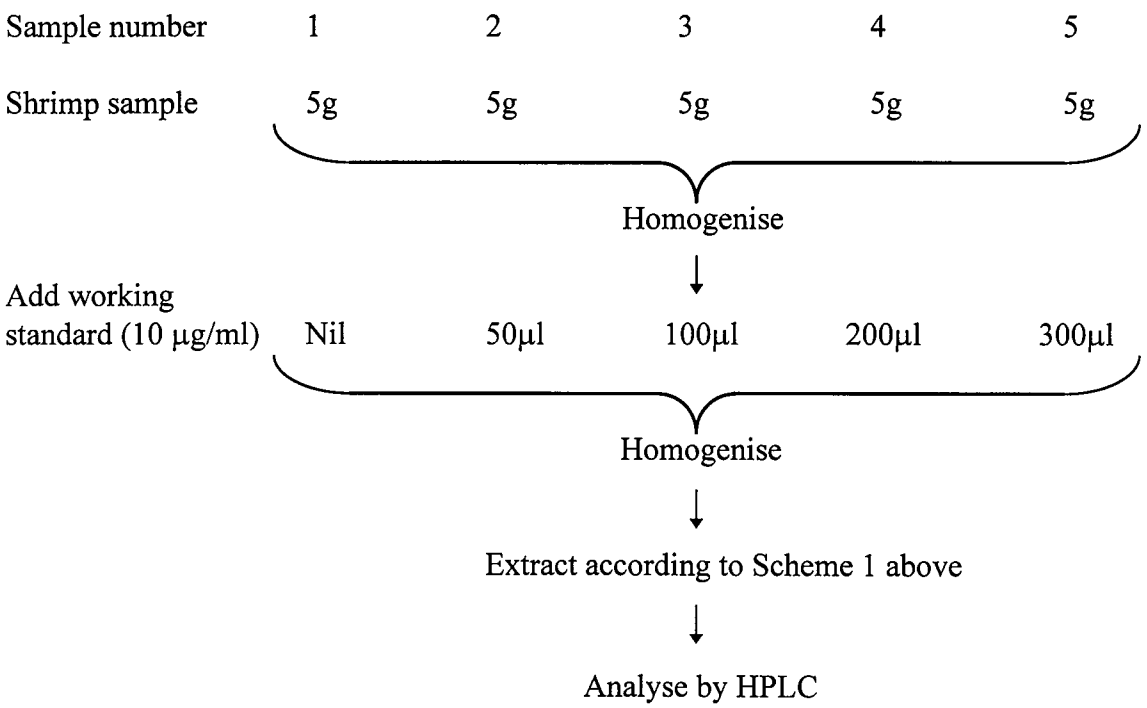
Sample Preparation:

Sample of large fish such as salmon and trout should be taken as steaks (minimum 5 samples) following the procedure outlined by AOAC. A representative sample from the product lot (shrimp, fish, crab, lobster etc.) should be collected and stored at -30 to -40°C to maintain sample integrity. The samples are prepared according to Scheme 1.

Scheme 1. Preparation of Samples for Extraction of Drug Residues



Scheme 2. Fortification and Extraction of Furazolidone from Spiked Shrimp Samples



Apparatus:

(1) HPLC System: The system used included a Waters 717 plus Autosampler,Waters 486 Tunable Absorbance Detector, Waters Temperature Control Module and Oven.

(2) Pump System: Waters 6510 HPLC pumps with capacity to generate rapid gradient and at least 4000 psi.

(3) Column: Vydac 201T54, 25 x 4.6 mm, Id., was used without a guard.

(4) Column Oven: Waters , Model Code CHM, Serial No. MX4MM7468M.

(5) Data Processing: Millennium 2010 Chromatography Manager equipped with Millennium computer software and coupled to COMPQ (Prolinear 4/66) Data Station with a NEC Printer (Model P1200) (NEC Technology, Hong Kong Ltd.).

(6) Effluent Monitor: Waters 486 Absorbance detector has sufficient sensitivity between 280-288nm and is an UV-Visible monitor.

(7) Syringes: Plastic disposable 5ml with 26g x 1.5 inch needle.

(8) Filters: Nylon 13mm syringe filters with 0.2 μ pore size.

(9) Centrifuge: Sorval model.

(10) Homogenizer: Brinkman Polytron.

(11) Glassware: Polyethylene centrifuge tubes, 50ml; glass centrifuge tubes, 50ml; round bottomed flasks, 50 or 100ml.

Reagents:

(1) Solvents: Acetonitrile (HPLC grade), glass distilled water, all other solvents ACS grade or better.

(2) Stock solutions of Standards (0.10 mg/ml): SDM and OMP standards were obtained from Hoffman-LaRoche while TMP and SDZ were products from Sigma Chemical Company. Weigh 10mg of each standard into a 100 ml volumetric flask. Dissolve the standard and bring each flask to volume with acetonitrile. TMP may require a drop or two of trifluoroacetic acid.

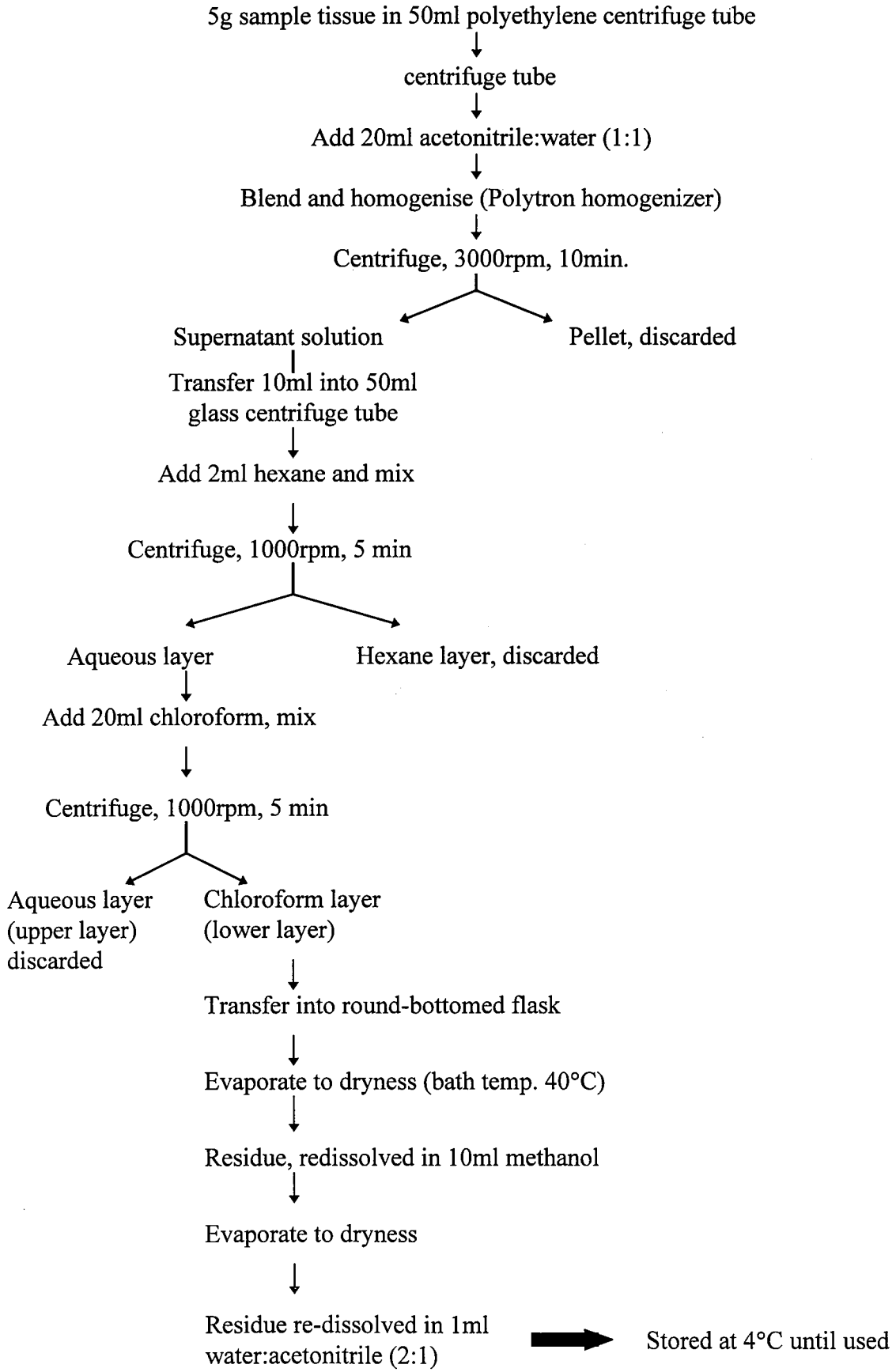
(3) Working standards (0.001 mg/ml): Pipette 1.0ml of each stock solution into a 100ml flask and dilute to volume with glass distilled water:acetonitrile (2:1).

(4) Mobile Phase: Solvent A - Glass distilled water, degassed.
Solvent B - Acetonitrile with 0.1% trifluoroacetic acid (TFA).

Procedure:

Extraction and clean up: See the flow diagram (*Scheme 3*).

Scheme 3


5g sample tissue in 50ml polyethylene centrifuge tube
centrifuge tube
Add 20ml acetonitrile:water (1:1)
Blend and homogenise (Polytron homogenizer)
Centrifuge, 3000rpm, 10min.
Supernatant solution
Pellet, discarded
Transfer 10ml into 50ml glass centrifuge tube
Add 2ml hexane and mix
Centrifuge, 1000rpm, 5 min
Aqueous layer
Hexane layer, discarded
Add 20ml chloroform, mix
Centrifuge, 1000rpm, 5 min
Aqueous layer (upper layer) discarded
Chloroform layer (lower layer)
Transfer into round-bottomed flask
Evaporate to dryness (bath temp. 40°C)
Residue, redissolved in 10ml methanol
Evaporate to dryness
Residue re-dissolved in 1ml water:acetonitrile (2:1)
Stored at 4°C until used

HPLC Analysis:

Preparation for HPLC: Use 0.2 μ syringe to directly filter sample into a 1.5ml autosampler vial.

Chromatographic conditions: The column system was operated at 35°C. The system was stabilised with 100% solvent A pumped at 1.0 ml/ml. The gradient used was as follows:

Time(min)	% Solvent A	% Solvent B
	100	0.0
0.1	92.0	8.0
7.0	85.0	15.0
24.0	30.0	70.0
30.0	100.0	0.0
35.0	100.0	0.0

After the gradient is over equilibrate for 11 min and then return the column to initial conditions over a period of 6 min and allow to equilibrate for 5 min before the next run is assumed.

Analysis: The system was standardised for peak height and retention time by at least two repeat injections of the mixed standard. Each sample (50μl) was injected and peaks identified by comparison of retention times with standards.

Calculations: To determine the μg of a drug in the sample formula of Burns et al[10] was used.

$$\frac{PH}{PHS} \times D = \text{Total drug in the original sample tissue}$$

where PH = Peak height/ 50μl injection

PHS = Peak height per μg standard

D = Dilution factor

The concentration of drug is normally expressed on a wet-basis such as μg/g wet weight (ww). To obtain this value simply divide the total analyte in μg by the weight of the original tissue extracted (W) in grams.

When 50 μl of sample is injected the dilution factor is 50.

$$\mu g \text{ drug/g ww} = \frac{PH \times 50}{PHS \times W}$$

Sample calculation for SDZ:

(1) $\frac{18.71}{19.20} \times \frac{50}{50} \times 100 = 97.4\,\%$

(2) $\frac{15.61}{19.20} \times \frac{100}{100} \times 100 = 81.3\,\%$

(3) $\frac{8.91}{19.20} \times \frac{200}{200} \times 100 = 81.4\,\%$

(4) $\frac{12.65}{19.20} \times \frac{300}{300} \times 100 = 66.0\,\%$

Results And Discussion:

Different concentrations of standard SDM, SDZ, TMP, and OMP were used to establish separately calibration curves. *Figures 1*, *2*, *3*, and *4* depict the calibrations curves. These curves indicate that the method used is sensitive enough to simultaneously detect residues of interest. The average retention times for SDZ, SDM, TMP and OMP were found to be, respectively, 9.61, 18.45, 21.43 and 21.91 minutes. The slopes of the calibration curves were linear with correlation coefficient values ranging between 0.9984 and 0.9988. Standard errors in these analysis were found to be 5.3%(TMP), 8.8%(SDZ), 10%(OMP), and 13.8%(SDM). *Figures 5*, *6*, *7*, and *8* depict elution profiles of these residues on a Vydac column used in this work. *Figure 9* is a chromatogram showing an elution pattern of the same analytes when injected as a mixture and chromatographed on the same HPLC column under similar conditions.

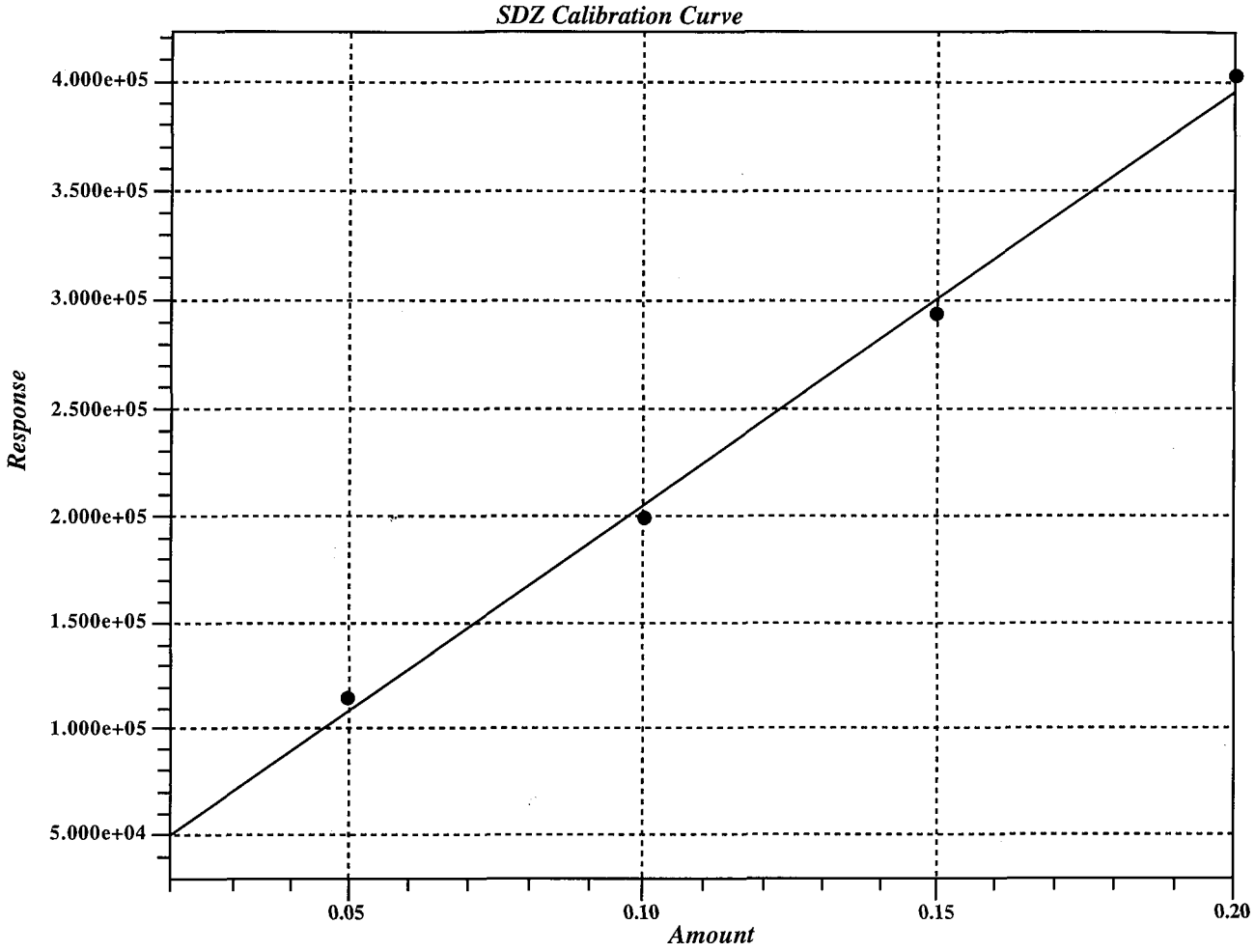
Table 1 summarises the results of an experiment in which sample of shrimp were fortified with known concentrations of the drug residues and then extracted using the approved protocol outlined above. Per cent recoveries of individual residues were calculated and were found to be 81.5%, 77.3%, 84.5%, and 109% for SDZ, SMZ, TMP, and OMP, respectively. Some component of shrimp origin which may have been co-extracted with the residues caused difficulty in estimating precisely the per cent recovery of TMP and OMP. Since these co-extractants perhaps absorbed light at

wavelengths similar to those for TMP and OMP the values calculated for their recoveries were higher than 100% as is evident from *Table 1*.

Table 1. Recovery of Analyte Residues from Fortified Shrimp Samples

Analyte	Amount added (ng)	Amount recovered (ng)	% Recovery
SDZ	50	48.7	97.4
	100	81.3	81.3
	200	162.7	81.4
	300	197.7	66.0
		Average: 81.5%	
SDM	50	30.0	60.0
	100	81.0	81.0
	200	152.0	76.0
	300	275.0	92.0
		Average: 77.3%	
TMP	50	32.3	64.5
	100	101.0	101.0
	200	316.0	158.0
	300	264.6	88.8
		Average: 84.5%	
OMP	50	34.4	68.6
	100	107.0	107.0
	200	337.0	168.0
	300	282.0	94.0
		Average: 109.4%	

Figure 1. Calibration Curve for Sulfadiazine (SDZ)



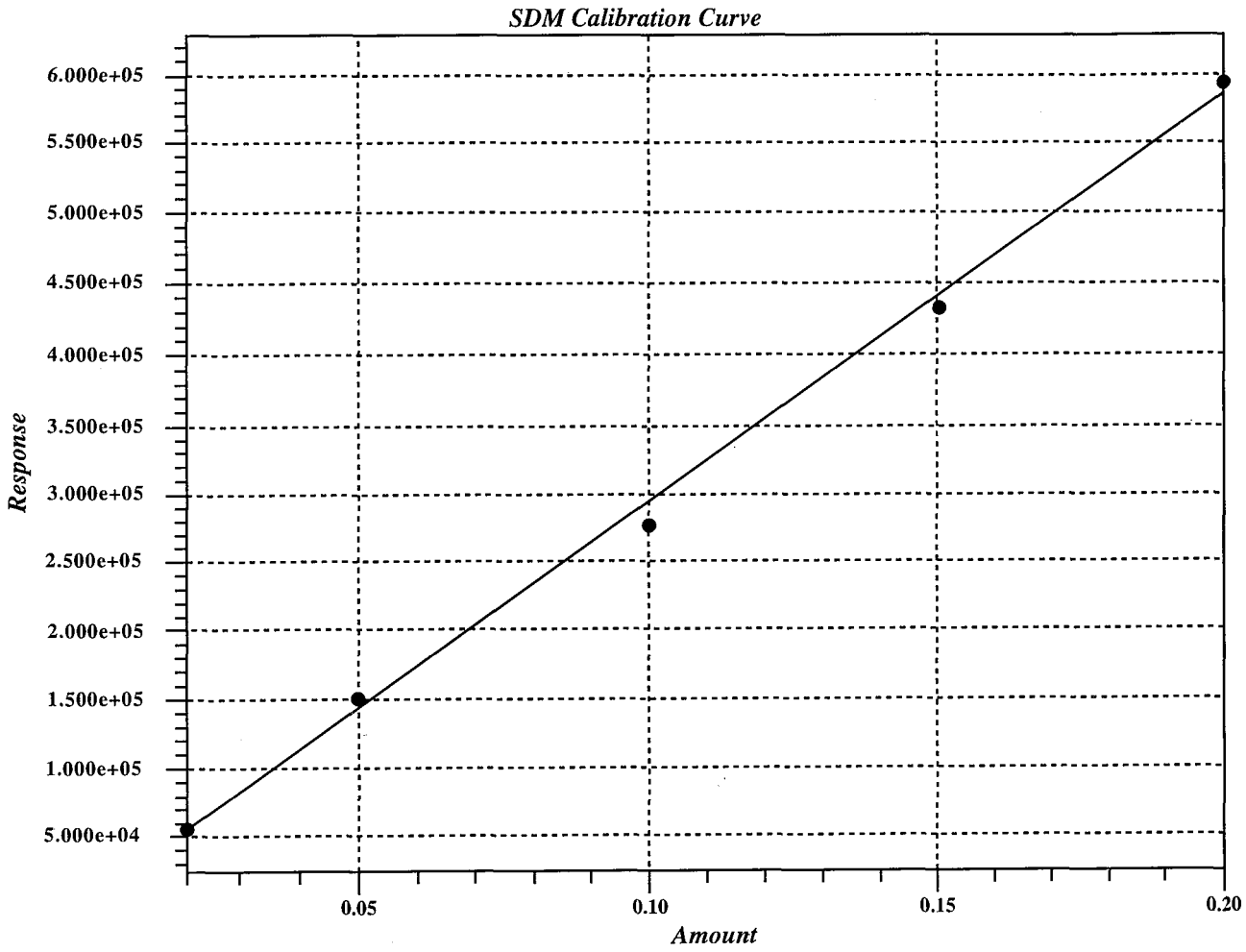
SDZ Calibration Information

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	17-DEC-96
Type	LC	Name	SDZ
Retention Time	9.733 min	Order	1
A	10536.256098	B	1935559.210600
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998537	R^2	0.997077
Standard Error	8835.200296		

SDZ Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.020000	49550.270000	0.020156	0.782	Yes
2	0.050000	114368.468000	0.053645	7.289	Yes
3	0.100000	197228.952000	0.096454	-3.546	Yes
4	0.150000	292343.647000	0.145595	-2.937	Yes
5	0.200000	405680.733000	0.204150	2.075	Yes

Figure 2. Calibration Curve for Sulfadizine (SDM)



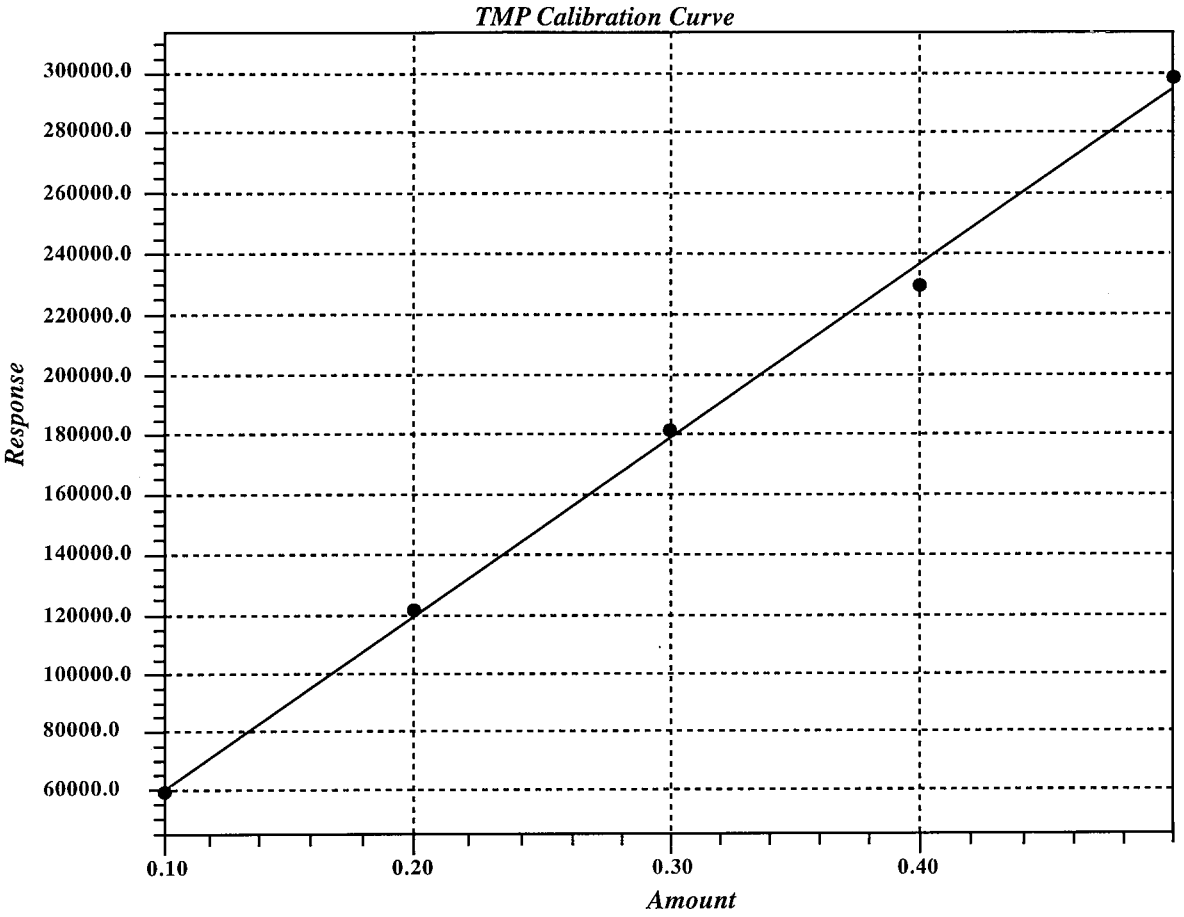
SDM *Calibration Information*

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	18-DEC-96
Type	LC	Name	SDM
Retention Time	18.433 min	Order	1
A	-5544.190171	B	2976050.376642
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998484	R^2	0.996970
Standard Error	13831.721025		

SDM Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.020000	56160.883000	0.020734	3.669	Yes
2	0.050000	154063.998000	0.053631	7.262	Yes
3	0.100000	276077.752000	0.094629	-5.371	Yes
4	0.150000	432549.913000	0.147207	-1.862	Yes
5	0.200000	600972.699000	0.203799	1.900	Yes

Figure 3. Calibration Curve for Trimethoprim (TMP)



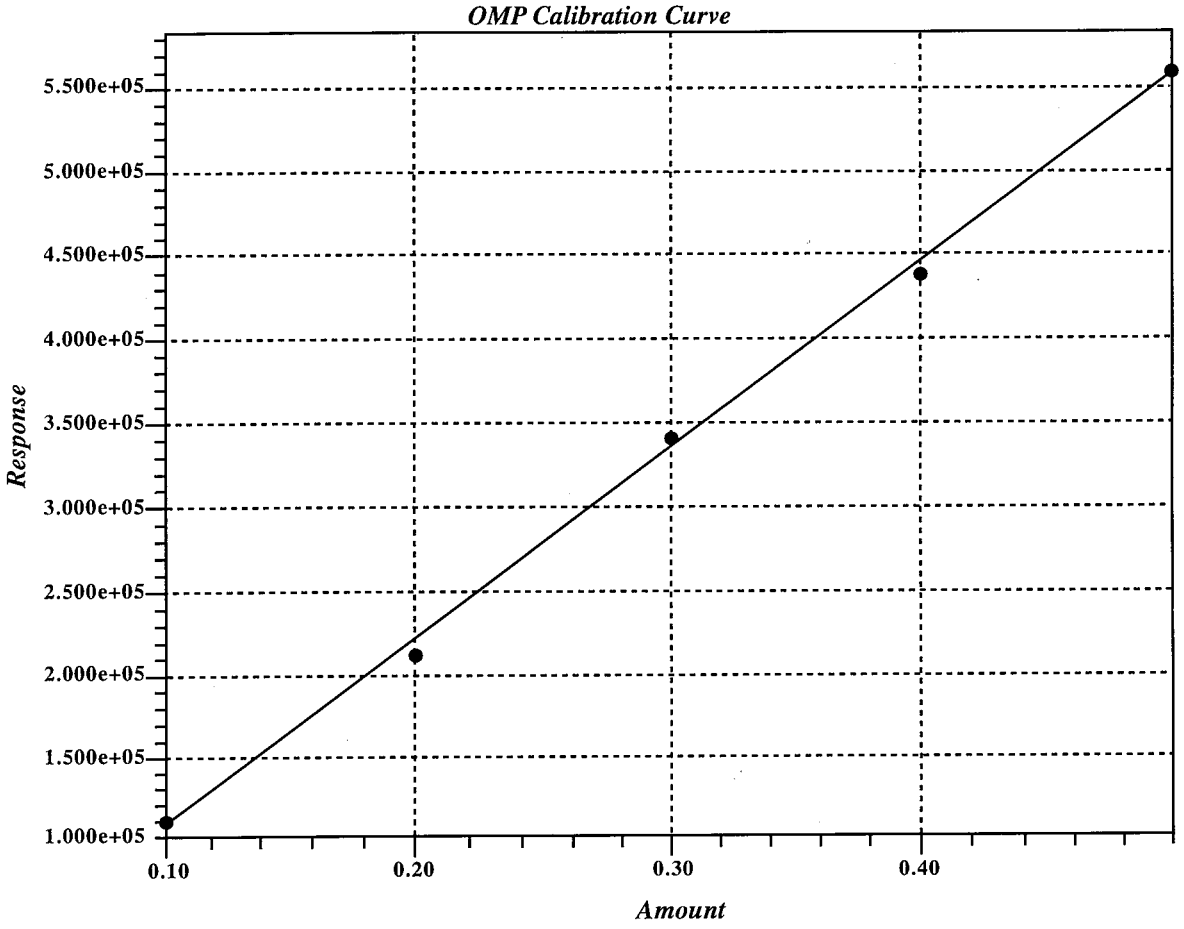
TMP Calibration Information

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	18-DEC-96
Type	LC	Name	TMP
Retention Time	21.150 min	Order	1
A	-825.516703	B	598510.737408
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998844	R^2	0.997689
Standard Error	5259.392120		

TMP Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.100000	57820.741791	0.097987	-2.013	Yes
2	0.200000	121226.679199	0.203926	1.963	No
3	0.300000	181338.263137	0.304362	1.454	No
4	0.400000	231126.776751	0.387549	-3.113	No
5	0.500000	302126.061719	0.506176	1.235	No

Figure 4. Calibration Curve for Ormetoprim (OMP)



OMP Calibration Information

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	18-DEC-96
Type	LC	Name	OMP
Retention Time	21.567 min	Order	1
A	-6583.610951	B	1125397.937000
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998610	R^2	0.997223
Standard Error	10843.623381		

OMP Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.100000	110052.021000	0.103639	3.639	Yes
2	0.200000	210282.364000	0.192702	-3.649	Yes
3	0.300000	340988.369744	0.308844	2.948	Yes
4	0.400000	431927.849000	0.389650	-2.587	Yes
5	0.500000	561928.247000	0.505165	1.033	Yes

Figure 5. Representative Chromatogram of 20ng of Sulfadiazine detected by UV Detector at 288nm under the conditions employed in this work.

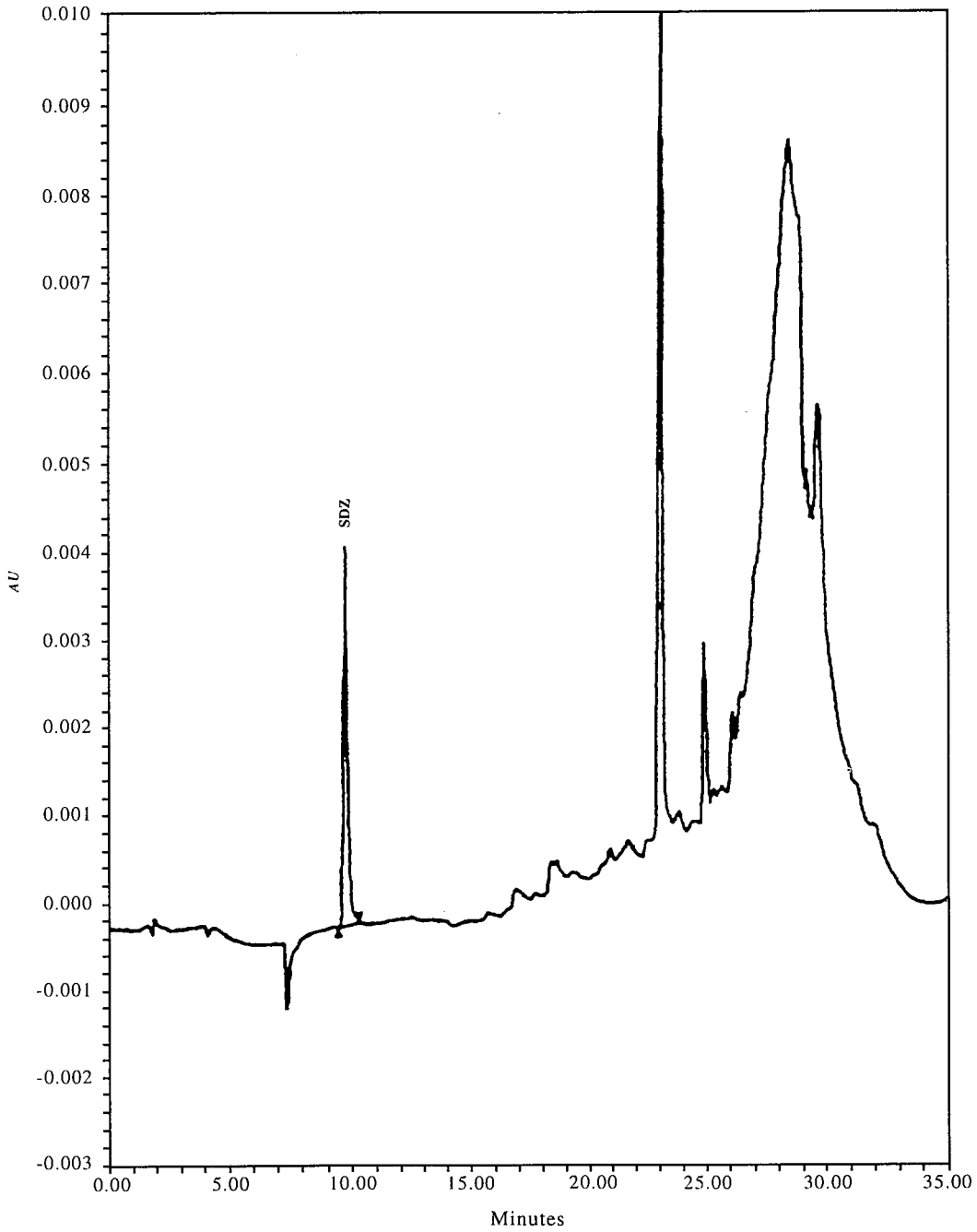


Figure 6. Representative Chromatogram of 20g of Sulfadimethoxin detected at 288nm under the conditions described in the text.

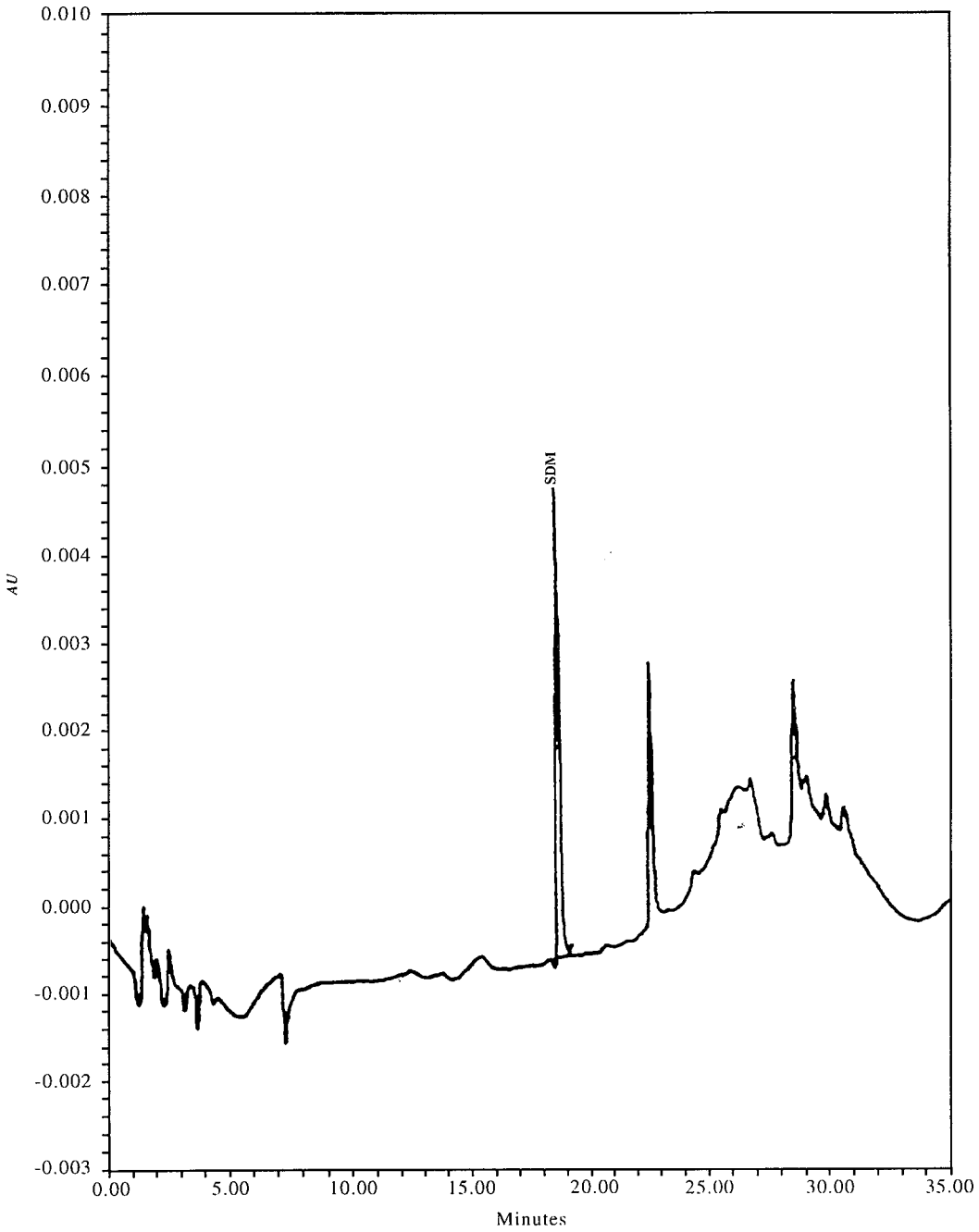


Figure 7. Representative Chromatogram of 200ng of Trimethoprim detected at 288nm under the conditions outlined in the text.

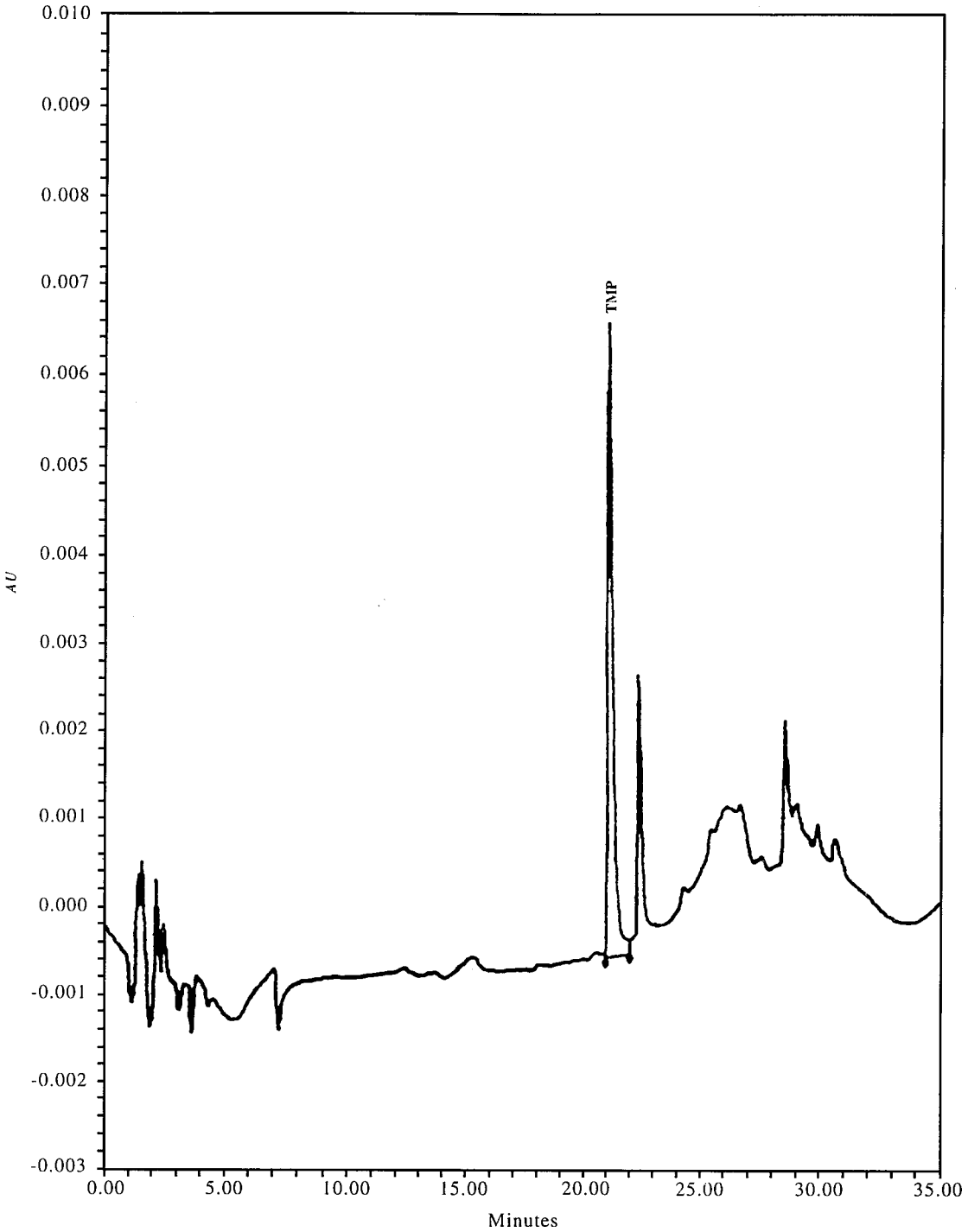


Figure 8. Representative Chromatogram of 200ng of Ormetoprim under the conditions described in the text.

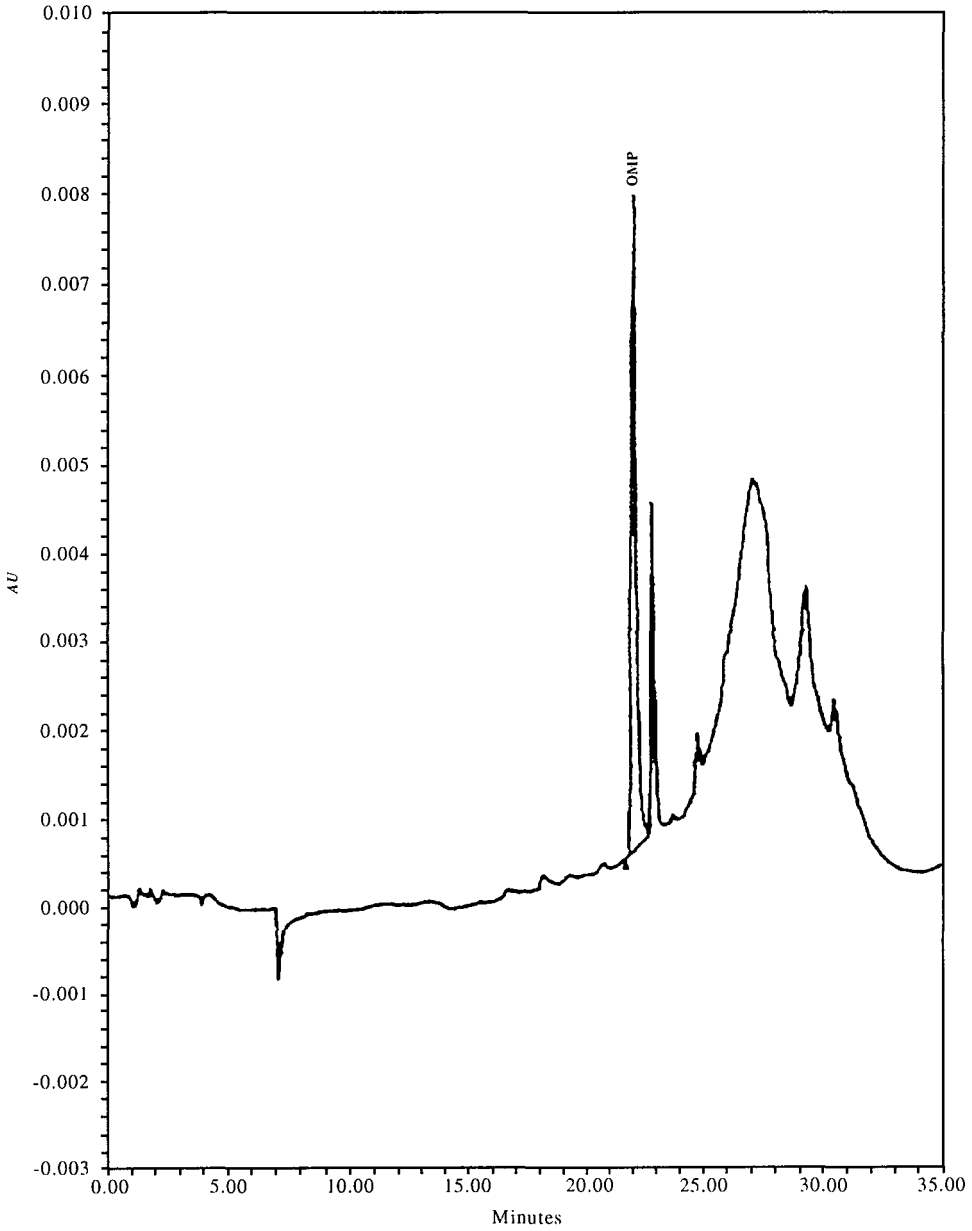
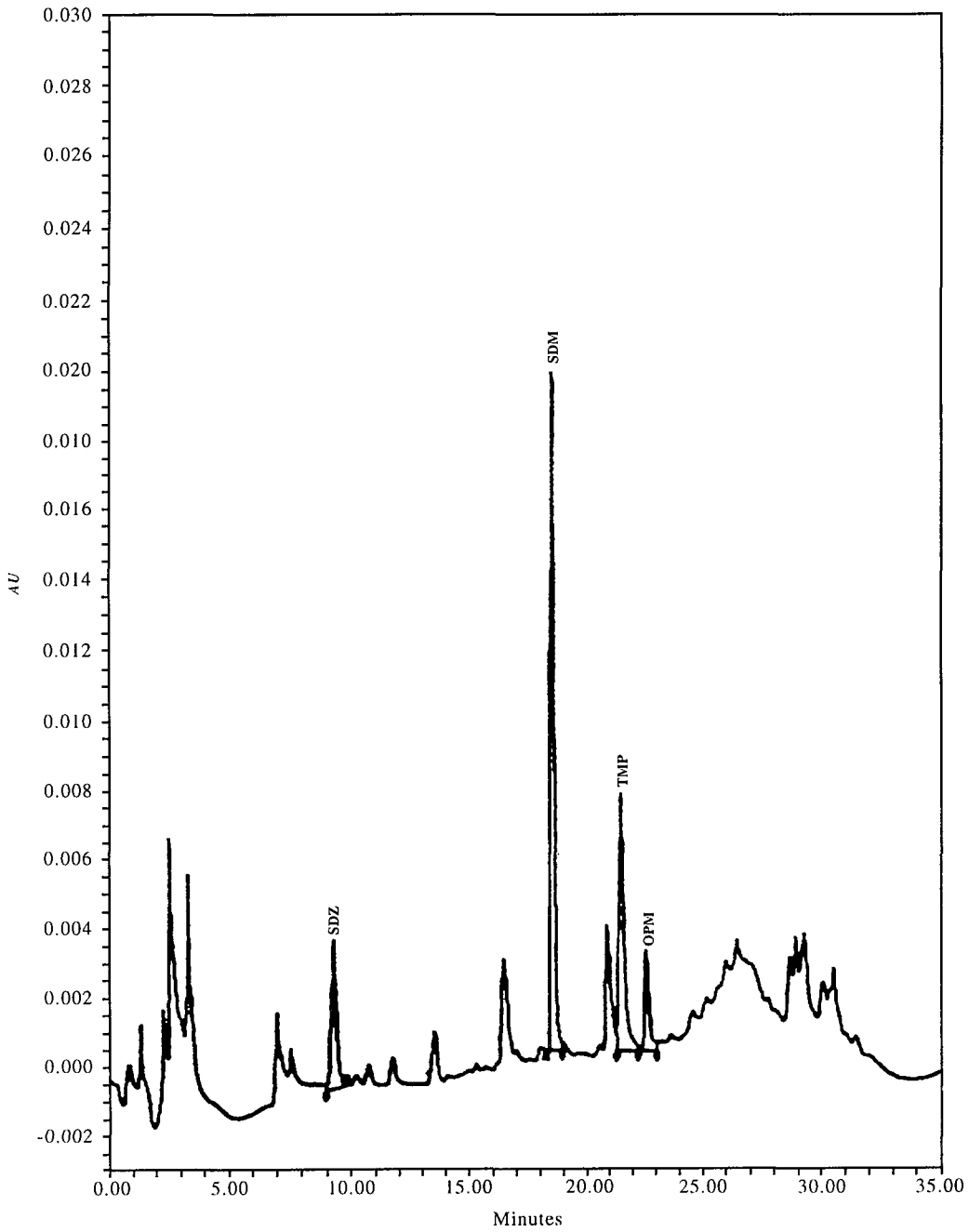


Figure 9. Liquid Chromatogram of 100ng of each of SDZ, SDM, TMP, and OPM obtained on Vyadac 201T54 Column under the conditions described in the text. UV Detection was monitored at 288nm.



REFERENCES:

(1) Boison, J. O. and J. D. MacNeil (1995). New Test Kit Technology. In Chemical Analysis for Antibiotics used in Agriculture. H. Oka, A. Nakazawa, K. Harada, and J. D. MacNeil (Eds).AOAC International, Arilington, VA, USA.

(2) de Kinkelin, P., and C. Michel (1992). the use of drugs in Aquaculture. Infofish International 4: 45

(3) Thomas, M.H., and et al (1983). Quantitative thin layer chromatographic multisulfonamide screening procedure. Collaborative Study. J. Asso. Off. Anal. Chem. 66: 884.

(4) Reimer, G.J., and A. Suarez, (1991). Development of screening method for five sulphonamides in Salmon muscle tissue using thin layer chromatography. J. Chromatogr. 555: 315

(5) Samson R. M. m F. B. Suhre, and J. W. Shaffer (1985) Quantitative gas chromatographic-mass spectrophotometric assay of four sulfonamide residues in animal tissues. J. Asso. Off. Anal. Chem. 68: 23.

(6) Reimer, G.J., and A. Suarez (1992). Liquid chromatographic confirmatory method for five sulfonamides in Salmon muscle tissue by matrix solid phase dispersion. J Asso. Off. Anal. Chem. & %: 979

(7) Weiss, G., P. D. Duke, L. Gonzales (1987). HPLC method for the simultaneous analysis of sulfadimethoxine and ormetoprim in tissue and blood of cattle, chickens, and catfish. J. Agric. Food Chem. 35: 905.

(8) Walisser, J. A., H. M. Burt, T. A. Valg (1990). High performance liquid chromatographic analysis of Romet 30 in Salmon following administration of medicated feed. J. Chromatogr. 518: 179.

(9) Horie, M., and et al (1991). simultaneous determination of residual synthetic antibacterials in fish by HPLC. J. Chromatogr. 538: 484

(10) Burns, B,G., and et al (1996). The simultaneous determination of the residual components of Romet 30 and Tribrissen in salmonid products by HPLC. Can. Technical Report of Fisheries and Aquatic Sciences, No. 2008.

C. DETERMINATION OF FURAZOLIDONE IN SHRIMP USING HPLC METHOD

Scope:

Furazolidone is a synthetic nitrofuran antimicrobial that is effective in treating bacterial and fungal infections in fish and shrimp[1]. The use of this is prohibited in United States and Canada because of its carcinogenic properties. There are no laws to restrict its use in many other countries. Thus, residues of this drug may be present in aquaculture products and may represent a threat to human health.

In recent years fish for food have been artificially cultivated on a large scale in both fresh water and sea water in many countries. Oxolinic acid, nalidixic acid, and piromidic acid are antibacterial agents that are widely used in the cultivation of fish such as salmon, rainbow trout, sweetfish, carp, eel, and yellowtail.

In Japan the Food Safety Law established a zero residue level for all antimicrobial agents in foods in 1971. Hence, routine screening of above drug residues in cultivated fish is necessary, but the available methods of analysis are limited. Microbiological assays for oxolinic acid lack sensitivity and specificity.

Chemical methods for quantitation of furazolidone in tissues of terrestrial and aquatic animals have been developed[2-5]. The method described here is one reported by Stehly et al.[5].

Principle:

The HPLC method described involves extraction of furazolidone from shrimp using acetonitrile, and the extract is taken to dryness. The residue is dissolved in acetonitrile , and the solution is passed through alumina and C-18 cleanup columns. The eluate is taken to dryness and re-dissolved in a suitable solvent for reverse phase (C-18) liquid chromatography with UV detection at 365nm.

Materials:

(1) Blender: Warring 2-speed commercial blender, 50-250 ml container.

(2) Centrifuge tubes: Polepropylene, 15 and 50ml capacity.

(3) Homogenizer: Brinkmann PT 10-35, (Brinkmann Instruments, Inc. Westbury, NY.)

(4) Boiling flasks: Round-bottom flask, 100ml, and a pear shaped flask, 25ml.

(5) Filter: Millex-FG, 0.2μm, 13mm filter unit (Millipore Products Division, Bedford, MA).

(6) HPLC system: The system included A Waters 717 plus Autosampler, Waters 486 Tunable Absorbance Detector, Water Temperature Control Module and Oven.

(7) Pump system: Waters 510 HPLC pumps with capacity to generate rapid gradient and at least 4000 psi.

(8) HPLC column: Beckman 5 um Ultrasphere (254 x 4.6mm, id.) with 5µm Adsorbosphere C18 (10 x 4.6mm) guard column (Althech Associates Inc., Deerfield, Il., USA)

(9) Solvents: LC reagent-grade acetonitrile (J. T. Baker Inc., Philipsburg, PA., USA)

(10) Sodium sulfate: Anhydrous powder, reagent grade (J. T. Baker)

(11) Standard: Furazolidone (Sigma Chemical Co., St. Louis, MO)

Procedure:

(a) Fortification and Recovery Experiment:

Sample No.	1	2	3	4	5
Amt. of homogenised shrimp	1g	1g	1g	1g	1g
Amt. of furazolidone added (1µg/ml)	0	50	100	150	200

Homogenize separately

↓

Incubate, 15 min

↓

Extracted according to the scheme given below

↓

Final preparation in ml of 0.01% H_3PO_4:acetonitrile (80: 0)

←

Inject 50µl into HPLC

Scheme 1

Sample Preparation

↓

Sample (cleaned shrimp)

↓

Remove tail and chitin shell

↓

Homogenize

↓

Weigh 1g in 50 ml polypropylene centrifuge tube

↓

Add 0.05g anhyd. Na_2SO_4 and 35 ml acetonitrile

↓

Incubate, 10 min

↓

Homogenize, 15 sec.

↓

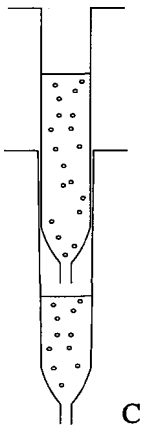
Wash homogenizer probe with 3 ml acetonitrile

↓

Homogenize, 15 sec.

↓

Filter homogenate through pre-washed extraction column (15 ml with 20 um, polyethylene frits)



↓

Collect effluent into 100 ml boiling flask

↓

Evaporate to dryness (35°C)

↓

Residue, redissolved in 2 ml acetonitrile

↓

Pass through prewashed C18 column connected below an alumina column

↓

Collect filtrate in a 25 ml flask (total 6 ml)

↓

Evaporate to dryness

↓

Residue, redissolved in 500 µl solution of 0.01% H_3PO_4 : acetonitrile (80 : 20)

↓

Sonicate (30-60 sec) and pass through Millex filter

←

Inject 50 µl into HPLC

(b) Liquid chromatography: Prepare standard solutions containing 5, 10, 40, 80, 100, and 150ng furazolidone in 0.01% H_3PO_4:acetonitrile (80:20). The solutions are stable for one week if protected from direct light and refrigerated when not in use.

Set UV detector at 365nm and use mobile phase 0.01% H_3PO_4 - acetonitrile (70 + 30) at a flow rate of 1 ml/min to elute furazolidone standards at ca. 6.5 min. Plot standard curve from 50µl injections of the standards. Determine concentrations of furazolidone in shrimp by comparing integrated peak area for injected extract with standard curve.

Temperature of the HPLC column was maintained at room temperature and effluent was monitored at 365nm using Water Tunable Absorbance Detector.

(c) Data Processing: Millennium 2010 Chromatography Manager equipped with Millennium computer software and coupled to COMPQ (Prolinear 4/66) Data Station with a NEC Printer (Model P1200) (NEC Technology, Hong Kong Ltd).

Results:

Table 2 shows recovery of furazolidone from spiked shrimp homogenates while *Table 3* summarises the results obtained with different concentrations of standard furazolidone. Recovery values calculated for spiked samples were consistent between 50 and 200ng/g range and averaged 83%. Other workers have reported recovery values ranging between 77 and 90%.

Although in the present work lower levels (5 - 40ng/g) were not tested Stehly et al[5] have successfully demonstrated that this method is suitable for the quantitation of low levels of furazolidone extracted from shrimp.

Retention time for standard furazolidone under the conditions used ranged between 6.2 and 6.53 min while the average value was found to be 6.53 min. Under the conditions the values obtained for the spiked samples ranged between 6.15 and 6.30 min with an average value of 6.2 min.

Figure 10 depicts an elution profile of 80ng sample of standard furazolidone injected into HPLC and a similar peak was obtained from the residue extracted from spiked shrimp samples as seen in *Figure 11*. A calibration curve obtained by injecting different concentration of standards is shown in *Figure 12*. The plot reveals a linear relationship between the concentration of the drug and the peak height. The correlation coefficient value obtained agrees with the theoretical value expected.

Table 2. Recovery of Furazolidone from Spiked Shrimp Homogenates

Sample No.	Amount Added (ng)	Peak Area	Peak height	% Recovery	Retention time (min)
1.	Nil	--	--	--	--
2.	50	4252	207	114	6.22
3.	100	5252	361	71	6.18
4.	150	9649	521	86	6.17
5.	200	8992	603	60	6.15
STD	50	3724	332	100	6.30

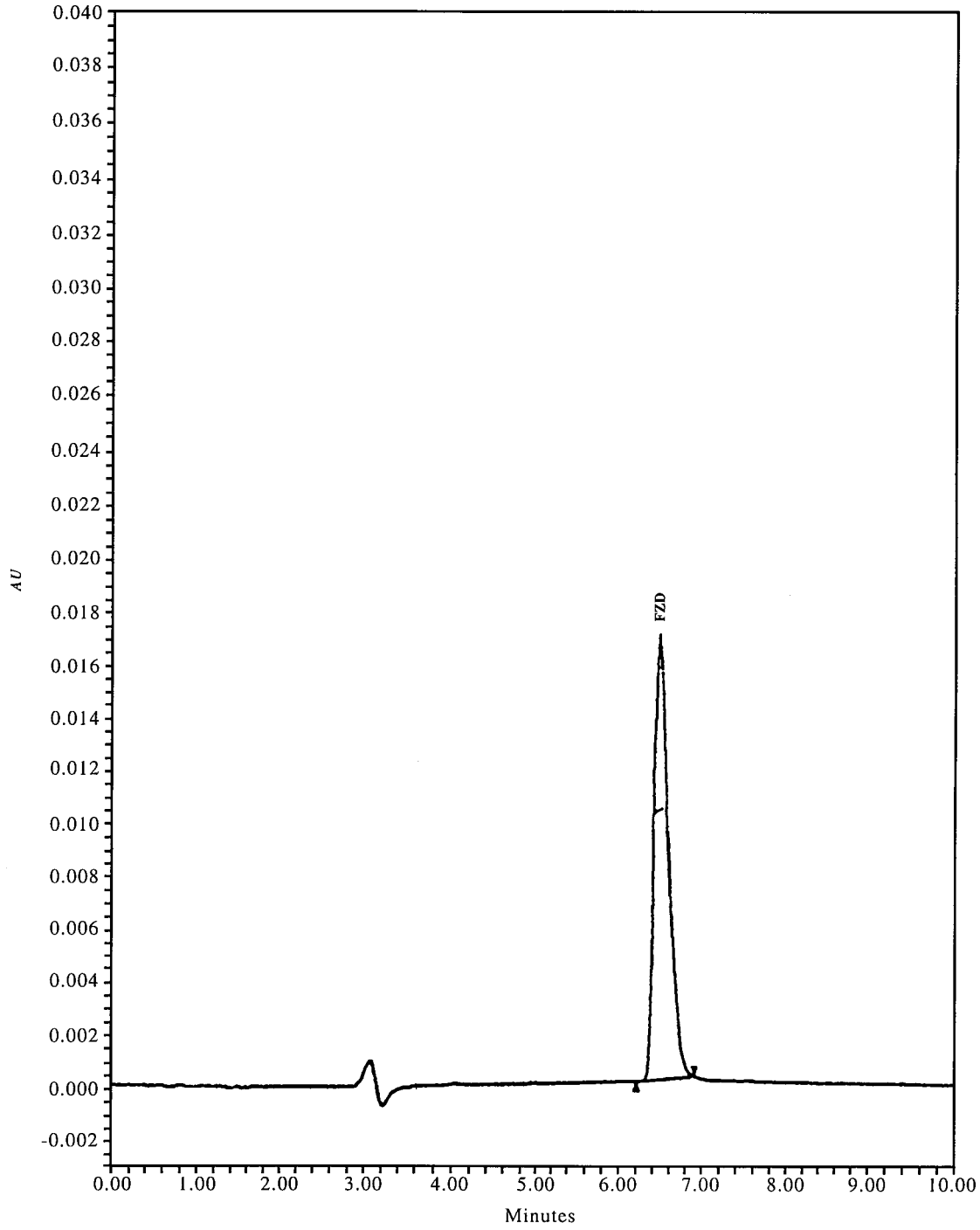
Average % Recovery = 83
Average retention time = 6.2 min

Table 3. Calibration Curve for the Standard Furazolidone

Amout injected (ng)	Peak height	Peak Area	Retention time (min)
5	971	11156	6.38
10	2134	24402	6.42
20	4211	48387	6.43
40	8536	102361	6.45
80	16838	206177	6.48
100	20204	256557	6.48
150	30104	390638	6.53

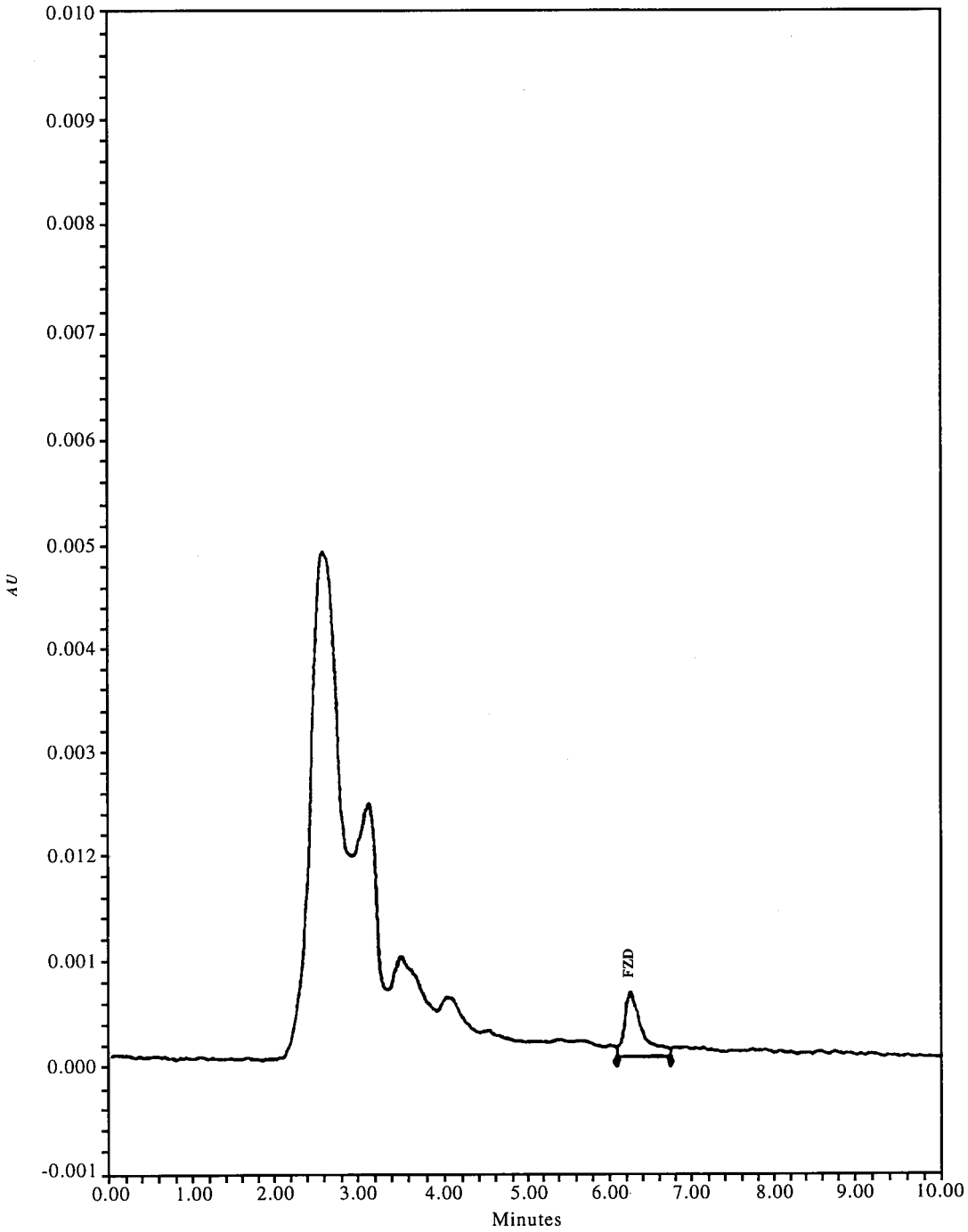
Correlation coefficient (R) = 0.9999
Average retention time = 6.53 min.
Coefficient of determination 9R (R) = 0.9999

Figure 10. Representative chromatogram of 80ng of Furazolidone from an Ultraphere column under the conditions described in the text. UV detection was monitored at 365nm.



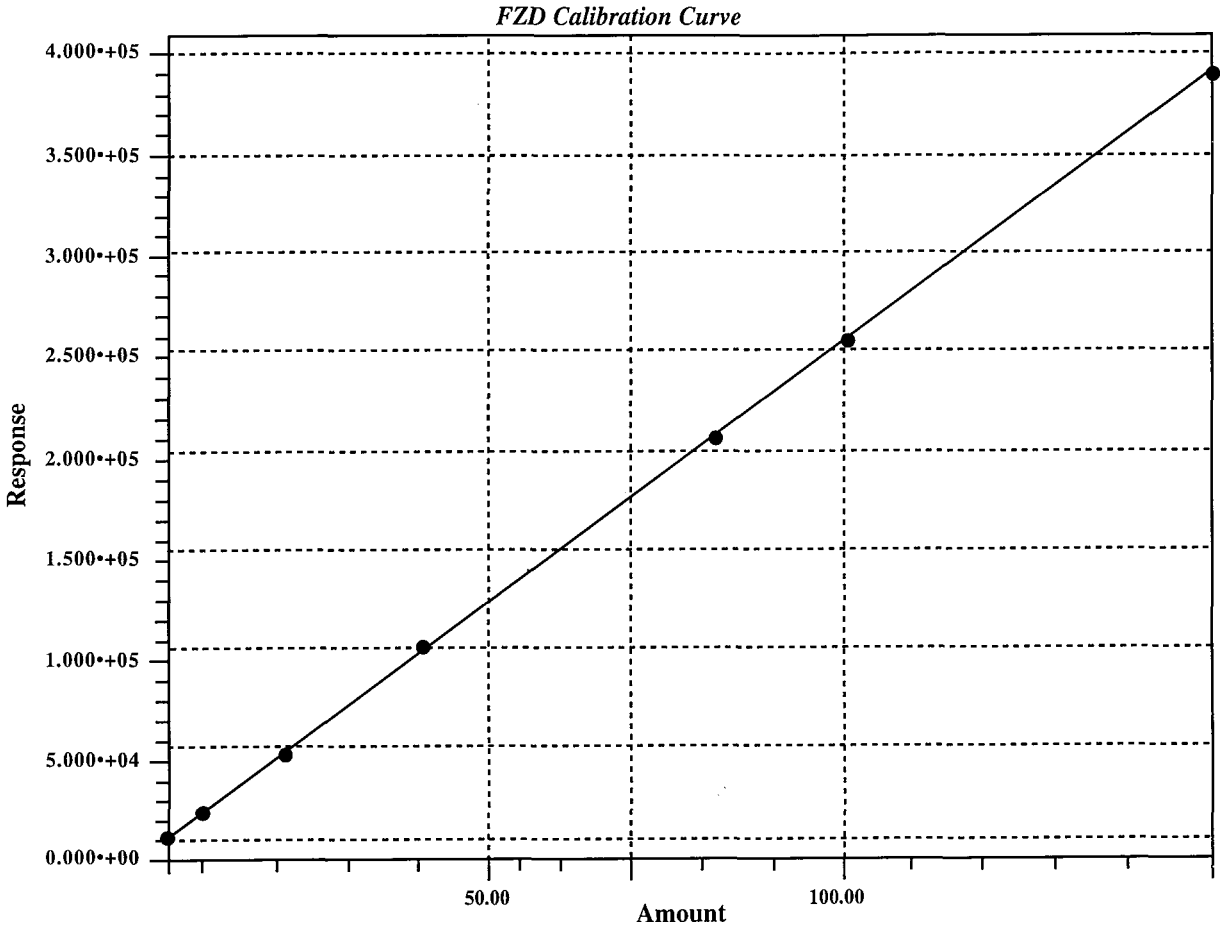
Sample Name: Std. FZD 80ng Vial: 1 Inj: 1 Ch: 486 Type: Standard

Figure 11. Representative chromatogram of spiked shrimp homogenate (20ng/g). UV detection was monitored at 365nm.



Sample Name: REC.4 200µl Vial: 6 Inj: 1 Ch: 486 Type: Unknown

Figure 12. Calibration Curve Obtained with Different Concentrations of Standard Furazolidone



OMP Calibration Information

Processing Method	Default	System	EDTA
Channel	486	Date	24-DEC-96
Type	LC	Name	FZD
Retention Time	6.383 min	Order	1
A	-2604.654023	B	2612.124164
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.999961	R^2	0.999922
Standard Error	1366.719624		

FZD Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	5.000000	11156.377529	5.26138	5.363	No
2	10.000000	24401.500333	10.338771	3.388	No
3	20.000000	48387.096757	19.521182	-2.394	No
4	40.000000	102360.997409	40.184021	0.460	No
5	80.000000	206177.158689	79.927982	-0.090	No
6	100.000000	256556.582025	99.214746	-0.785	No
7	150.000000	390637.995539	150.515160	0.363	No

REFERENCES

(1) Williams, R.R., and D. V. Lighter (1988). J. World Aqua. Soc. 19: 188

(2) Samuelson, O.B.(1990) J. Chromatogr. 528: 495

(3) Horie, M., and et al.(1991) J. Chromtogr. 538: 484

(4) Long, A.R. and et al. (1991) J. Assoc. Off. Anal. Chem. 74: 292

(5) Stehly, G.R. and et al (1994) J. Assoc. Off. Anal. Chem. 77: 901.

D. DETECTION AND QUANTITATION OF MALACHITE GREEN IN SPIKED SHRIMP SAMPLES

Scope:

Malachite green (MG) has been used as an effective treatment for external fungal and protozoan infections in fish[1-2]. MG belongs to the triphenylmethane class of dyes, some of which are carcinogens[3]. Scientists have demonstrated that MG causes significant development of abnormalities when administered to eggs of rainbow trout and pregnant New Zealand white rabbits.

In animals, MG is reduced to its colourless form, leuco MG[4-5]. The leuco form of MG is a precursor of the chemical during production and could be a contaminant in commercially prepared dye.

Principle:

The chromatic and leuco forms of MG have been determined simultaneously by Bauer et al[6]. The authors split the fish tissue samples into half and oxidised one half with the lead oxide (PbO_2). The oxidised sample was analysed for chromatic MG. The amount of leuco MG in the sample was determined by the difference between unaltered and oxidised subsamples. Chromatic MG and leuco MG can be analysed by HPLC system using visible spectrophotometry after postcolumn oxidation of leuco form to chromatic form. The method described here is that of Allen et al[7] and involves conversion of leuco to chromatic form by the treatment with lead dioxide.

Apparatus:

LC System:	Waters 717 system with Autosampler, Waters 486 tunable Absorbance Detector, Waters Temperature Control Module and Oven.
LC Pump system:	Waters 6510 pumps with capacity to generate rapid gradient and at least 4000 psi.
LC Column:	uBondapak C18, 300 x 3.9mm, id., particle size 10μm.
Spectrophotometer:	Waters 486 Tunable Absorbance Detector. Mg was monitored at 600nm instead of at 618nm because of the specification of the detector used.
Postcolumn reactor:	Postcolumn oxidation of leuco form. Stainless steel tube 32 x 4mm, id, packed with 10% lead dioxide (PbO_2) suspended in Celite 545 (PbO_2 is previously dry-mixed with Celite to give uniform mixture) and capped with 2μm frits. As the reactor is being packed with PbO_2 in Celite, gently tap it to prevent the formation of voids. Place postcolumn reactor in line between the HPLC column and the spectrophotometer detector.

Homogenizer:	Any high speed homogenizer.
Blender:	Warring or equivalent, equipped with a stainless steel cup.
Filtration column:	Bakerbond spe 6ml disposable filtration columns.

Chemicals And Reagents:

LC Mobile Phase:	Consisting of 85 + 15 mixture of methanol to aqueous acetate buffer (0.05 M sodium acetate and 0.1M glacial acetic acid in water)
Malachite green oxalate:	Cat. No. 1264 (Eastman Kodak Co., Rochester, NY)
Leuco malachite green:	Cat. No. 3620 (Eastman Kodak).
Anhydrous acetic acid:	cat.No.24,124-5 (Aldrich Chemical Co., Inc., Milwaukee, WI)
Anhydrous sodium bicarbonate:	Cat. No.S-8875 (Sigma Chemical Co., St. Louis, MO)
Sodium acetate (acetic acid, sodium salt, anhydrous):	Cat. No. 24,124-5 (Aldrich)
Anhydrous sodium sulfate:	Cat.No.7757-82-6 (Fisher Scientific, Pittsburgh, PA)
Solvents:	LC grade solvents, including glacial acetic acid, water, methanol, chloroform, and acetonitrile (J. T. Baker Inc.)
Lead oxide:	Cat. No.5727, ACS grade (Mallinckrodt Inc., Science Product Division, St. Louis, MO)
Celite 545:	Cat. No. C-212 (Fisher Scientific)
Malachite green oxalate and leuco malachite green stock solutions:	1mg/ml in methanol. Prepare standard solutions of malachite oxalate and leuco malachite green at concentrations of 200, 400, 800 and 1000ng/ml fresh daily in mobile phase for LC retention time markers.

Test Sample Preparation (fortification) and Extraction (recovery):

Sample No.	1	2	3	4	5
Fish sample (homogenised)	5g	5g	5g	5g	5g
Malachite green (ng)	0	200	400	800	1000

Homogenize

↓

Incubate, 30 min, room temperature

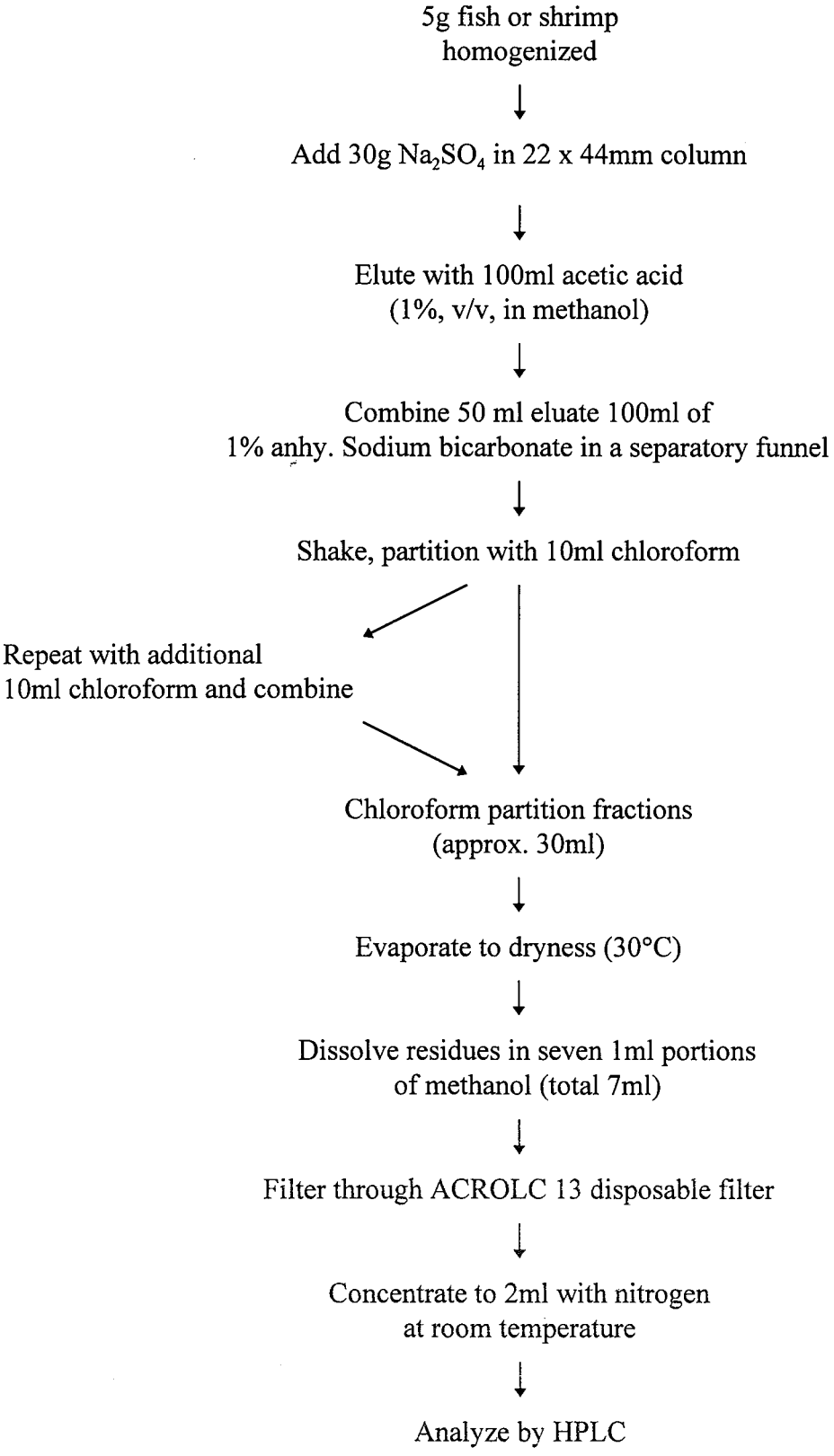
↓

Extract according to the scheme shown below

↓

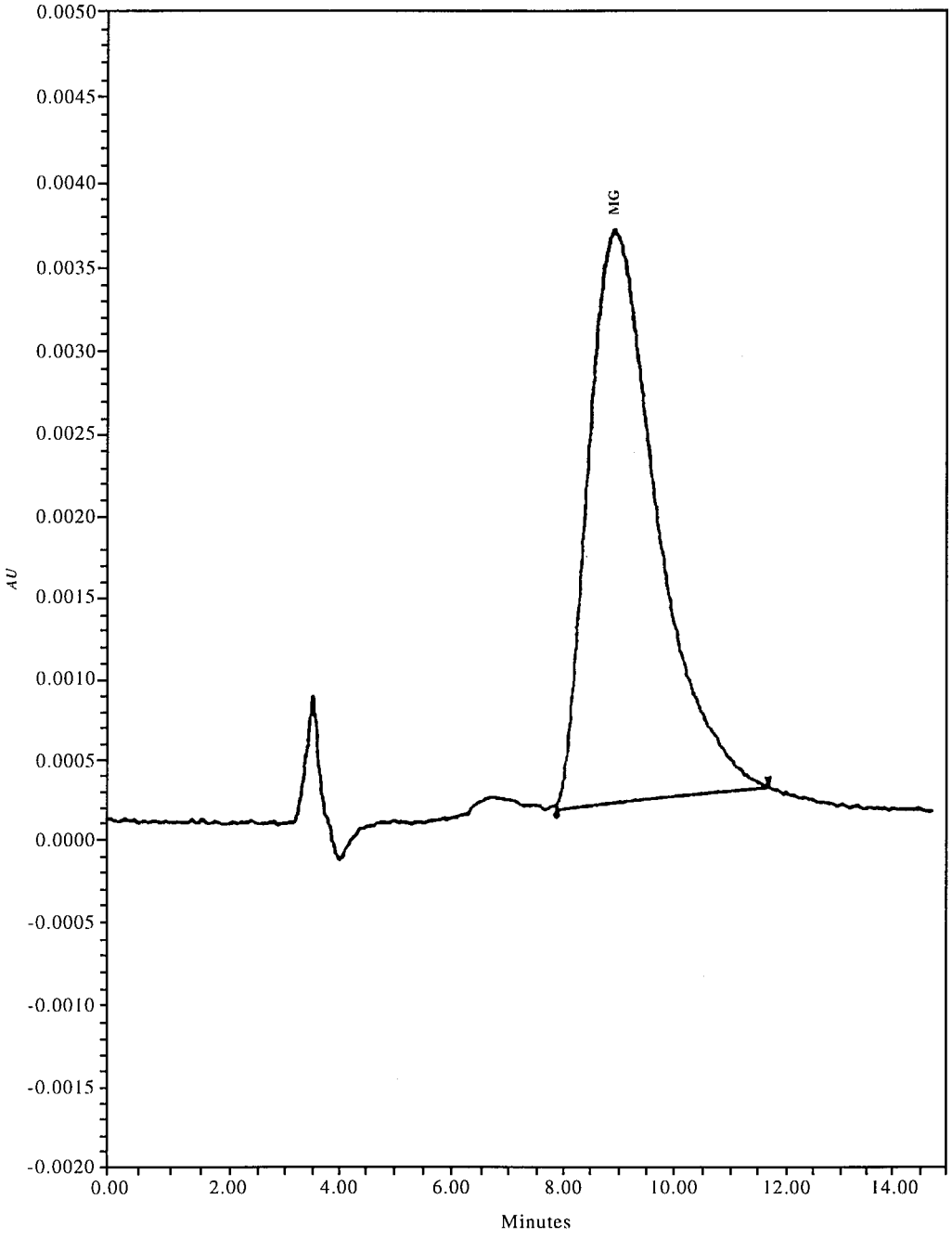
Final preparation
HPLC analysis

Sample Preparation:



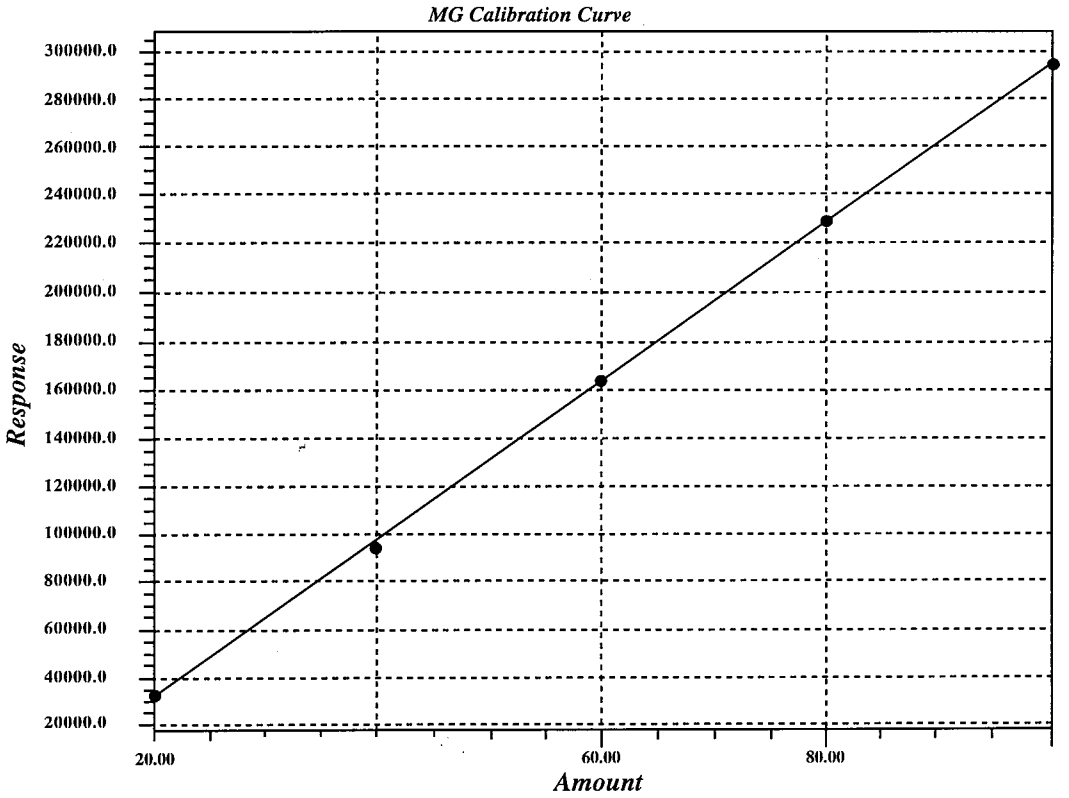
Results:

Figure 13. Chromatogram of 100ng of chromatic malachite green on a uBondapak C18 column with 1 ml/min of methanol-aqueous acetate buffer (85:15)



Sample Name: Std. MG 100 Vial: 1 Inj: 1 Ch: 486 Type: Standard

Figure 14: Calibration Curve for Malachite Green



MG Calibration Information

Processing Method	MG	System	EDTA
Channel	486	Date	02-JAN-97
Type	LC	Name	MG
Retention Time	9.333 min	Order	1
A	-34205.456400	B	3295.140210
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.999891	R^2	0.999782
Standard Error	1775.816900		

MG Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	20.000000	33189.682000	20.452889	2.264	Yes
2	40.000000	95094.384000	39.239556	-1.901	Yes
3	60.000000	163828.323000	60.098741	0.165	Yes
4	80.000000	230302.994000	80.272290	0.340	Yes
5	100.000000	295099.398000	99.936523	-0.063	Yes

Fortification and Recovery:

Total Residue Recoveries As Percent Of Total Fortification For Extracts Of Rainbow Muscle Tissue

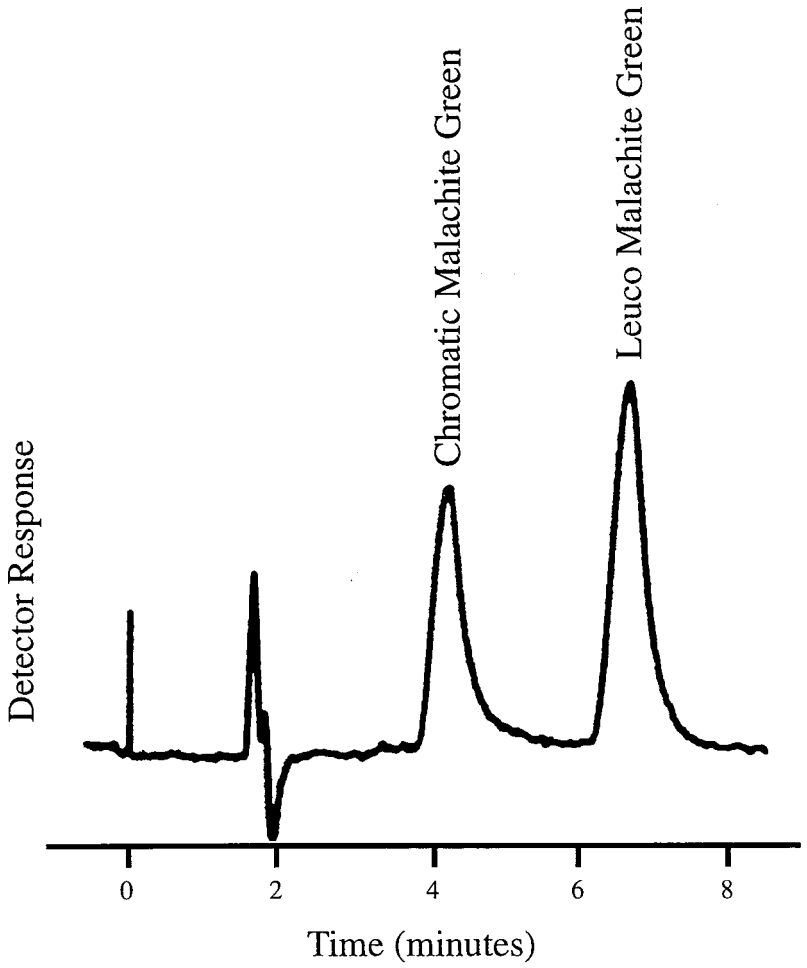
Fortification (ng/g)	Sample No.	Leuco MG	Chromatic MG
1000	1	85	15
	2	100	00
	3	100	00
	4	75	25
	5	70	30
	6	94	06

Source: Allen et al (1994). J.AOAC, 77: 553

It is interesting to note that the mean recovery of leuco MG and chromatic MG in Allen et al[7] were 89% and 11%, respectively. The standard deviation for the two residues were about 10.5%. The living cells in fish tissue tends to convert chromatic form into leuco form and hence the recovery of the former is usually low. However use of postcolumn oxidation of leuco form by lead dioxide chromatic form can facilitate the determination of chromatic as well as leuco form by the difference.

Allen et al[7] have successfully chromatographed simultaneously leuco and chromatic forms of MG using methanol-water (81:19), buffered with 0.05M sodium acetate and 0.05M glacial acetic acid.

Chromatogram of 10ng each of chromatic MG, and leuco MG on C18 column with 1.5ml/min of methanol:water (81:19), buffered with 0.05M sodium acetate and 0.05 M glacial acetic acid at 618nm.



REFERENCES:

(1) Alderman, D. J.(1985) J. Fish Dis. 8: 289
(2) Bailey, T. A. (1983) J. Fish Dis. 6: 91
(3) Rushing, L.G., and M. C. Bowman (1980).J. Chromatogr. Sci. 18: 224
(4) McDonald, J. J. and C. E. Cerniglia, C. E.(1983) Drug Metab. Displs. 12:330
(5) Poe, W. E. and R. P. Wilson (1983) Prog. Fish. Cult. 45: 228.
(6) Bauer, K and et al (1988) Lebensmittelhyg 39: 97.
(7) Allen J.L. and et al (1994) J. Assoc. Off. Anal. Chem. Intl. 77: 553