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Rapid method for the determination of organochlorine pesticides in milk

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ABSTRACT

   This method involved one step solvent extraction of milk with ethyl acetate–acetone–methanol by ultrasonication. The supernatants were further cleaned-up and enriched by solid-phase extraction using octadecyl (C_{18})-bonded silica cartridges, then assayed by capillary gas–liquid chromatography with electron capture detection. The recoveries of eleven organochlorine pesticides (OCPs) from raw milks were quantitative, ranging from 90–110% at 10 times the limit of detection (LOD). The LOD ranged from 0.5 μg/l whole milk for α-hexachlorocyclohexane to 2.5 μg/l whole milk for 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane. The day-to-day variation of the method was evaluated over 7 days using 3 different pools of spiked cow milks (at the LOD, 5 and 10 times the LOD). The coefficient of variations (C.V.s) were 16 ± 6, 10 ± 2 and 9 ± 3% (mean S.D.), respectively.
   The method showed no emulsion problems common with conventional non-polar solvent extraction, and the use of solid-phase extraction considerably reduced the sample clean-up process compared with the existing methods. The method also showed that OCPs in milk could be extracted quantitatively without extraction of total fat, and that OCPs spiked into cows milk could be used to construct calibration curves for human milk determinations.

INTRODUCTION

   Organochlorine pesticide (OCP) pollutants in human milk have been of interest since residues were detected in ethnic American mothers in 1951 [1]. Since then methodology for studying pesticide residues in milk has been subject to some controversy because of the results from inter-laboratory comparisons [2]. Conventional methods to extract fat soluble compounds from fatty matrices often involve complete extraction of fat from the matrices, followed by extensive clean-up before chrmomtographic or other analysis. These methods are often lengthy, labour intensive and costly. The fat content and composition in milk is influenced by several physiological factors [2–4], for instance there are differences in fat content both between individuals and with milk from one individual before and after feeds. These factors have made collection of single samples of human milk for exposure studies unrepresentative [5]. However, increasing understanding of human lactation and milk composition has parallelled both the improvement of the analytical techniques, and greater awareness of the need to monitor occupational and environmental exposure to chemicals.
   Several approaches to extraction and clean-up of milk have been reported. A

typical procedure for milk samples would involve treatment with concentrated sul-
phuric acid, extraction with non-polar solvents such as petroleum ether or *n*-hexane
followed by clean-up of extracts using column adsorption chromatography [6–8].
High-performance gel permeation chromatography has been reported as showing
some advantages over conventional adsorption chromatography, for the separation
of large molecular components of fat from OCPs and polychlorinated biphenyls
(PCBs) [9]. The extraction of milk fat with a mixture of *n*-hexane–acetone (1:1) is
common as this allows total fat determination using gravimetric methods. Following
this initial extraction, the fats were then redissolved in *n*-hexane before further clean-
up, with concentrated sulphuric acid [10,11], or by adsorption chromatography
[12,13]. However, solvent extraction of OCPs and other organochlorine compounds
from milk using more polar solvent mixtures has been reported [14,15]. This work
showed low fat content in these extracts compared with the other approaches. Suzuki
et al. [14] used a mixture of hexane–acetonitrile–ethanol (20:5:1) to extract a 10-ml
milk sample followed by Florisil minicolumn chromatography for clean-up. There
have been reports that the fat in high fat containing raw milk is more readily extracted
than lower fat milks. Muccio *et al.* [15] modified Suzuki's method by using Chem Elut
cartridges to extract OCPs from a mixture of milk–acetonitrile–ethanol by eluting
with light petroleum (b.p. 40–60°C)–acetonitrile–ethanol (100:25:5). The eluates were
concentrated and further cleaned-up using a Florisil minicolumn.

     Ultrasonication has proved useful in milk fat homogenisation [16], since fat
soluble compounds in milk could possibly be trapped or bound in milk fat globules,
thereby affecting the reproducibility of recovery. This problem is overcome if milk fat
globules are completely broken down during the extraction process using for example
ultrasonication.

     The objective of the present study was to develop a rapid and simple method to
determine OCPs in milk such that studies in humans could be undertaken. Ideally, all
method development and calibration would be carried out using uncontaminated
human milk, but this is very difficult to obtain [17,18]. Consequently, dairy milk was
considered suitable for much of the programme.

EXPERIMENTAL

Standards and reagents

     Hexachlorocyclohexane (HCH) isomers: α-HCH, β-HCH, γ-HCH (lindane);
1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane   (*p,p'*-DDT);   1,1-dichloro-2,2-bis(*p*-
chlorophenyl)ethylene (*p,p'*-DDE); 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-
DDD); endrin; dieldrin; aldrin and heptachlor were from Polyscience (USA). Hepta-
chlor epoxide and 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethylene (*o,p'*-
DDE were products of Promochem (Germany). Octadecyl (C_{18}) bonded silica car-
tridges (500 mg/2.8 ml) were products of Analytichem (USA). Organic solvents (pes-
ticide residue grade), acetonitrile, acetone, methanol, *n*-hexane and ethyl acetate were
obtained from BDH (Poole, UK); 2,2,4-trimethylpentane (isooctane) was obtained
from J. T. Baker (Hayes, UK).

     Standard pesticide mixture: a stock standard solution (approximately 0.5 mg/
ml) of each pesticide was prepared separately in isooctane, except β-HCH which was
prepared in *n*-hexane–toluene (1:1). Spiking solutions of all OCPs were prepared in

TABLE I

CONCENTRATION OF SPIKING STANDARD MIXTURES

OCPs	Spiking concentrations (μg/ml)						
	I	II	III	IV	V	VI	VII
α-HCH	0.1	0.2	0.3	0.4	0.5	0.7	1.0
β-HCH	0.2	0.4	0.6	0.8	1.0	1.4	2.0
γ-HCH	0.1	0.2	0.3	0.4	0.5	0.7	1.0
Heptachlor	0.2	0.4	0.6	0.8	1.0	1.4	2.0
Aldrin	–	–	–	–	–	–	–
Heptachlor epoxide	0.2	0.4	0.6	0.8	1.0	1.4	2.0
o,p'-DDE	–	–	–	–	–	–	–
p,p'-DDE	0.3	0.6	0.9	1.2	1.5	2.1	3.0
Dieldrin	0.3	0.6	0.9	1.2	1.5	2.1	3.0
Endrin	0.4	0.8	1.2	1.6	2.0	2.8	4.0
p,p'-DDD	0.3	0.6	0.9	1.2	1.5	2.1	3.0
p,p'-DDT	0.5	1.0	1.5	2.0	2.5	3.5	5.0

ethyl acetate. The concentrations of these are shown in Table I. Aldrin (5 ng/ml milk) and o,p'-DDE (10 ng/ml milk) were used as internal standards. Working pesticide mixture solutions were prepared in isooctane the concentration of these are shown in Table II.

Apparatus and materials

The capillary gas chromatograph with ^{63}Ni electron capture detector (ECD), was Hewlett-Packard Model 5890A with an automatic injector, Hewlett-Packard

TABLE II

CONCENTRATION OF WORKING STANDARD MIXTURES

OCPs	Working concentrations (ng/ml)						
	1	2	3	4	5	6	7
α-HCH	1	2	3	4	5	7	10
β-HCH	2	4	6	8	10	14	20
γ-HCH	1	2	3	4	5	7	10
Heptachlor	2	4	6	8	10	14	20
Aldrin	10	10	10	10	10	10	10
Heptachlor epoxide	2	4	6	8	10	14	20
o,p'-DDE	20	20	20	20	20	20	20
p,p'-DDE	3	6	9	12	15	21	30
Dieldrin	3	6	9	12	15	21	30
Endrin	4	8	12	16	20	28	40
p,p'-DDD	3	6	9	12	15	21	30
p,p'-DDT	5	10	15	20	25	35	50

(Wokingham, UK). Capillary fused-silica column (10 m × 0.32 mm I.D.) 1-μm SE 52/4 phase was from SAC Chromatography (Cambridge, UK). The ultrasonicator was from Radleys (Saffron Walden, UK). Pyrex test tubes with PTFE-lined screw caps, size 100 × 16 mm and 120 × 16 mm were from FSA (Loughborough, UK).

PROCEDURE

Spiked cows milk

Both pooled pasteurised and raw cows milk were stirred in warm water (at 37°C) for half an hour then 250 μl of spiking solutions were added to 50 ml of well-mixed milk. This was stirred in warm water for another half an hour. Spiked milk (2 ml) was aliquoted into glass tubes and stored at −20°C in a freezer. These spiked cows milk were used for calibration purposes when determining OCP levels in human milk.

Solvent extraction

All 2-ml milk samples were thawed and mixed well in warm water before processing. Internal standard mixture (50 μl of aldrin 0.2 ng/μl, and *o,p'*-DDE 0.4 ng/μl) were added to human milk samples, spiked and unspiked cows milk. All milk samples (2 ml each) were extracted with 10 ml ethyl acetate–methanol–acetone (2:4:4) and vortexed for 1 min. All sample tubes were then placed in an ultrasonic bath for 20 min. The tubes were centrifuged at 2000 rpm, for 15 min at 20°C and the total supernatant aspirated into 500-ml conical flasks. The average volume of supernatant was 11.5 ml.

Solid phase extraction

After the initial solvent extraction, solid-phase extraction cartridges were used to further clean up and concentrate samples. C_{18}-cartridges (500 mg) were pre-conditioned with 2 × 1 ml isooctane, 2 × 1 ml ethyl acetate, 2 × 1 ml methanol and 2 × 1 ml distilled water. The vacuum was turned off, and care was taken to keep the cartridges wet. An aliquot of 13 ml of distilled water was added to the supernatants. The diluted supernatants were then passed through the cartridges at a flow-rate of 6–8 ml/min. The conical flask was rinsed with 2 × 1 ml distilled water. The cartridges were washed with 2 × 1 ml 25% acetonitrile–water and dried by pulling air through the cartridges for 3 min. The OCPs were eluted from the cartridges with 2 × 0.5 ml isooctane.

Capillary gas–liquid chromatography

Operating conditions were: injection port 200°C; detector oven (ECD) 320°C; column oven, initial temperature 80°C for 1 min, increased by 10°C/min to 250°C and held for 10 min; inlet pressure 4.3 p.s.i. which was 40 cm/s gas velocity; splitless injection with purge off for 1 minute, injection volume was 1 μl.

Quantitation

Every series of determinations contained one blank sample of unspiked pasteurised cows milk, pasteurised cows milk spiked at seven known concentrations, eight human milk samples (for OCP determination), three spiked raw cows milk (for

quality control) and one human milk for control. Peak area ratios of each pesticide were calculated. The calibration curve for extracted OCPs was obtained from linear regression analysis. The OCPs in human milk samples, human milk control and spiked raw cows milk controls were calculated from the calibrations obtained with the extracted OCPs.

## Total milk fat content determination

Milk samples (2 ml) were extracted with 8 ml of *n*-hexane–acetone (1:1), by vortexing for 1 minute and ultrasonification for 20 min [10,11]. After centrifugation at ca. 1000 *g*, for 15 min at 20°C, the supernatants were aspirated into the pre-weighed glass tubes. The solvent extraction was repeated and the supernatants combined. The supernatants were then dried under nitrogen at 40°C to constant weight and weighed. The total fat content was obtained by difference.

RESULTS AND DISCUSSION

The novel sample preparation procedure described for pasteurized and raw cows milk and human milk showed no problems with emulsions or viscous supernatants which might clog the solid-phase extraction. The fat content of pasteurized and raw cows milk was about 0.74% using the present method, and about 4.1% using the microscale (total fat extraction) method. The fat content in human milk extracts from the present method was 0.60% and from the microscale total fat extraction method was 1.78%. Thus the fat content in solvent extracts from both types of cows milk was around 18% of total fat. It was not necessary to extract total fat to get quantitative recovery of OCPs. Chromatograms of pure OCP standards, unspiked and spiked cows milk, and human milk are shown in Fig. 1. Aldrin (5 ng/ml whole milk) and *o,p'*-DDE (10 ng/ml whole milk) were evaluated as internal standards in this method. Both were suitable, *i.e.* they were well separated from the other OCPs

TABLE III

RECOVERY OF OCPs FROM SPIKED COWS MILK

| Compound	Low		Medium		High	
	Concentration (ng/ml)	Recovery (%)	Concentration (ng/ml)	Recovery (%)	Concentration (ng/ml)	Recovery (%)
α-HCH	0.5	83	2.5	105	5	103
β-HCH	1	119	5	91	10	97
γ-HCH	0.5	80	2.5	86	5	96
Heptachlor	1	82	5	88	10	91
Aldrin	5	(100)	5	(100)	5	(100)
Heptachlor epoxide	1	100	5	88	10	95
p,p'-DDE	1.5	93	7.5	92	15	90
Dieldrin	1.5	96	7.5	95	15	97
Endrin	2	116	10	108	20	110
p,p'-DDD	1.5	132	7.5	111	15	104
p,p'-DDT	2.5	101	12.5	112	25	97
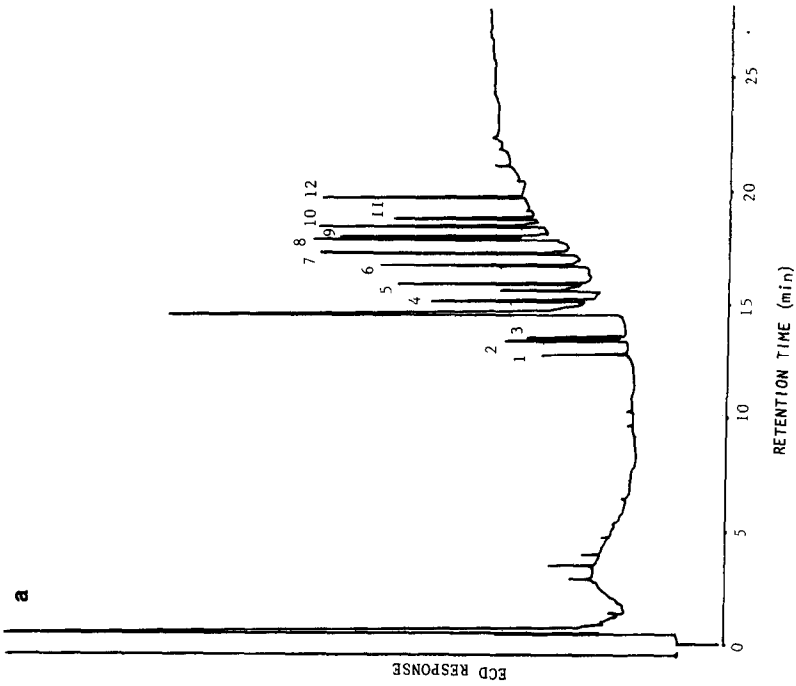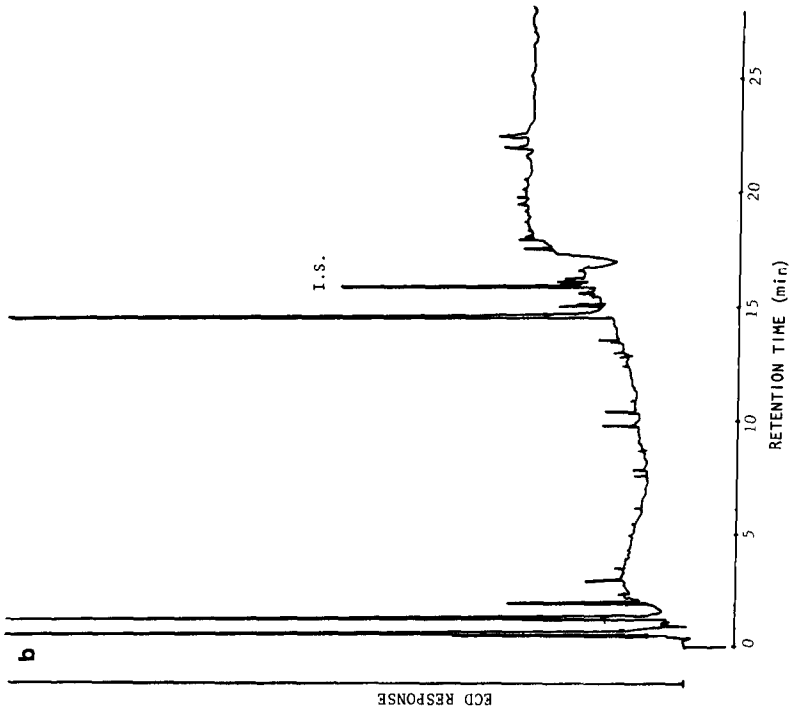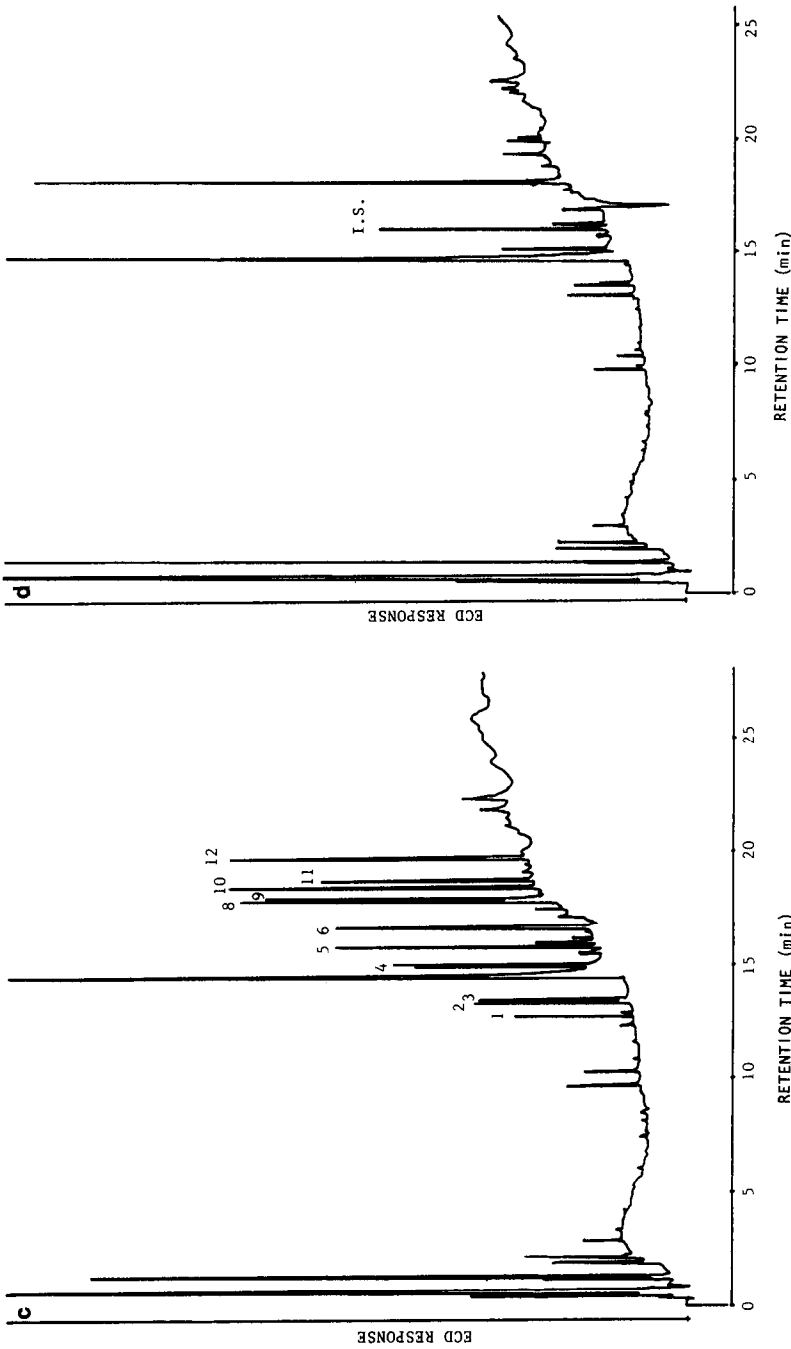
Fig. 1. Specimen chromatograms of (a) OCP standards, (b) unspiked cows milk, (c) spiked cows milk, (d) human milk sample. Peak identification: 1 = αHCH; 2 = β-HCH; 3 = γ-HCH; 4 = Heptachlor; 5 = Aldrin; 6 = Heptachlor epoxide; 7 = o,p'-DDE; 8 = p,p'-DDE; 9 = Dieldrin; 10 = Endrin; 11 = p,p'-DDD; 12 = p,p'-DDT. Chromatographic conditions: Column: 10 m × 0.3 mm I.D.; 1-μm SE52/4; detector: ECD; temperature: 80°C for 1 min then rise 10°C per min to 250°C for 10 min; injector: 200°C; detector: 320°C.

TABLE IV

LINEARITY OF CALIBRATION LINES

Compound	Pure OCP standard		Extracted OCP standard		
	Slope	r	Slope	r	% Recovery OCP
α-HCH	0.0929	0.9984	0.0731	0.9866	79
β-HCH	0.0579	0.9984	0.0546	0.9964	94
γ-HCH	0.1052	0.9992	0.0945	0.9919	89
Heptachlor	0.0881	0.9935	0.0595	0.9983	68
Aldrin	as internal standard				
Heptachlor epoxide	0.0954	0.9970	0.0885	0.9801	93
p,p'-DDE	0.0716	0.9971	0.0709	0.9964	99
Dieldrin	0.0774	0.9914	0.0721	0.9954	93
Endrin	0.0541	0.9981	0.0635	0.9978	117
p,p'-DDD	0.0526	0.9986	0.0511	0.9961	97
p,p'-DDT	0.0389	0.9944	0.0538	0.9957	136

and eluted in the middle of the chromatograms among those OCPs of interest. There was often some interference in human milk samples at the retention time corresponding to o,p'-DDE, but not at that of aldrin. Hence, aldrin has been used as internal standard for most of this work. So far, using this method for human milk there has been no interference at the retention time of aldrin. Aldrin is rapidly metabolised to dieldrin by a wide range of organisms, including man [19] so this could explain why no aldrin residues occur. A large peak appeared at a retention time of 14.5 min, this was a "ghost" peak from the GC system and did not interfere with the assay. The gas chromatographic (GC) conditions chosen including the temperature programme rate were optimised. No peaks were seen in human milk other than DDE and DDT.

TABLE V

LIMIT OF DETECTION AND REPRODUCIBILITY OF OCPs IN COWS MILK

Compound	LOD (ng/ml milk)	Low		Medium		High	
		ng/ml milk	% C.V.[a] (n = 7)	ng/ml milk	% C.V. (n = 7)	ng/ml milk	% C.V. (n = 7)
α-HCH	0.5	0.5	9	2.5	8	5	2
β-HCH	1	1	14	5	8	10	7
γ-HCH	0.5	0.5	25	2.5	9	5	8
Heptachlor	1	1	23	5	14	10	14
Aldrin	I.S.[b]	5	–	5	–	5	–
Heptachlor epoxide	1	1	18	5	7	10	9
p,p'-DDE	1.5	1.5	13	7.5	9	15	8
Dieldrin	1.5	1.5	12	7.5	12	15	8
Endrin	2	2	25	20	10	20	10
p,p'-DDD	1.5	1.5	11	7.5	11	15	11
p,p'-DDT	2.5	2.5	13	25	11	25	12

[a] C.V. = Coefficient of variation.
[b] I.S. = Internal standard.

The recovery of eleven OCPs from spiked cow milks is shown in Table III. Recoveries of 80% or greater were achieved for all OCPs. The limit of detection (LOD) of this method for 11 OCPs (shown in Table III as the low value) ranged from 0.5–2.5 ng/ml with only 2 ml of milk sample required. This is favourable when compared with the existing methods which employed a larger milk volume [2]. The linearity of calibration curves of eleven OCPs in pure and processed standards were greater than 0.99 (Table IV) except for α-HCH (0.9866) and heptachlor epoxide (0.9801) which sometimes showed chromatographic interferences. The recovery of OCPs calculated from the slopes of the two calibration curves is also shown in Table IV. The day-to-day variations (for 7 days) of three different pools of spiked cows milk (0.5–2.5; 2.5–12.5 and 5–25 ng/ml whole milk) are shown in Table V.

CONCLUSIONS

This method allows the assay of 40 samples per week, with 2 h for sample preparation and only 1–2 ml human milk samples. It is simpler than current published methods and will allow a survey of OCP exposure to infants via mothers milk to be carried out.

ACKNOWLEDGEMENTS

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