MIN 312/014272

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Sponsor

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3M Corporate Toxicology,
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MN 55133-3220,
USA.

Research Laboratory

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Report issued: 7 October 2005

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APPENDICES

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The UK Good Laboratory Practice regulations 1999 (Statutory Instrument No 3106) as amended by Statutory Instrument 2004 No. 994.

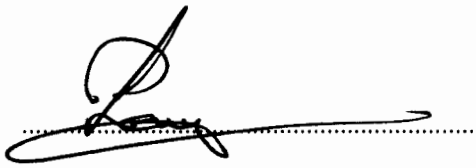
OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98)17.

EC Commission Directive, 1999/11/EC of 8 March 1999 (Official Journal No L 77/8), as amended by EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

These principles of Good Laboratory Practise are accepted by the regulatory authorities and the United States of America and Japan on the basis of Intergovernmental agreements.

An expiry date for the test substance was not supplied. The material was assumed to be stable for the duration of the study.

No claim of compliance is made with regard to the scanning electron microscope examination and X-ray microanalysis.



7 October 2005

Terence J. Kenny, B.Sc. (Hons.),
Study Director,
Huntingdon Life Sciences Ltd.

Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study:

Study Phases Inspected	Date of Inspection	Date of Reporting
Protocol Audit	21 August 2001	21 August 2001
Study Preparation	13 September 2001	14 September 2001
Exposure	13 September 2001	14 September 2001
Sampling	13 September 2001	14 September 2001
Clinical signs	13 September 2001	14 September 2001
Test item control disposition	13 September 2001	14 September 2001
Post Mortem	18 September 2001	18 September 2001
SEM X-RAY - EM	27 September 2001	28 September 2001
Report Audit	10-17 December 2001 28 September 2005	17 December 2001 29 September 2005

Protocol: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Study based inspections: Inspections and audits of phases of this study were conducted and reported to the Study Director and Company Management as indicated above.

Process based inspections: At or about the time this study was in progress inspections and audits of other routine and repetitive procedures employed on this type of study were carried out. These were promptly reported to appropriate Company Management.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

Analytical phases of this study conducted by Exygen Research were subjected to Quality Assurance monitoring and audit according to Exygen's own procedures. Details are presented in the Analytical Phase Report.

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Tracy Scarfe, FRQA,
Group Manager,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

7 October 2005

Date

CONTRIBUTING SCIENTISTS

STUDY MANAGEMENT

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SUMMARY

One group of rats (each of 5 males and 5 females) of the Crl:CD®BR strain were exposed to POSF, 6 hours a day for 5 consecutive days using a snout-only exposure system. A second group, acting as control, was exposed to air only.

The study mean analysed concentration was 316.95 ppm.

The following comments are made in summary:

Mortality and clinical signs

There were no unscheduled deaths or any treatment related signs seen during the course of the study.

Bodyweight, food and water consumption

Following 5 days of treatment with POSF there was reduced bodyweight gain, food consumption and water consumption in treated males.

Organ weights

Bodyweight adjusted liver weights were higher in treated animals, with statistical significance ($p \leq 0.05$) being attained in females.

Absolute lungs and bronchi weights were higher in treated animals with statistical significance being achieved in males and in bodyweight adjusted female lung and bronchi weights.

Macroscopic and microscopic pathology

There were no treatment-related macroscopic or microscopic findings.

Conclusion

A no-effect level was not established for this study.

INTRODUCTION

The purpose of this study performed at Huntingdon Life Sciences Limited, Huntingdon, England was the assessment of systemic toxic potential in a 1-week inhalation study in rats, by snout-only administration of the test substance POSF, for 6 hours a day, for 5 consecutive days.

The test substance was administered by inhalation, a possible route of accidental exposure in man. The rat was the species of choice due to requirement for a rodent species by regulatory agencies and the strain was selected on account of the availability of comprehensive background data, relating to clinical and pathological parameters, at our laboratories.

RELEVANT STUDY DATES

Approved by:

Study Director:	17 August 2001
HRC Management:	17 August 2001
Study Sponsor:	20 August 2001

Animals arrived at HRC:	5 September 2001
Exposures commenced:	13 September 2001
Serum/urine sampling:	18 September 2001
Terminal kill:	18 September 2001
Experimental completion date:	28 September 2002

TEST SUBSTANCES

Sponsor's identification:	Perfluorooctanesulfonyl fluoride
Other names:	POSF, T-7661.1, FX-8B
Storage conditions:	In a refrigerator (*ca* 4°C)
Date received:	14 June 2001
Supplier:	Sponsor
Batch number:	040227
Expiry date:	Assumed to be stable for the duration of the study
Purity:	>99.5%

A small sample (1 ml) was sealed in a suitable contained and stored in Archives at an appropriate temperature.

EXPERIMENTAL PROCEDURE

ANIMALS

Twenty rats (10 male and 10 female) aged approximately 6 weeks, of the Crl:CD®BR, a caesarean derived strain of Sprague-Dawley origin, were obtained from Charles River (UK) Limited, Manston Road, Margate, Kent, on 5 September 2001.

The latest Health Screen Report published by the animal supplier was provided to Huntingdon Life Sciences (HLS). In addition, the additional consignments of animals included a health screen relating to the current status of the breeding colony. These documents were sent to HLS Veterinary Services immediately upon receipt for review and subsequent archiving.

Random assignment to experimental groups took place on arrival. The animals were then uniquely identified by numbers tattooed on the tail.

The identification of individual rats in the 2 groups together with the target exposure level were as follows:

Group	Target exposure level (ppm)	Animal numbers	
		Male	Female
1 (Air control)	-	1-5	11-15
2 (POSF)	300	6-10	16-20

ACCOMMODATION

The rats were housed 5 of the same sex to a cage in suspended stainless steel cages fitted with mesh front, back and floor with stainless steel sheet sides. Plastic trays lined with absorbent paper were placed below each cage to collect animal excreta and the paper was changed daily. Each cage had a coloured label identifying the group and the numbers of the animals contained within it. The rats were kept in a single room and, additionally, after the start of the exposure period, each group was positioned on an individual cage battery. Exposure took place in the same room.

The temperature and relative humidity of the holding room were recorded using a Kent Clearspan recorder. The study holding room temperature and relative humidity were set to be maintained within limits of 21 ± 2°C and 55 ± 15% respectively. Recorded ranges were 19.0 to 21.0°C and 40 to 72% humidity. Minor deviations from these ranges were of relatively short duration and considered not to have affected the scientific integrity of the study.

Lighting was controlled to give 12 hours light (0600 - 1800 hours) and 12 hours dark per 24 hours.

DIET

While in their cages, all rats had access to a weighed quantity of standard quality-controlled laboratory rat food (SDS Rat and Mouse No. 1 SQC modified maintenance diet, Special Diets Services, Witham, Essex).

There was no information available to indicate that any non-nutrient substance likely to influence the effect of the test compound could reasonably be expected to be present in the diet. The analytical data have been lodged in Huntingdon Life Sciences Archives.

Tap water was available from moulded polypropylene water bottles at all times while the rats were in the cages. The water bottles were rinsed and refilled daily and thoroughly cleaned at intervals during the study.

There was no information available to indicate that any substance likely to influence the effect of the test system could reasonably be expected to be present in the drinking water.

Results of the routine physical and chemical analyses of water at source (sampling point, Grafham Final Water) as conducted by the supplier, Anglian Water Services Ltd, have been made available to Huntingdon Life Sciences. Anglian Water takes its guidelines on water quality from the EEC directive relating to water for human consumption, *viz.* Council Directive 80/778/EEC.

The analytical data have been lodged in Huntingdon Life Sciences Archives.

ADMINISTRATION

The test substance was administered for 6 hours a day, for 5 consecutive days.

The test material was delivered to an all glass vapouriser and generated as a droplet atmosphere, into a stream of air for administration to the rats by inhalation from snout only exposure chambers. The test substance was metered to the vaporiser from an infusion pump. The vapour/air mixture passed directly into the exposure chamber. The target chamber concentration was achieved by using different liquid feed rates controlled by the infusion pump and using syringes of an appropriate value.

The target concentrations for treated rats was 300 ppm. Control rats received air only.

The rats were exposed to the control/test atmosphere using ADG snout-only exposure chambers (ADG Developments Ltd, Hitchin, Hertfordshire, England) of a modular construction in aluminium alloy comprising a base unit, 3 sections each having 20 exposure ports, and a top section incorporating a central inlet with a tangential air inlet.

All animals (including reserves) were subjected to restraint procedures and exposed to air only ('Sham dosing'), for 5 consecutive days, in order to accustom animals to the restraining procedure prior to study initiation. The rats were restrained once per day, increasing the 'Sham dosing' period progressively (0.5, 1, 2, 4 and 6 hours for Days -5 to -1 respectively).

Details of administration and analysis of the test atmospheres together with the results obtained are presented in **ADMINISTRATION OF POSF BY INHALATION TO RATS** appended to this report.

CLINICAL INVESTIGATIONS

Dated and signed records of all activities relating to the day to day running and maintenance of the study, as well as to the group observations and examinations outlined in this procedure were recorded in the Study Daybook. Individual dated and signed records noting nature and severity, date and time of onset and duration and progress of observed clinical signs were maintained for each animal.

Mortality

Throughout the study, all cages were checked in the morning and again at the end of the normal working day for dead or moribund animals.

Clinical signs

Dated and signed records of appearance, change and disappearance of clinical signs were maintained. Individual animal records were maintained on the basis of:

- any observation, considered to be of possible importance, made at any time during the study;
- any observation, considered to be of possible importance, made during transfer to restraining tubes (prior to exposure), during exposure (although severely restricted due to tube restraint), on return to holding cages (after exposure) and as late a possible in the working day.

During the acclimatisation period, observations of the animals and their cages were recorded at least once a day.

BODYWEIGHT

The weight of each rat was recorded a week prior to the start of exposures. During the treatment period, bodyweight was recorded on the day that treatment commenced, daily thereafter, and also prior to necropsy.

FOOD CONSUMPTION

The quantity of food consumed by each cage of rats was recorded on a daily basis. Food intake per rat (g/rat/day) was calculated using the total amount of food given to and left by each cage in each group and the number of rats surviving in each cage.

WATER CONSUMPTION

The quantity of water consumed by each cage of rats was recorded on a daily basis, commencing 1 week before the start of exposures.

Water intake per rat (g/rat/day) was calculated using the total amount of water given to and left by each cage in each group and the number of rats surviving in each cage.

TERMINAL STUDIES

Pre-terminal urine sampling

Individual urine samples were collected from animals within 2 hours following lights on in the animal holding room where possible. For animals failing to produce a specimen during this interval, urine was collected at necropsy. The urine samples were immediately assayed for pH using a microelectrode, and prepared SEM stubs despatched to Plymouth University for calculi and crystal analysis by SEM X-ray element identification.

Test substance/metabolite analyses

Samples of blood (for serum) were obtained at necropsy by cardiac/aorta puncture while the rats were held under terminal sodium pentobarbitone anaesthesia.

The blood samples (up to 4 ml) were collected, run into tubes, allowed to clot at room temperature and the serum separated and frozen prior to despatch to the Sponsor for subsequent analysis at Exygen Research.

Necropsy

All animals were killed on Day 6 of the study, following 5 days of exposure. Animals were killed by an intraperitoneal injection of sodium pentobarbitone followed by exsanguination from the brachial arteries.

All study rats were subjected to a macroscopic *post mortem* examination.

The following procedures applied:

> All superficial tissues were examined visually and by palpation and the cranial roof removed to allow observation of the brain, pituitary gland and cranial nerves. After ventral mid-line incisions and skin reflection, all subcutaneous tissues were examined. The condition of the thoracic viscera was noted, with due attention to the thymus, lymph nodes and heart.
>
> The abdominal viscera were examined before and after removal; the urinary bladder was examined externally and by palpation.

The gastro-intestinal tract was examined as a whole and the stomach, caecum and portions of duodenum, jejunum, ileum, colon and oesophagus were incised and examined. The lungs were removed and all pleural surfaces examined. The liver was sectioned at intervals of a few millimetres; the kidneys were incised and examined. Any abnormalities in the appearance and size of the gonads, adrenals, uterus, intra-abdominal lymph nodes and accessory reproductive organs were recorded.

Sections of liver samples were immediately frozen by immersion in liquid nitrogen and stored at -70°C prior to dispatch to the Sponsor for subsequent analysis at Exygen Research.

The following organs from all surviving animals were dissected free of fat and weighed. For bilateral organs, left and right organs were weighed together.

adrenals	liver	heart
kidneys	lungs	

Preservation of tissues

During *post mortem* examination, samples or the whole of the tissues listed below, from all study animals, were preserved in 10% neutral buffered formalin; except the eyes which were preserved in Davidson's fixative and testes/epididymides were fixed in Bouin's solution and then transferred to 70% alcohol. The lungs were infused with fixative prior to immersion. The nasal cavity was flushed with fixative prior to immersion.

abnormalities	kidneys (with pelvic region)*	pancreas	sternum
adrenals	lachrymal glands	pituitary	stomach
aorta (thoracic)	larynx (2 levels)	prostate	testes
brain	liver	rectum	thymus
caecum	lungs (section from all lobes including bronchi)	salivary glands (submandibular/ sublingual)	thyroid with parathyroids
colon	lymph nodes: mandibular mesenteric tracheobronchial	sciatic nerves	tongue
duodenum	mammary area (caudal)	seminal vesicles	trachea (2 levels)
epididymides	nasal turbinates	skeletal muscle (thigh)	urinary bladder*
eyes	oesophagus	skin	uterus with cervix
femur (longitudinal section through joint)	optic nerves	spinal cord (transverse and longitudinal sections at the cervical, lumbar and thoracic levels)	vagina
harderian glands	ovaries	spleen	
head			
heart			
ileum			
jejunum			

* To be examined microscopically

Histopathology examination

The tissues in the list above were examined using a light microscope as annotated. Tissues were embedded in paraffin wax and sections approximately 4 - 5 micrometres were cut, processed and stained with haematoxylin and eosin.

PCNA Staining for cell proliferation

Sections of urinary bladder were taken from all animals for PCNA immunostaining in order to assess the degree of cell proliferation present. A total of 3,000 cells were counted from 3 separate sections (1,000 cells/section) in order to determine the cell proliferation index. Positive PCNA staining cells were counted, and examined by the pathologist to assess the sections and count S-phase positive cells, if practicable.

In addition, sections of the duodenum from each animal were taken and stained to act as positive controls for the immunostaining methodology.

STATISTICAL ANALYSIS

For categorical data, the proportion of animals were analysed using Fisher's exact test for each treated group versus the control.

For continuous data, Bartlett's test was first applied to test the homogeneity of variance between the groups. Using tests dependent on the outcome of the Bartlett's test, treated groups were then compared with the control group, incorporating adjustment for multiple comparisons where necessary.

ARCHIVING

All specimens, raw data and study-related documents generated during the course of the study at Huntingdon Life Sciences, together with a copy of the final report were lodged in Huntingdon Life Sciences, Archives.

Such specimens and records will be retained for a minimum period of five years, from the date of issue of the final report. At the end of the five-year retention period the Sponsor will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the Sponsor's knowledge.

DEVIATION FROM PROTOCOL

Due to some slight tissue damage it was not possible to count 1000 cells/section on a number of urinary bladder sections stained for PCNA analysis. The number of sections affected was low and there was no effect on the outcome of the study.

RESULTS

CHAMBER ATMOSPHERE CONDITIONS

Chamber analysed concentration of POSF

The data are presented in **ADMINISTRATION OF POSF TO RATS BY INHALATION** appended to this report.

The mean chamber concentrations (ppm) are summarized below:

Exposure No.	Concentration ppm
1	316.04
2	313.69
3	323.33
4[a]	
5	314.74
Mean	316.95
sd	4.361
cv	1.4

Sd Standard deviation
CV Coefficient of variation (%)
[a] No values recorded due to hardware failure, however nominal concentrations were similar for all days therefore the actual exposure levels were deemed to be on target

Study mean analysed concentration was 316.95 ppm which was in good agreement with the target concentration of 300.

CLINICAL OBSERVATIONS

Mortality

There were no unscheduled deaths.

Clinical signs

The data are presented as follows:

Appendix 1 - individual findings

There were no treatment-related signs.

Signs observed post dose included red staining around eyes, brown staining on head and body and wet fur. These signs were seen in a proportion of Test and Control animals during the shamming and treatment periods and were associated with the method of restraint used.

Bodyweight

The data are presented as follows:

Figure 1 - group mean values (g)
Table 1 - group mean values (g)
Appendix 2 - individual values (g)

Over the five days of treatment, treated rats gained less weight than controls. On Days 2 and 3 of exposure the male rats lost weight.

Food consumption

The data are presented as follows:

Table 2 - group mean values (g/rat)

Over the five days of treatment, a small reduction in food consumption was seen in treated males, the greatest effect being on Days 2 and 3 of exposure.

Water consumption

The data are presented as follows:

Table 3 - group mean values (g/rat)

On Days 2 and 3 of exposure water consumption in treated males was reduced. At other times consumption was comparable with that of control rats.

TERMINAL INVESTIGATIONS

Organ weights

The data are presented as follows:

Table 4 - group mean values
Appendix 3 - individual values

Bodyweight adjusted liver weights were higher in treated animals, with statistical significance ($p \leq 0.05$) being attained in females.

Absolute lungs and bronchi weights were higher in treated animals with statistical significance being achieved in males and in bodyweight adjusted female lung and bronchi weights.

Macroscopic pathology

The data are presented as follows:

Table 5 - incidence summary
Appendix 4 - individual pathological findings

The macroscopic examination performed at termination revealed no changes attributable to treatment with POSF.

The incidence and distribution of all the findings were considered to fall within the background range of macroscopic changes.

Microscopic pathology

The data are presented as follows:

Table 6 - incidence summary
Appendix 4 - individual pathological findings

No treatment related microscopic findings were reported.

All microscopic findings were considered to be incidental and of no toxicological importance.

Urinary pH

The data are presented as follows:

Appendix 5 - individual values

Differences between the groups were minimal and considered to be of no toxicological significance.

Cell proliferation

The data are presented as follows:

Appendix 6 – individual and mean values.

The mean cell proliferation index (% positive cells) value for treated males was greater than the control value, this was principally due to differences at levels 1 and 2 only. However, intragroup differences were large such that a proportion of the individual values for the treated group lay within the range of values seen for the control group. Differences between control and treated males at level 3 and at all levels in females were minimal. It is considered that the differences seen were not of toxicological significance.

SEM and X-ray examination of urine and bladder tissue

The results of examination together with SEM photomicrographs are presented in the Scanning Electron Microscope Examination and X-ray Microanalysis report appended to this main report.

Test substance and metabolite analysis

The methods and results of analyses are presented in the **ANALYTICAL PHASE REPORT** appended to this main report.

DISCUSSION

Administration of a POSF to rats at a dosages of 300 ppm a day over 5 consecutive days produced no mortalities and transient clinical effects which were considered to be treatment-related. There was a reduction in bodyweight gain, food and water consumption amongst treated males principally on Days 2 and 3 of exposure. There were no macroscopic pathology changes. Lung weights were higher in treated animals as were bodyweight adjusted liver weights. The toxicological importance of the organ weight changes is unclear given the absence of macroscopic and microscopic findings.

There were no differences in urinary pH or cell proliferation index considered to be of toxicological importance.

CONCLUSION

A no-effect level was not established for this study.

REFERENCES

BARTLETT, M.S. (1937), Properties of sufficiency and statistical test, *Proc. Roy. Soc* **A 160**: 268 -282.

FISHER, R.A., *Statistical Methods for Research Workers*, Para. 21.02, Oliver and Boyd, Edinburgh.

FIGURE 1

Bodyweight - group mean values (g)

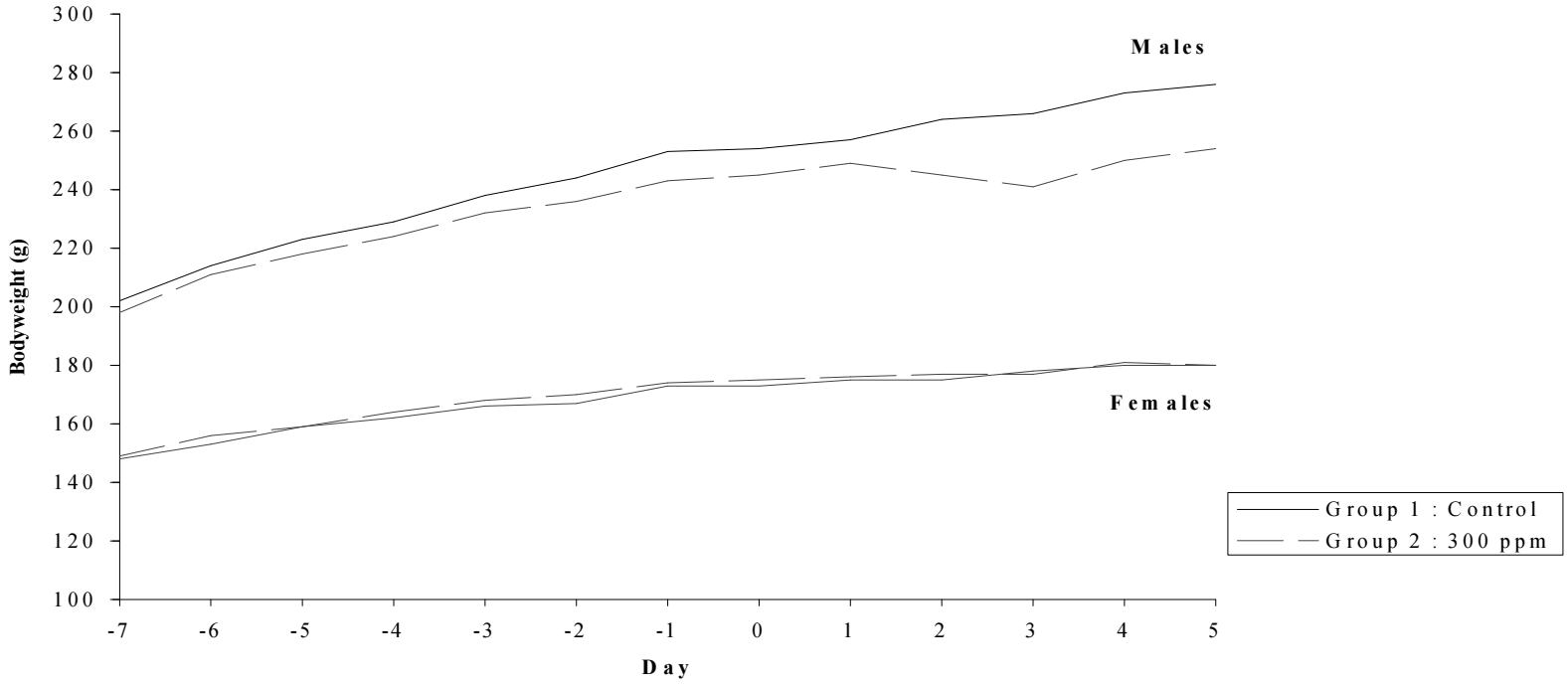

TABLE 1

Bodyweight - group mean values (g)

Print No: 0001

Printed: 21-SEP-01

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

SEX:	MALE		FEMALE	
GROUP:	1	2	1	2
DAY				
-7	202	198	148	149
-6	214	211	153	156
-5	223	218	159	159
-4	229	224	162	164
-3	238	232	166	168
-2	244	236	167	170
-1	253	243	173	174
0	254	245	173	175
1	257	249	175	176
2	264	245	175	177
3	266	241	178	177
4	273	250	180	181
5	276	254	180	180
		++		
Gain Day 0-5	22	9	7	5
% of Control	-	41	-	71

Level of significance – all comparisons made with Vehicle Control:

Student's 't' test: ++ $p \leq 0.01$

TABLE 2

Food consumption - group mean values (g/rat)

Print No: 0002

Printed: 21-SEP-01

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

SEX:	MALE		FEMALE	
GROUP:	1	2	1	2
DAY				
-7	29	27	19	21
-6	29	28	20	21
-5	30	28	19	23
-4	30	28	19	21
-3	28	28	18	23
-2	36	26	19	25
-1	19	25	16	13
1	26	24	18	18
2	27	17	17	17
3	27	15	19	18
4	28	22	18	19
5	29	25	18	19
Cumulative Days 1-5	137	103	90	91
% of Control	-	75	-	101

TABLE 3

Water consumption - group mean values (g/rat/)

Day	Group/dosage			
	1M (Control)	2M (POSF)	1F (Control)	2F (POSF)
-7	30	30	21	22
-6	31	30	21	20
-5	32	32	23	23
-4	33	30	25	24
-3	34	31	22	24
-2	32	30	21	21
-1	33	29	22	23
1	31	32	21	23
2	32	21	20	21
3	31	19	21	21
4	35	32	24	27
5	32	32	23	23
Cumulative 1 to 5	161	135	110	116
% of Control	-	84	-	106

TABLE 4

Organ weights - group mean values (g)

Print No: 0004

Printed: 21-SEP-01

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

	ABSOLUTE VALUES		BODYWEIGHT ADJUSTED VALUES	
SEX:	MALE		MALE	
GROUP:	1	2	1	2
NUMBER:	5	5	5	5
TERMINAL BODY WEIGHT (g)				
N	5	5		
MEAN	274.3	252.3		
sd	14.4	12.7		
ADRENALS				
N	5	5		
MEAN	0.050	0.054		
sd	0.005	0.007		
HEART				
N	5	5		
MEAN	1.173	1.062		
sd	0.103	0.186		
KIDNEYS				
N	5	5		
MEAN	2.04	1.94		
sd	0.14	0.08		
LIVER			**LIVER**	
N	5	5	5	5
MEAN	12.91	12.65	12.29	13.27
sd	1.00	0.82		

No differences of statistical significance

TABLE 4

(Organ weights - continued)

Print No: 0004

Printed: 21-SEP-01

GROUP	:	1	2
COMPOUND	:	CONTROL	POSF
DOSAGE (PPM)	:	0	300

Xybion protocol number: MIN 312

ABSOLUTE VALUES		
SEX:	-------MALE-------	
GROUP:	---1---	---2---
NUMBER:	5	5
LUNGS & BRONCHI		
		+++
N :	5	5
MEAN :	1.112	1.828
sd :	0.128	0.204

Level of significance - all comparisons made with Vehicle Control:

Student's 't' test: +++ $p \leq 0.001$

TABLE 4

(Organ weights - continued)

Print No: 0005

GROUP : 1 2 — Printed: 21-SEP-01
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

	ABSOLUTE VALUES		BODYWEIGHTS ADJUSTED VALUES	
SEX:	------FEMALE------		FEMALE	
GROUP:	---1---	---2---	---1---	---2---
NUMBER:	5	5	5	5
TERMINAL BODY WEIGHT (g)				
N	5	5		
MEAN	179.5	180.4		
sd	8.7	12.5		
ADRENALS				
N	5	5		
MEAN	0.054	0.056		
sd	0.004	0.006		
HEART			**HEART**	
N	5	5	5	5
MEAN	0.814	0.893	0.816	0.891
sd	0.114	0.091		
KIDNEYS			**KIDNEYS**	
N	5	5	5	5
MEAN	1.36	1.44	1.37	1.44
sd	0.11	0.14		
LIVER			**LIVER +**	
N	5	5	5	5
MEAN	7.97	9.16	8.00	9.12
sd	0.77	1.31		

Level of significance – all comparisons made with Vehicle Control:

Student's 't' test: + $p \leq 0.05$

TABLE 4

(Organ weights - continued)

Print No: 0005

Printed: 21-SEP-01

GROUP	:	1	2
COMPOUND	:	CONTROL	POSF
DOSAGE (PPM)	:	0	300

Xybion protocol number: MIN 312

	ABSOLUTE VALUES		BODYWEIGHTS ADJUSTED VALUES	
SEX:	------FEMALE------		FEMALE	
GROUP:	---1---	---2---	---1---	---2---
NUMBER:	5	5	5	5
	LUNGS & BRONCHI		LUNGS & BRONCHI	
				+++
N :	5	5	5	5
MEAN :	0.891	1.478	0.895	1.474
sd :	0.070	0.159		

Level of significance - all comparisons made with Vehicle Control:

Student's 't' test: +++ $p \leq 0.001$

TABLE 5

Macroscopic pathology - incidence summary

Print No: 0006

Printed: 21-SEP-01

GROUP	:	1	2
COMPOUND	:	CONTROL	POSF
DOSAGE (PPM)	:	0	300

Xybion protocol number: MIN 312

		--- NUMBER-OF-ANIMALS-AFFECTED ---			
	SEX:	--MALE--		-FEMALE-	
	GROUP:	-1-	-2-	-1-	-2-
ORGAN AND KEYWORD(S) OR PHRASE	NUMBER:	5	5	5	5
** TOP OF LIST **					
KIDNEYS	NUMBER EXAMINED:	5	5	5	5
PELVIC DILATION		0	0	1	0
LN MANDIBULAR	NUMBER EXAMINED:	5	5	5	5
CONGESTED		1	0	0	0
ENLARGED		0	1	0	0
LN MESENTERIC	NUMBER EXAMINED:	5	5	5	5
ENLARGED		0	0	0	1
SKIN	NUMBER EXAMINED:	5	5	5	5
SCAB(S)		0	0	3	0
STOMACH	NUMBER EXAMINED:	5	5	5	5
ANTRUM WHITE NODULE(S)		2	1	1	0
TEETH	NUMBER EXAMINED:	5	5	5	5
INCISOR(S) PALE		0	0	0	2
UTERUS	NUMBER EXAMINED:	0	0	5	5
FLUID DISTENTION		0	0	1	1
** END OF LIST **					

TABLE 6

Microscopic pathology - incidence summary

Print No: 0011

Printed: 02-NOV-01

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

		--- N U M B E R - O F - A N I M A L S - A F F E C T E D ---			
SEX:		--MALE--		-FEMALE-	
GROUP:		-1-	-2-	-1-	-2-
ORGAN AND FINDING DESCRIPTION	NUMBER:	5	5	5	5
** TOP OF LIST **					
KIDNEYS	NUMBER EXAMINED:	5	5	5	5
--CORTICAL TUBULAR BASOPHILIA		3	3	3	3
--DILATED TUBULE WITH EPITHELIAL HYPERPLASIA		0	1	0	1
--CORTICAL TUBULES WITH HYALINE DROPLETS		2	2	0	0
--CORTICAL INFLAMMATORY CELL INFILTRATE		1	2	0	1
--CORTICAL MINERALISATION		0	1	0	0
--HYPERPLASIA, PELVIC EPITHELIUM		0	0	1	0
--PELVIC DILATATION		0	0	1	0
URINARY BLADDER	NUMBER EXAMINED:	5	5	5	5
--REFLUXED SEMINAL COLLOID PLUG		1	3	0	0
** END OF LIST **					

APPENDIX 1

Daily dose observations - individual findings

Group	Sign No.	Animal No.	Day							
			-3	-2	-1	1	2	3	4	5
1M (Control)	Wet fur	1	√		√	√	√		√	√
		2	√	√	√	√	√	√		√
		3	√	√	√	√	√	√		√
		4	√		√	√	√	√	√	
		5	√	√	√	√	√	√	√	√
	Red staining around eyes	1				√	√	√	√	√
		2				√	√		√	√
		3					√	√		
		4				√	√		√	
		5			√		√		√	
	Brown staining head	1								√
		2		√						
		3				√				
		5		√						
	Brown staining muzzle	5							√	
2M (POSF)	Wet fur	6	√	√	√	√	√	√	√	√
		7	√		√	√		√	√	√
		8	√	√	√	√		√	√	√
		9	√	√	√	√		√	√	√
		10	√	√	√	√	√	√	√	√
	Red staining around eyes	7					√		√	
		9				√				
	Brown staining head	6					√			
		7		√						
		8			√					
	Brown staining muzzle	6				√			√	
		7							√	

Only animals showing signs are presented
√ sign present

APPENDIX 1

(Daily dose observations - continued)

Group	Sign No.	Animal No.	Day							
			-3	-2	-1	1	2	3	4	5
1F (Control)	Wet fur	11	√	√	√	√	√	√	√	√
		12	√	√	√	√	√	√		
		13	√	√	√	√	√	√		√
		14	√	√	√	√	√	√		
		15	√	√	√	√	√	√		
	Red staining around eyes	11				√	√			
		12				√	√	√		
		13				√	√	√	√	
		14					√			√
		15				√	√			√
	Brown staining head	11				√				√
		12						√		√
		13			√					√
		14			√					
		15				√				
	Brown staining muzzle	11							√	√
2F (POSF)	Wet fur	16	√	√	√	√	√	√	√	√
		17	√		√	√	√	√	√	√
		18	√		√	√	√	√	√	√
		19	√	√	√	√	√	√	√	√
		20			√	√	√	√	√	√
	Brown staining head	17								√
		19				√				
		20			√	√	√			
	Brown staining muzzle	17							√	
		18							√	
		20						√	√	

Only animals showing signs are presented
√ sign present

APPENDIX 2

Bodyweights - individual values (g)

Print No: 0007

Printed: 08-OCT-01

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

GROUP	ANIMAL	DAY -7	DAY -6	DAY -5	DAY -4	DAY -3	DAY -2	DAY -1	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
1M	1	210	228	237	245	251	254	264	263	265	270	275	281	282
	2	194	208	215	215	222	226	233	233	235	241	246	252	254
	3	195	206	217	223	233	237	247	248	251	262	263	273	276
	4	204	209	220	227	240	248	258	261	265	270	271	279	284
	5	206	221	228	236	245	256	261	264	269	276	275	282	283
2M	6	200	212	215	222	233	235	243	240	246	233	221	232	240
	7	190	203	213	213	217	220	228	228	236	241	233	241	244
	8	207	222	232	240	248	255	260	264	263	262	259	268	269
	9	196	208	210	219	228	231	238	243	246	241	240	249	250
	10	197	210	220	227	236	240	246	252	252	250	252	259	265
1F	11	152	152	155	159	163	161	169	167	169	166	170	172	171
	12	152	159	164	168	172	175	182	182	184	188	191	192	190
	13	143	151	156	158	160	164	169	169	170	172	172	177	178
	14	140	149	160	162	165	165	170	172	173	170	174	174	175
	15	154	156	160	162	170	170	173	176	177	181	181	184	185
2F	16	151	158	163	169	175	177	179	182	179	185	185	183	188
	17	153	159	165	168	171	174	180	183	181	178	186	190	189
	18	153	162	163	170	174	177	181	185	186	184	184	190	188
	19	147	153	153	159	161	164	166	166	167	170	170	173	172
	20	142	148	153	154	159	160	165	160	165	167	163	168	165

APPENDIX 3

Absolute organ weights - individual values (g)

Print No: 0010

Printed: 08-OCT-01

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

GROUP	ANIMAL	TERMINAL BODY WT (g)	ADRENALS	HEART	KIDNEYS	LIVER	LUNGS & BR
1M	1	281.1	0.052	1.250	2.23	13.56	1.161
	2	249.8	0.045	1.012	1.94	11.29	0.908
	3	273.6	0.047	1.206	2.02	12.78	1.161
	4	285.5	0.057	1.263	2.12	13.87	1.246
	5	281.5	0.051	1.133	1.88	13.06	1.082
2M	6	239.0	0.051	0.973	1.90	11.66	1.600
	7	244.1	0.048	1.392	1.90	11.96	2.095
	8	268.7	0.058	1.019	2.05	12.81	1.900
	9	247.2	0.064	0.963	1.85	13.26	1.901
	10	262.7	0.049	0.963	1.99	13.56	1.645

APPENDIX 3

(Absolute organ weights - continued)

Print No: 0010

Printed: 08-OCT-01

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

GROUP	ANIMAL	TERMINAL BODY WT (g)	ADRENALS	HEART	KIDNEYS	LIVER	LUNGS & BR
1F	11	169.5	0.050	0.798	1.33	7.28	0.816
	12	189.3	0.059	1.008	1.52	8.96	1.006
	13	176.7	0.052	0.785	1.23	7.10	0.891
	14	174.3	0.055	0.769	1.44	8.34	0.869
	15	187.9	0.053	0.709	1.31	8.15	0.871
2F	16	188.1	0.058	0.994	1.57	9.72	1.455
	17	190.5	0.063	0.980	1.55	10.10	1.584
	18	189.0	0.050	0.866	1.48	10.45	1.690
	19	171.6	0.051	0.845	1.38	7.96	1.343
	20	162.8	0.061	0.782	1.24	7.55	1.318

APPENDIX 4

Individual pathological findings

The initial examination was undertaken by the study pathologist, the results of which were then subjected to a routine peer review by a second pathologist. The diagnoses reported here represent the consensus opinions of both pathologists.

Study pathologist: Takahito Kambara, B.V.Sc., M.V.Sc., Ph.D.,
Pathologist
Department of Pathology

Peer review: Samuel G. McCormick, M.V.B., M.R.C.V.S., Ph.D., F.R.C.Path.,
Director of Pathology
Department of Pathology

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0001 SEX: MALE DOSE GROUP: 1 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 281.1 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

Printed: 02-NOV-01

```
GROUP              :         1          2
COMPOUND           :      CONTROL      POSF
DOSAGE (PPM)       :         0         300
                                                                                                                    Xybion protocol number: MIN 312
-------------------------------------------------------------------------------------------------------------------------------------------------------
ANIMAL NUMBER: 0002           SEX: MALE           DOSE GROUP: 1       SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01      STUDY DAY OF DEATH: 6       STUDY WEEK OF DEATH: 1        TERMINAL BODY WEIGHT:   249.8 GRAMS
-------------------------------------------------------------------------------------------------------------------------------------------------------
                                          P A T H O L O G Y   O B S E R V A T I O N S
                              NECROPSY                                                    HISTOPATHOLOGY
-------------------------------------------------------------------   ---------------------------------------------------------------
                                                                      KIDNEYS :
                                                                        -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL

                                                                      URINARY BLADDER :
                                                                        -REFLUXED SEMINAL COLLOID PLUG,-PRESENT

-  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
-------------------------------------------------------------------------------------------------------------------------------------------------------

    *** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

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APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP	:	1	2	Printed: 02-NOV-01
COMPOUND	:	CONTROL	POSF	
DOSAGE (PPM)	:	0	300	

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0003　SEX: MALE　DOSE GROUP: 1　SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01　STUDY DAY OF DEATH: 6　STUDY WEEK OF DEATH: 1　TERMINAL BODY WEIGHT: 273.6 GRAMS

P A T H O L O G Y　O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL -CORTICAL INFLAMMATORY CELL INFILTRATE,-MINIMAL, FOCAL

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

Printed: 02-NOV-01

```
GROUP              :         1          2
COMPOUND           :      CONTROL      POSF
DOSAGE (PPM)       :         0         300
```

Xybion protocol number: MIN 312

```
-------------------------------------------------------------------------------------------------------------------------------
ANIMAL NUMBER: 0004          SEX: MALE          DOSE GROUP: 1       SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01     STUDY DAY OF DEATH: 6       STUDY WEEK OF DEATH: 1        TERMINAL BODY WEIGHT:   285.5 GRAMS
-------------------------------------------------------------------------------------------------------------------------------
                                        P A T H O L O G Y   O B S E R V A T I O N S
                          NECROPSY                                                  HISTOPATHOLOGY
-------------------------------------------------------------------   ---------------------------------------------------------
                                                                      KIDNEYS :
                                                                        -CORTICAL TUBULES WITH HYALINE DROPLETS,-MINIMAL

LN MANDIBULAR :
  -CONGESTED

STOMACH :
  -ANTRUM WHITE NODULE(S);  MUCOSA, ONE, NEAR TO LIMITING
   RIDGE, 1MM.

-------------------------------------------------------------------------------------------------------------------------------
```

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

Printed: 02-NOV-01

```
GROUP              :         1          2
COMPOUND           :      CONTROL      POSF
DOSAGE (PPM)       :         0         300
```

Xybion protocol number: MIN 312

```
-------------------------------------------------------------------------------------------------------------------------------
ANIMAL NUMBER: 0005           SEX: MALE           DOSE GROUP: 1       SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01      STUDY DAY OF DEATH: 6       STUDY WEEK OF DEATH: 1        TERMINAL BODY WEIGHT:   281.5 GRAMS
-------------------------------------------------------------------------------------------------------------------------------
                                        P A T H O L O G Y   O B S E R V A T I O N S
                              NECROPSY                                                HISTOPATHOLOGY
-------------------------------------------------------------------   ---------------------------------------------------------
                                                                      KIDNEYS :
                                                                        -CORTICAL TUBULES WITH HYALINE DROPLETS,-MINIMAL

STOMACH :
  -ANTRUM WHITE NODULE(S);  MUCOSA, ONE, NEAR TO LIMITING
   RIDGE, 1MM.

-------------------------------------------------------------------------------------------------------------------------------
```

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

Printed: 02-NOV-01

GROUP	:	1	2
COMPOUND	:	CONTROL	POSF
DOSAGE (PPM)	:	0	300

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0006 SEX: MALE DOSE GROUP: 2 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 239.0 GRAMS

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

*** ANIMAL HAS NO MICROSCOPIC FINDINGS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

Printed: 02-NOV-01

```
GROUP              :        1          2
COMPOUND           :     CONTROL      POSF
DOSAGE (PPM)       :        0         300
                                                                                                                    Xybion protocol number: MIN 312
-------------------------------------------------------------------------------------------------------------------------------------------------------
ANIMAL NUMBER: 0007          SEX: MALE          DOSE GROUP: 2       SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01     STUDY DAY OF DEATH: 6       STUDY WEEK OF DEATH: 1        TERMINAL BODY WEIGHT:   244.1 GRAMS
-------------------------------------------------------------------------------------------------------------------------------------------------------
                                          P A T H O L O G Y   O B S E R V A T I O N S
                              NECROPSY                                                    HISTOPATHOLOGY
-------------------------------------------------------------------   ---------------------------------------------------------------
                                                                      KIDNEYS :
                                                                        -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL

                                                                      URINARY BLADDER :
                                                                        -REFLUXED SEMINAL COLLOID PLUG,-PRESENT

-  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
-------------------------------------------------------------------------------------------------------------------------------------------------------

    *** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

-------------------------------------------------------------------------------------------------------------------------------------------------------
```

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0008 SEX: MALE DOSE GROUP: 2 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 268.7 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULES WITH HYALINE DROPLETS,-MINIMAL
LN MANDIBULAR : -ENLARGED; RIGHT, ONE.	
STOMACH : -ANTRUM WHITE NODULE(S); MUCOSA, ONE, NEAR TO LIMITING RIDGE, 1MM.	
	URINARY BLADDER : -REFLUXED SEMINAL COLLOID PLUG,-PRESENT

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0009 SEX: MALE DOSE GROUP: 2 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 247.2 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL -CORTICAL INFLAMMATORY CELL INFILTRATE,-MINIMAL, FOCAL -CORTICAL MINERALISATION,-MINIMAL, FOCAL

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0010 SEX: MALE DOSE GROUP: 2 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 262.7 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL -DILATED TUBULE WITH EPITHELIAL HYPERPLASIA,-MINIMAL, FOCAL -CORTICAL TUBULES WITH HYALINE DROPLETS,-MINIMAL -CORTICAL INFLAMMATORY CELL INFILTRATE,-MINIMAL, FOCAL >NOTE:>FIBROSIS ALSO SEEN IN THE LESION OF TUBULAR HYPERPLASIA URINARY BLADDER : -REFLUXED SEMINAL COLLOID PLUG,-PRESENT

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0011 SEX: FEMALE DOSE GROUP: 1 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 169.5 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
UTERUS :	
-FLUID DISTENTION, MODERATE	

*** ANIMAL HAS NO MICROSCOPIC FINDINGS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

```
GROUP              :        1          2                                                        Printed: 02-NOV-01
COMPOUND           :     CONTROL      POSF
DOSAGE (PPM)       :        0         300
                                                                                       Xybion protocol number: MIN 312
-------------------------------------------------------------------------------------------------------------------------------------
ANIMAL NUMBER: 0012        SEX: FEMALE        DOSE GROUP: 1      SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01      STUDY DAY OF DEATH: 6       STUDY WEEK OF DEATH: 1        TERMINAL BODY WEIGHT:   189.3 GRAMS
-------------------------------------------------------------------------------------------------------------------------------------
                                              P A T H O L O G Y   O B S E R V A T I O N S
                            NECROPSY                                                             HISTOPATHOLOGY
----------------------------------------------------------------   ----------------------------------------------------------------
SKIN :
  -SCAB(S);  TAIL, A FEW, 1MM.

STOMACH :
  -ANTRUM WHITE NODULE(S);  MUCOSA, ONE, NEAR TO LIMITING
   RIDGE, 1MM.

- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
-------------------------------------------------------------------------------------------------------------------------------------

    *** ANIMAL HAS NO MICROSCOPIC FINDINGS RECORDED ***

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```

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2 Printed: 02-NOV-01
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0013 SEX: FEMALE DOSE GROUP: 1 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 176.7 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL
SKIN : -SCAB(S); TAIL, MULTIPLE, 1MM.	

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0014 SEX: FEMALE DOSE GROUP: 1 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 174.3 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
KIDNEYS : -PELVIC DILATION, MINIMAL; RIGHT.	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL -HYPERPLASIA, PELVIC EPITHELIUM,-MINIMAL, FOCAL -PELVIC DILATATION,-SLIGHT

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2 Printed: 02-NOV-01
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0015 SEX: FEMALE DOSE GROUP: 1 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 187.9 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL
SKIN : -SCAB(S); TAIL, A FEW, 1MM.	

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

Printed: 02-NOV-01

```
GROUP              :         1          2
COMPOUND           :      CONTROL      POSF
DOSAGE (PPM)       :         0         300
                                                                                                                    Xybion protocol number: MIN 312
-------------------------------------------------------------------------------------------------------------------------------------------------------
ANIMAL NUMBER: 0016           SEX: FEMALE         DOSE GROUP: 2       SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01      STUDY DAY OF DEATH: 6       STUDY WEEK OF DEATH: 1        TERMINAL BODY WEIGHT:   188.1 GRAMS
-------------------------------------------------------------------------------------------------------------------------------------------------------
-------------------------------------------------------------------------------------------------------------------------------------------------------

    *** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

    *** ANIMAL HAS NO MICROSCOPIC FINDINGS RECORDED ***

-------------------------------------------------------------------------------------------------------------------------------------------------------
```

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0017 SEX: FEMALE DOSE GROUP: 2 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 190.5 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL

*** ANIMAL HAS NO GROSS OBSERVATIONS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2 Printed: 02-NOV-01
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0018 SEX: FEMALE DOSE GROUP: 2 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 189.0 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
TEETH : -INCISOR(S) PALE; LOWER, RIGHT.	

*** ANIMAL HAS NO MICROSCOPIC FINDINGS RECORDED ***

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

GROUP : 1 2
COMPOUND : CONTROL POSF
DOSAGE (PPM) : 0 300

Printed: 02-NOV-01

Xybion protocol number: MIN 312

ANIMAL NUMBER: 0019 SEX: FEMALE DOSE GROUP: 2 SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01 STUDY DAY OF DEATH: 6 STUDY WEEK OF DEATH: 1 TERMINAL BODY WEIGHT: 171.6 GRAMS

P A T H O L O G Y O B S E R V A T I O N S

NECROPSY	HISTOPATHOLOGY
	KIDNEYS : -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL -DILATED TUBULE WITH EPITHELIAL HYPERPLASIA,-MINIMAL, FOCAL -CORTICAL INFLAMMATORY CELL INFILTRATE,-MINIMAL, FOCAL >NOTE:>FIBROSIS ALSO SEEN IN THE LESION OF TUBULAR HYPERPLASIA
TEETH : -INCISOR(S) PALE; LOWER.	

APPENDIX 4

(Individual pathological findings - continued)

Print No: 0012

Printed: 02-NOV-01

```
GROUP              :         1          2
COMPOUND           :      CONTROL      POSF
DOSAGE (PPM)       :         0         300
```

Xybion protocol number: MIN 312

```
-------------------------------------------------------------------------------------------------------------------------------
ANIMAL NUMBER: 0020          SEX: FEMALE         DOSE GROUP: 2       SACRIFICE STATUS: SCHEDULED, TERMINAL SACRIFICE
DATE OF DEATH: 18-SEP-01     STUDY DAY OF DEATH: 6       STUDY WEEK OF DEATH: 1        TERMINAL BODY WEIGHT:   162.8 GRAMS
-------------------------------------------------------------------------------------------------------------------------------
                                        P A T H O L O G Y   O B S E R V A T I O N S
                          NECROPSY                                                  HISTOPATHOLOGY
-------------------------------------------------------------------   ---------------------------------------------------------
                                                                      KIDNEYS :
                                                                        -CORTICAL TUBULAR BASOPHILIA,-MINIMAL, FOCAL

LN MESENTERIC :
  -ENLARGED, MINIMAL;  ONE.

UTERUS :
  -FLUID DISTENTION, MODERATE

-------------------------------------------------------------------------------------------------------------------------------
```

APPENDIX 5

pH values for terminal urine - individual values

Group	Males		Females	
	Animal number	pH	Animal number	pH
1 (Control)	1	NS	6	INS
	2	8.7	7 [a]	8.6
	3	8.6	8 [a]	8.8
	4	9.1	9	8.9
	5	9.3	10	INS
2 (POSF)	11 [a]	9.0	16 [a]	8.7
	12 [a]	8.9	17	7.4
	13	8.3	18 [a]	9.1
	14 [a]	8.1	19	6.9
	15	8.4	20 [a]	8.8

[a] Samples obtained in animal room following procedure provided by Sponsor, remaining samples obtained immediately prior to or during post mortem examination

INS Sample volume < 8 µl required to obtain accurate pH reading

NS No sample obtained

APPENDIX 6

PCNA staining in the urinary bladder – individual and mean values

	Level 1				Level 2				Level 3				Combined Levels			
Animal No	**Positive**	**Negative**	**Total**	**C.P.I.***	**Positive**	**Negative**	**Total**	**C.P.I.***	**Positive**	**Negative**	**Total**	**C.P.I.***	**Positive**	**Negative**	**Total**	**C.P.I.***
1	14	644	658	2.1	15	837	852	1.8	6	248	254	2.4	11.7	576.3	588.0	2.1
2	63	937	1000	6.3	53	947	1000	5.3	37	870	907	4.1	51.0	918.0	969.0	5.2
3	18	982	1000	1.8	20	980	1000	2.0	16	984	1000	1.6	18.0	982.0	1000.0	1.8
4	5	995	1000	0.5	5	995	1000	0.5	4	996	1000	0.4	4.7	995.3	1000.0	0.5
5	6	994	1000	0.6	3	997	1000	0.3	5	995	1000	0.5	4.7	995.3	1000.0	0.5
mean				**2.3**				**2.0**				**1.8**				**2.0**
6	4	996	1000	0.4	11	989	1000	1.1	6	994	1000	0.6	7.0	993.0	1000.0	0.7
7	65	935	1000	6.5	77	923	1000	7.7	31	969	1000	3.1	57.7	942.3	1000.0	5.8
8	47	953	1000	4.7	49	951	1000	4.9	27	973	1000	2.7	41.0	959.0	1000.0	4.1
9	17	983	1000	1.7	15	985	1000	1.5	7	993	1000	0.7	13.0	987.0	1000.0	1.3
10	98	902	1000	9.8	83	917	1000	8.3	41	959	1000	4.1	74.0	926.0	1000.0	7.4
mean				**4.6**				**4.7**				**2.2**				**3.9**
11	3	997	1000	0.3	4	996	1000	0.4	2	998	1000	0.2	3.0	997.0	1000.0	0.3
12	9	991	1000	0.9	16	984	1000	1.6	9	991	1000	0.9	11.3	988.7	1000.0	1.1
13	7	904	911	0.8	6	994	1000	0.6	1	864	865	0.1	4.7	920.7	925.3	0.5
14	6	994	1000	0.6	5	995	1000	0.5	1	999	1000	0.1	4.0	996.0	1000.0	0.4
15	2	998	1000	0.2	7	993	1000	0.7	1	999	1000	0.1	3.3	996.7	1000.0	0.3
mean				**0.6**				**0.8**				**0.3**				**0.5**
16	1	999	1000	0.1	1	999	1000	0.1	3	997	1000	0.3	1.7	998.3	1000.0	0.2
17	1	999	1000	0.1	0	926	926	0.0	1	999	1000	0.1	0.7	974.7	975.3	0.1
18	6	994	1000	0.6	2	998	1000	0.2	4	763	767	0.5	4.0	918.3	922.3	0.4
19	11	475	486	2.3	4	382	386	1.0	4	416	420	1.0	6.3	424.3	430.7	1.4
20	6	863	869	0.7	2	696	698	0.3	4	557	561	0.7	4.0	705.3	709.3	0.6
mean				**0.8**				**0.3**				**0.5**				**0.5**

*: cell proliferation index (%positive cells)

Mean cell proliferation index (%positive cells)

Group	Sex	Mean	SD	CV(%)
1	Male	2.0	1.95	97.1
2		3.9	2.86	74.2

Group	Sex	Mean	SD	CV(%)
1	Female	0.5	0.34	63.9
2		0.5	0.53	99.8

CV (%) Coefficient of variation (sd × 100 / mean)

ADMINISTRATION OF POSF

BY INHALATION TO RATS

Author
Simon Moore

CONTENTS

Page

TEST SUBSTANCE AND ADMINISTRATION

TEST SUBSTANCE

The test substance, POSF, is a liquid with boiling point of 154°C.

A consignment of the Test Article (4 x 20 kg, Lot number 040227), was received from the Sponsor on 14 June 2001. The test substance was stored securely in the original containers at room temperature until it was transferred to the atmosphere generation system.

The stated purity was >95.5%. Information regarding the purity and stability of the test substance is the responsibility of the Sponsor.

ADMINISTRATION

The test material was administered to the rats by snout-only exposure chambers described below:

The chamber atmosphere was produced by metering the liquid test substance into a glass vapour generator through which dried air was passed at a flow rate of 29 l/minute for administration to the rats, by inhalation from snout only exposure chambers. The target chamber concentration was achieved by metering the test substance from polypropylene syringes mounted on a syringe driver. This atmosphere gave the final chamber concentration of POSF.

The setting of the test substance metering system required to obtain the target chamber concentration was determined during preliminary generation trials without animals present and based on the Fourier Transform Infrared (FT-IR) analysis of chamber atmosphere samples. Minor adjustments were made to the test material delivery rates in order to maintain the chamber concentration close to target.

Animals assigned to Group 1 (Air control) received an exposure to air only, from the same compressed air source as used for the generation of the test atmospheres.

The duration of administration was a single 6-hour exposure, daily, for 5 days.

The usage of POSF was determined, for each day of treatment, for the lone test group.

TEST ATMOSPHERE GENERATION

The vapour delivery system for the dose group comprised a polypropylene syringe located on a syringe driver (Precidor, Model 5003), which delivered the liquid test material to a glass frit contained in a glass vessel via PolyTetraFluoroEthylene (PTFE) tubing. The syringe size and syringe driver settings required to achieve the target concentrations were established during the preliminary phase of the study. Air was passed through the vapouriser at a rate of 29 l/minute. The vapour/air mixture passed out of the vapouriser into the chamber inlet ducting through a 22 mm diameter flexible pipe. All equipment was housed in an extracted cabinet.

The air control group received clean air only at a rate of 29 l/minute.

EXPOSURE CHAMBERS (Figure A)

The inhalation exposure system comprised a snout-only inhalation exposure chamber and rats restraining tubes. Accessories included air supply and extract lines, which attached to the top and bottom of the chamber respectively. A filtration system was incorporated into the extract line.

A schematic diagram of an exposure system is shown in Figure A. The component parts of the system are described in further detail below:

Inhalation chamber

ADG snout-only inhalation chamber (ADG Developments Ltd, Hitchin, Hertfordshire, England). This is a modular apparatus of aluminium alloy construction comprising of a base unit, a variable number of animal exposure sections, each having 20 exposure ports, and a top section incorporating a central aerosol inlet surrounded by a tangential air inlet.

The chambers used on this study were assembled using 3 rodent exposure sections, identified as levels 1 (top) to 3 (bottom). All exposure sections had 20 ports and formed a 28 cm diameter cylinder with a volume of approximately 47 litres. During dosing each chamber was housed in an enclosed ventilated cabinet.

Rats restraining tubes

Moulded polycarbonate tubes tapered at one end to allow the snout only to project from the tapered end. The other end is normally closed by insertion of an expanded plastic bung. A push rod passes through the centre of the bung and is adjustable to maintain the position of the rats during restraint. Tubes are attached to a chamber by means of push-fit "O" ring seals located in the exposure ports of the animal exposure sections. The restraining tubes were attached to chamber level two.

Chamber level two was used to expose the animals. All exposure ports not in use were sealed with an expanded plastic bung.

Air supply and extract

Air supplies were provided by a compressor. The air was filtered to remove any residual particulate and was dried (dew point ~2ºC). A 1 l/minute differential was maintained between the inlet and outlet airflows to provide a small negative pressure within the exposure system. The in-line flowmeters were calibrated daily against high quality tapered tube rotameters measuring the free flow of air at points of attachment of the supply and extract lines to each chamber. The airflows used for each group are detailed in Table A.

The calibrated exhaust airflow of each exposure system was passed through a trapping system comprising a Fluosorber carbon vapour filter and a silica gel column before it was passed to atmosphere. The exhaust flows used for each group are detailed in Table A.

PROCEDURE

Two exposure systems were used, one for each group. The procedure followed for each group was similar except that, for the control group, the rats in the control group received air only. Therefore in the following description the comments relating to the sampling and the Fourier Transform Infrared spectrophotometer apply to the test group only.

The FT-IR data capture programme was initialised and the path difference was inspected to ensure the value was zero, subsequently, a background scan was recorded and the cell path length was calibrated.

A syringe was filled with the test substance, the weight of liquid was recorded and the syringe was mounted on the syringe driver. The syringe was connected to the vapouriser by PTFE tubing. The pump was turned on briefly in order to engage the gearing and to move the liquid along the PTFE tubing almost to the surface of the glass frit. The injection rate to be used was then set.

The rats were removed from their cages and placed into restraining tubes, which were then attached to their assigned level of the chamber. Unused exposure ports were sealed with blanking plugs. The rats were removed from their cages and placed into restraining tubes, which were colour coded for the treatment group and numbered for each animal. The tubes were then attached to level 2 of the chamber utilising 5 ports on either side of the 20 port section. Unused exposure ports were sealed with blanking plugs.

The inlet and outlet airflows of the exposure system were calibrated using precision made tapered glass tube flowmeters.

The connections of the generation and extract systems to the chamber were checked to ensure they were correct and working.

The barometric pressure was recorded manually.

Generation commenced as the syringe driver was turned on and 15 minutes into the exposure, the chamber sampling system was activated (see below). At intervals of 30 minutes, any reactions by the rats to exposure, together with checks of generation and chamber operational parameters including temperature and inlet and outlet air flowrate were manually recorded. The airflows were measured throughout the exposures using in-line flowmeters.

A sample pump continually delivered the chamber atmosphere from the test chamber to the FT-IR system at a flow rate of 1 l/minute. Analysis for the determination of POSF concentration occurred at exactly 30 mimute intervals throughout each exposure. The chamber atmosphere was returned to the chamber downstream of the sampling point.

At the end of six hours generation, the syringe driver was turned off and the weight of the remaining syringe contents was recorded. Generation airflow was turned off and the chamber extract allowed to clear the vapour for 5 - 10 minutes (equilibration time t_{99} is 7 minutes). The data capture program halted automatically after collecting a specific number of samples.

At the end of this time, the rats were unloaded from the chambers and returned to their respective holding cages.

The chambers were washed with hot water.

The IR cell path length was retested to ensure that the initial value prior to the exposure was consistent throughout the exposure.

ANALYSIS OF THE TEST ATMOSPHERE (FIGURE B)

The concentration of POSF in air within the test inhalation chamber was measured using a Fourier Transform Infrared (FT-IR) Spectrophotometer. Definitive details of the FT-IR, its standardisation and validation are given in Appendix A.

The Spectrophotometer was located adjacent to the exposure chambers.

CHAMBER MONITORING SYSTEM

A PC running the AutoQuant 3.01 software was used to monitor and record the system performance during each exposure. The data collection sequence and display were controlled by a personal computer (PC) and all information collected was displayed on a monitor. Simultaneously, the data was stored electronically. This program was composed of three basic stages of operation: an initial setting up (pre-exposure) phase, an exposure monitoring phase and the post exposure data collation and presentation phase. The program and FTIR hardware were loaned by the Sponsor for the duration of the study. Raw data printed as a hard copy.

Setting-up phase

In the initial phase, prompted by the program screen display, the instrument response was checked, followed by a background scan. An ultra pure nitrogen cylinder was used for this purpose. The pathlength was then calibrated using a certified cylinder of ethylene. A regulated flowrate of 2 l/minute was used for the nitrogen and ethylene cylinders, the analyser drew 1 l/minute of the gas stream and the remainder was vented to waste. The flow meters used in conjunction with the cylinders were monitored using a calibrated in-line tapered tube gas flowmeter.

The study details including the daily pathlength, number of scans and samples and frequency of samples are entered and stored into the data capture program prior to the exposure.

Exposure monitoring phase

This phase was started 15 minutes later than the start of atmosphere generation. The test chamber's environment was monitored during a 30-minute cycle when the analysed concentration was recorded. The data were displayed on screen, printed and stored on the local hard drive of the computer and an external zip disc. A total of twelve sample points were recorded, each of which represented 32 coadded scans.

Post exposure phase

At the end of the exposure, the data collected during exposure was collated and printed. The mean values, together with standard deviation were calculated for each parameter recorded. The first set of chamber concentration data was included in the calculation of the mean and standard deviation because chamber conditions stabilised within the 15 minutes from the start of exposure (equilibration time, t_{99} was 7 minutes) before the analysis was started.

Midac Grams 32 version 4.11 software was solely used to generate a hard copy of the infrared data.

The IR cell path length calibration of the FT-IR was reassessed to ensure that the initial value prior to the exposure was consistent throughout the exposure.

Details of the analytical methodologies used are given in Appendix A.

TARGET CONCENTRATIONS

The target concentrations of POSF was:

Group	Designation	Concentration (ppm)
2	Test Group	300

The target concentrations were selected in consultation with the Sponsor, following the review of available data.

EXPOSURE CHAMBER CONDITIONS

Chamber analysed concentration of POSF

The test atmosphere was sampled from one point within the test atmosphere chamber. This was continually drawn through a transfer line, which was therefore in equilibrium with the mean concentration from the test chamber. The chamber atmosphere was returned to the exposure chamber downstream of the sampling point.

Every 30 minutes, the software for automated analysis and data logging activated the FT-IR.

The methodology is presented in Appendix A.

Chamber spatial distribution

All the animals and the analytical sampling point were situated on level two of the three level exposure chamber.

Nominal concentration of chamber atmospheres

The chamber nominal concentrations were calculated from the amount of liquid used over the six-hour exposure period, the mass of the liquid and the exposure mean airflow. The formulae used were as follows:

$$\text{Concentration} = \frac{V}{Va + V} \times 1{,}000{,}000\ \text{ppm} \qquad (1)$$

$$V = \frac{W \times R \times T}{M} \times \frac{760\ \text{mm Hg}}{Atm} \qquad (2)$$

where

	V	=	gaseous volume of POSF (L)
	W	=	mass of POSF (g)
	M	=	molecular weight of POSF (502.14 g/mole)
	R	=	Gas constant (0.08205 L atm $mmol^{-1}$ K^{-1})
	T	=	temperature (K)
	Atm	=	atmospheric pressure (mmHg)
	V_a	=	volume of air (L)

Airflow and temperature

These parameters were recorded manually, as described above under the Test Atmosphere generation and Procedure sections.

RESULTS

VAPOUR CONCENTRATION

Analysed concentration of POSF

The data are presented as follows:

Daily mean values	Table B
Individual values	Appendix B

The study mean concentration (the mean of daily mean values) for each group exposed to POSF are presented below:

Group	Chamber concentration (ppm)	
	Target	Analysed
2 (Test Group)	300	317

Analysed concentration was in good agreement with target concentration. The coefficient of variation of the daily mean was 1.4 % for Group 2.

The uncertainties relating to the analysed and room air concentrations were estimated by the data capture program based on the least squares statistics.

Nominal concentration of POSF

The data are presented in Table C and is summarised below:

Group	Nominal concentration (ppm)	A/N ratio (%)
2 (Test Group)	283	111.5

$$\text{A/N} = \left(\frac{\text{Analysed concentration}}{\text{Nominal concentration}} \right) \times 100$$

For Group 2, the nominal concentration for each exposure was calculated from the following parameters:

The mass of POSF delivered into each vapour generator;
The mean chamber temperature;
The barometric (atmospheric) pressure;
The molecular weight of POSF;
The gas constant;
The chamber airflow;
The exposure duration.

The equations used for the calculation of the nominal concentration are detailed in Table C.

The nominal concentration for each exposure period was calculated, for the test group from the mass of POSF delivered into the generator. The daily ratios of analysed to nominal concentration (A/N), expressed as a percentage, were between 109 and 115%, with a coefficient of variation of only 2.1%. Possible reasons for this unexpectedly high A/N ratio are discussed below.

Chamber Temperature

The daily mean chamber temperatures are presented in Table D.

The chamber temperatures were similar for both groups on each day of the study.

DISCUSSION

Control of the POSF vapour delivery to the exposure chambers was excellent, with a coefficient of variation of less than 1.5%, and a study mean concentration, which was within 6% of the target value for the test group. As no analytical data was available for exposure 4 due to a computer problem, it seems reasonable (using the consistent A/N ratio) that the animals were dosed with a concentration around *ca.* 313 ppm.

The ratio between the average analysed and the nominal chamber concentrations (A/N ratio) showed a definite discrepancy between these two values, with a mean of 111.5%. The coefficients of variation for the analysed and nominal values are 1.4% and 1.5% respectively, which shows that the discrepancy is consistent throughout the five day long exposure. The estimate of the nominal chamber concentration was lower than the analysed value and this is attributed to a combination of uncertainties as follows:

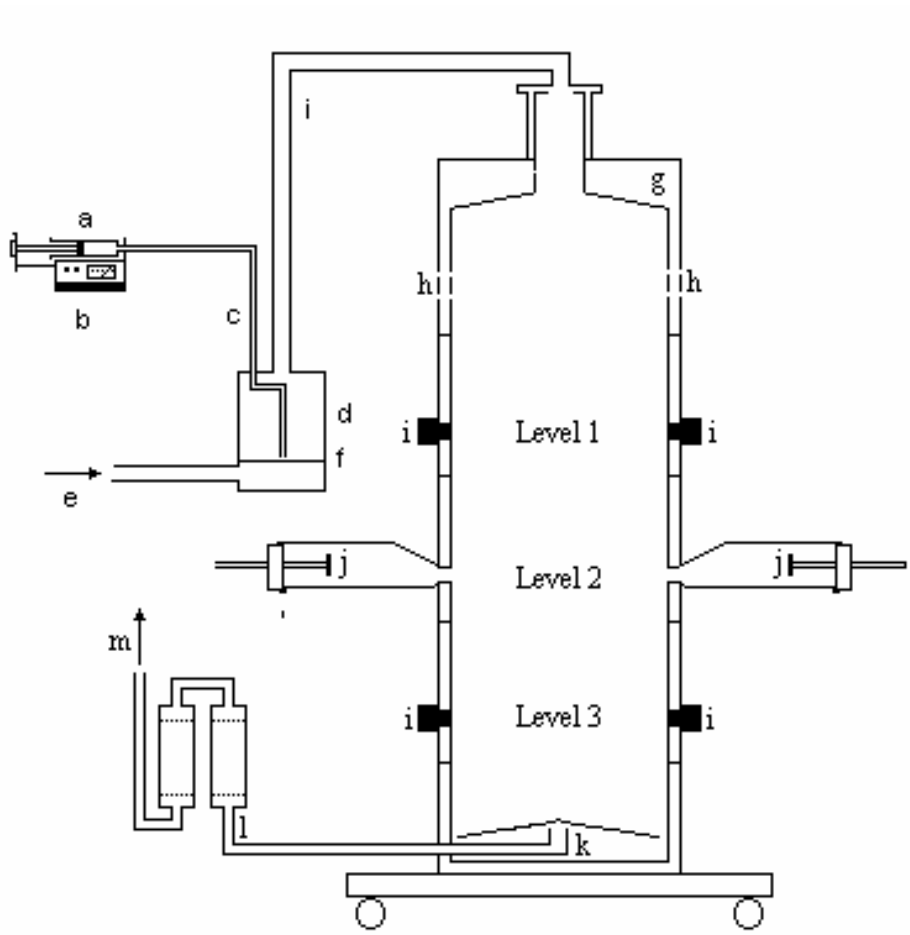
- POSF reference spectra and subsequent AutoQuant method calculations. These have errors of 5% associated with them (responsibility of the Sponsor);
- The cylinder of ethylene standard has certified errors of 2%;
- Uncertainties in airflow measurements. A 1 l/minute difference in the airflow would produce a 3% change in the nominal concentration;
- The test material has a stated purity of >95.5% POSF. other components of the test material were various perfluoroalkyl sulfonyl fluorides (<4.5%) and their absorbance may interfere with that of POSF;
- The effect of using ultra pure nitrogen for the background scans (rather than the control chamber atmosphere) is unknown.

CALCULATIONS

In order to minimise the cumulative errors, which result from repeated rounding of numbers, much of the data in this report has been calculated continuously using unrounded numbers and only rounded for printing. Consequently, these rounded numbers may include rounding errors in the last significant figure, possibly leading to small apparent discrepancies with other data in the report.

FIGURE A

Schematic of a rodent inhalation dosing system

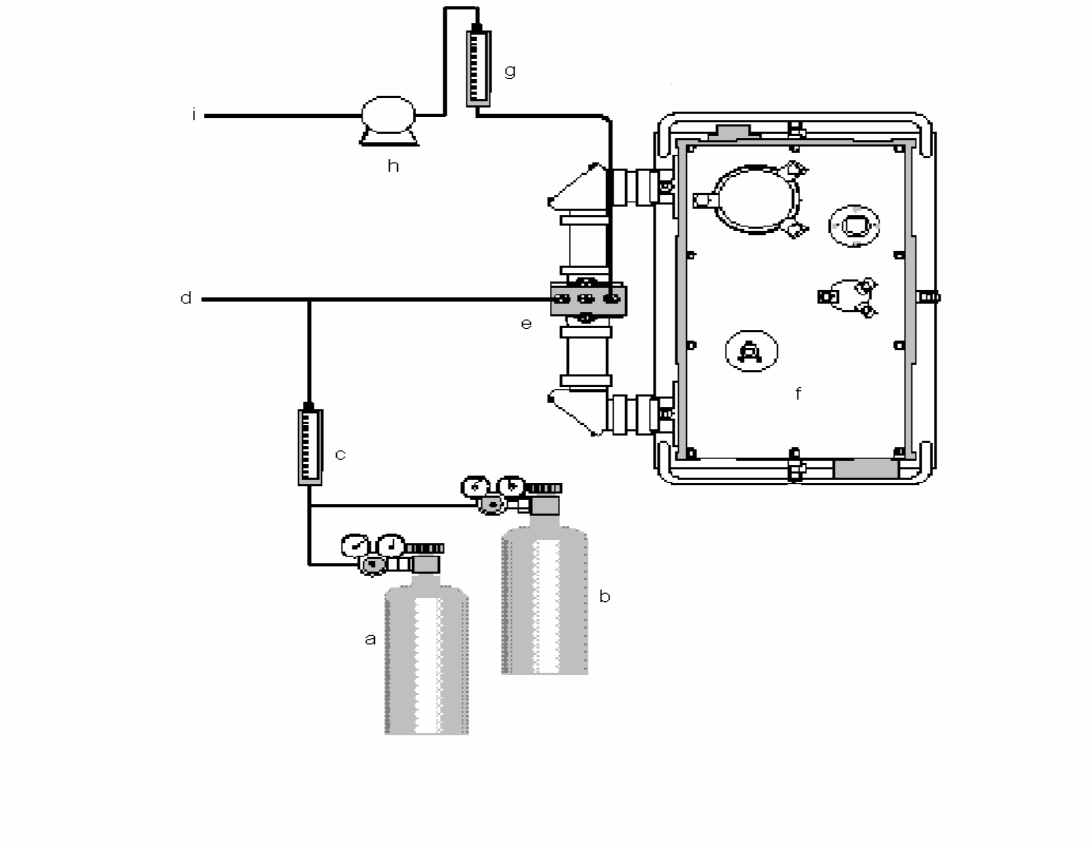


Key

a Polypropylene syringe
b Syringe driver
c Test compound feed line
d Glass Vapouriser
e Air supply (29 l/minute)
f Sinter diameter (114 mm)
g Exposure chamber
h Observation port
i Blanking plugs
j Rodent restraining tubes (standard sections with 20 exposure ports on levels 1 to 3
k Exhaust plenum
l Filtration
m Air extract (30 l/minute)

FIGURE B

Schematic of a Fourier Transform Infrared spectrophotometer



Key

a Nitrogen cylinder
b Ethylene cylinder
c Flowmeter regulated to 1 l/minute
d Test compound sample line from chamber
e 10 cm IR cell
f FT-IR spectrophotometer
g Flowmeter regulated to 1 l/minute
h Diaphragm pump
i Sample line return to chamber

TABLE A

Operating conditions for the inhalation exposure system

Parameter	Group	
	1 (Air control)	2 (Test Group)
Target concentration of POSF (ppm)	0	300
Chamber airflows (l/minute)		
Elutriator output (chamber inlet)	29	29
Chamber extract	30	30
Vapour generator settings		
Test material feed	N/A	Precidor 5003 syringe pump
Syringe size (ml)	N/A	50
Syringe pump setting (Speed, mm/min):		
Exposure 1	N/A	0.145
Exposures 2-5	N/A	0.140

N/A Not applicable

TABLE B

Chamber concentration of POSF (ppm) - daily mean values

Exposure No.	Analysed Concentration		Room Air		Concentration minus Room Air (ppm)
	ppm	Error [a]	ppm	Error	
1	316.04	0.85	0.398	0.004	315.65
2	313.69	0.83	0.373	0.004	313.32
3	323.33	0.82	0.289	0.003	323.04
4 [b]					
5	314.74	0.83	0.428	0.004	314.31
Mean	316.95	0.83	0.372	0.004	316.58
sd	4.361	0.009	0.0598	0.0007	4.414
CV (%)	1.4	1.1	16.1	19.9	1.4

[a] Error value reported by Autoquant software.

[b] No analytical data collected for exposure 4 due to computer problem with data capture program

sd Standard deviation

CV Coefficient of variation (sd × 100/mean)

TABLE C

Nominal concentrations of POSF (ppm) - individual exposure values

Group 2 (Test Group) - Target concentration 300 ppm

Exposure		Barometric pressure (mmHg)	POSF usage (g)	Chamber airflow (l/min)	Chamber concentration		A/N ratio (%)
No.	Duration (min)				Nominal [c] (ppm)	Analysed (ppm)	
1	360	753	64.6	30.0	289	316	109.1
2	360	763	63.0	30.0	278	313	112.6
3	360	757	63.5	30.0	283	323	114.2
4	360	762	63.3	30.0	280	[a]	[a]
5	360	766	64.8	30.0	285	314	110.3
Mean of Means	360	760	63.8	30.0	283	317	111.5
sd	0.0	5.2	0.81	0.0	4.4	4.4	2.32
CV (%)	0.0	0.7	1.3	0.0	1.5	1.4	2.1

[a] No analytical data collected due to computer problem with data capture program

[c] Nominal concentrations were calculated from the following equations:

$$\text{Concentration (ppm)} = \frac{V}{V_a + V} \times 10^6$$

$$V = \frac{W \times R \times T}{M} \times \frac{760\ \text{mm Hg}}{\text{Atm}}$$

where
- V = gaseous volume of POSF
- W = mass of POSF
- M = molecular weight POSF (502.14 g/mole)
- R = gas constant (0.08205 l atm mol^{-1} K^{-1})
- T = temperature (K), = temperature (°C, see Table D) + 273
- Atm = atmospheric pressure (mmHg)
- V_a = volume of air (litres) passing through the chamber during the exposure

A/N Analysed/nominal concentration ratio expressed as a percentage
sd Standard deviation
CV Coefficient of variation (sd × 100/mean)

TABLE D

Chamber temperature - exposure mean values

Exposure	Temperature (°C)	
	Group 1 (Control)	Group 2 (Test Group)
1	20.0	20.2
2	20.0	20.0
3	20.0	20.0
4	19.9	20.0
5	19.9	19.9
Mean	20.0	20.0
sd	0.06	0.11
CV (%)	0.3	0.5

sd standard deviation
CV Coefficient of variation (sd × 100/mean)

APPENDIX A

Methods of sample collection and analysis for POSF

SAMPLE COLLECTION

Chamber concentration

The atmosphere sample was continually drawn from the test chamber to the FT-IR system at a regulated flow rate of 1 l/minute using a laboratory pump positioned downstream of the IR cell. The concentration of POSF was determined at exactly 30 minute intervals throughout each exposure. The sampled atmosphere was returned to the exposure chamber downstream of the sampling point. The airflow to the spectrophotometer was monitored throughout each of the exposures using a calibrated in-line tapered tube gas flowmeter. Gas sampling lines were PTFE tubing (0.6 cm diameter).

METHOD OF ANALYSIS

Chamber atmosphere samples were analysed by extractive FT-IR. The method of sample analysis is detailed, together with a summary of the method validation, in the Inhalation Analytical Procedure at the end of this Appendix.

APPENDIX A

(Methods of sample collection and analysis for POSF - continued)

CALCULATIONS

FT-IR analysis

The samples of chamber atmosphere were passed through the IR cell, which was calibrated using nominal vapour standards. The method for calculating the concentration of POSF from the mass used to prepare each vapour standard using the nominal feed rate is given below in equations 1 and 2.

$$\text{Concentration} = \frac{V}{Va + V} \times 1{,}000{,}000 \text{ ppm} \qquad (1)$$

$$V = \frac{W \times R \times T}{M} \times \frac{760 \text{ mm Hg}}{Atm} \qquad (2)$$

where

V	=	gaseous volume of POSF (ml)
W	=	mass of POSF (g)
M	=	molecular weight of POSF (502.14 g/mole)
R	=	Gas constant (0.08205 ml atm $mmol^{-1}$ K^{-1})
T	=	temperature (K)
Atm	=	atmospheric pressure (mmHg)
V_a	=	volume of air (ml)

APPENDIX A

(Methods of sample collection and analysis for POSF - continued)

COMPOUND SPECIFIC INHALATION ANALYTICAL PROCEDURE FOR POSF

The analysis of POSF (Perfluorooctane sulfonyl fluoride) in air sample substrate

The method outlined in this document has been validated and is considered fit for the purpose of monitoring conditions in an Inhalation Toxicology study.

This document details the basic procedures for the analysis of POSF sampled by extractive FT-IR from test atmospheres. The resulting samples, of approximate concentration 240 to 360 ppm, are analysed using the MIDAC I-Series spectrophotometer and AutoQuant software. Study specific amendments and additions will be detailed within a supplementary document.

NOTE Throughout this document, the symbol **§** indicates that the relevant information is not available at present, but will be included in a Study specific supplement.

EFFECTIVE FROM:	**29 August 2001**

Test substance

POSF, perfluorooctane sulfonyl fluoride has the following formula: $C_8F_{17}SO_2F$.

Appearance	Clear liquid
Storage	Ambient temperature

Reagents

Air	In House	Compressed
Ethylene	Sponsor	Calibration gas (certified ± 2%)
Nitrogen	Sponsor	Calibration gas (certified >99.9995%)
Liquid nitrogen	BOC	Cooling Medium

APPENDIX A

(Methods of sample collection and analysis for POSF - continued)

Equipment

Balance and Data printer	Sartorius	R160P with YDP-01
Flow meter	Gilmont Instruments	0-4 Lpm
FT-IR	Midac Corporation	I2001 series (serial no. 151)
Computer	Compaq	LTE5400
Sotware	Midac Corporation	AutoQuant Version 3.01 Midac Grams 32 Version 4.11
Chamber	ADG	3 level snout only
Syringe Driver	Precidor	Type 5003
UV Quartz Cells	Midac Corporation	ZnSe Gas Cell

General laboratory glassware

Accessories also employed are: a vacuum pump; cylinder regulators, power connectors; regulated exhaust pumps.

Consumables

Syringes	Aldrich	50 ml polypropylene

Method of sample extraction

A volume of POSF is dispensed from the test drum into a 50ml syringe. The syringe driver is set to give a concentration of 300ppm in an airstream of 30 l/minute airflow, mixed thoroughly in the vapouriser and fed directly into the chamber. The test atmosphere is drawn along the sample line at a flow rate of 1 l/minute directly through the IR cell.

Production of a Background Scan

A flowrate of 1l/minute ultra-pure nitrogen (cylinder No. C0C7700219 (nitrogen >99.9995 ± 2%) is drawn though the IR cell. After a period of 3-5 minutes, an FT-IR single beam spectrum is generated approximately every 2 seconds and continuously collected for 32 scans to produce a more definitive spectrum.

APPENDIX A

(Methods of sample collection and analysis for POSF - continued)

Storage of standards and samples

Stability experiments were not required for on-line analysis.

Calibration and Quantification

For each sample measurement, the absorbance determines the amount present in the sample using the equation below (Beer's Law):

$$\text{Amount}(ppm) = \frac{A}{a \times b}$$

Where

A = absorbance at a given frequency of POSF in the sample spectrum
a = absorption coefficient (absorptivity) of POSF in the sample spectrum, which is derived from the reference calibration transfer standard spectrum
b = path length of the cell derived from calibration data

Calibration of Standards

Calibration is performed daily before each exposure (see "MIN/312 Operating Procedure").

A certified concentration of ethylene (cylinder No. C0C9700874 (ethylene = 2020ppm ± 2%) is passed through the sampling system prior to sample collection to check instrument response and determine the calibrated cell path-length. Cell path-length is a critical input to the analytical method used to perform each spectral analysis. The ethylene value is read from the test certificate on the calibration cylinder.

A test atmosphere of ethylene at a flowrate of 1 l/minute is drawn through the IR cell. After a period of 3-5 minutes, a FT-IR single beam spectrum is generated approximately every 2 seconds and continuously collected for 32 scans to produce a less noisy spectrum. This produces a calibrated path length. This value is then entered into the POSF method file and manually recorded.

QC **Path length Limits: -**The calibration will be repeated by the user if no peak is present in the 1400-650 cm^{-1} region or if the path length value is outside the range 0.100 to 0.115 m. If the calibration still does not conform, all of the calibration set up parameters will be checked.

QA **Instrument Calibration Check:** The calibration is reverified and subsequently recorded at the end of the exposure. Instrument calibration checks are within ±5%. Repetition of two four hour periods showed that the final calibration gave the path length values differed by less than 2% from the initial calibration value of the ethylene cylinder (cylinder No. C0C9700874 (ethylene = 2020ppm ± 2%). The 2% value is inside the 5% tolerance limits allowed as quality check standards.

The temperature of the test gas stream and the barometric pressure will be monitored manually.

APPENDIX A

(Methods of sample collection and analysis for POSF - continued)

FT-IR analyser specification

The Midac I2000 series FT-IR spectrophotometer has a wavelength accuracy of 0.5cm^{-1}. A MCT detector monitors the absorbance of various chemical bonds. AutoQuant version 3.01 (MIDAC, Irvine, CA) software is used for all infrared data acquisition and quantification.

The length of the IR cell or path length (approximately 10 cm) dictates the concentration range that will give a linear relationship between absorbance and concentration.

The optimum flow rate for the IR cell is 1 l/minute, which is set by a flow meter and all the analysis is conducted at that flow rate to avoid differences of cell pressure. Further details can be obtained from the operating procedure stored proximal to the FT-IR system. The analyser draws 1 l/minute from the gas stream and the remainder is returned to the exposure system exhaust. All exhaust lines are monitored by precision stainless steel and glass in-line rotameters.

Raw Data

Raw data is defined as the first hard copy output obtained immediately following data acquisition by the FT-IR system. This will be signed and dated immediately after printing. An electronic backup copy of this is saved to the local zip drive. If a run time hard copy was not obtained through a printer fault, the electronic copy from the zip drive directory may be used to generate a hard copy, which will be signed and dated. Exposure data is stored in the file format YYMMDD.txt.

Computer hardware:	Computers and VDUs are maintained by the Sponsor.
	Printer is maintained by the IT department.
FT-IR System hardware:	The Sponsor has supplied the hardware. Arrangements for servicing and maintenance would be made in consultation with the Sponsor.
Software:	Documentation is the responsibility of the Sponsor.

Safety

The COSHH assessment provides details regarding the toxicity of the test material POSF. It is considered that the standard handling procedures used within the department are suitable to prevent exposure to the test material.

In case of a total power failure, switch off the FT-IR and test compound generation equipment immediately.

APPENDIX A

(Methods of sample collection and analysis for POSF - continued)

Summary of method validation

The raw data for the method validation is located in study MIN/312.

Comparison of test blanks and test samples showed that the analyte was well resolved from any potential interfering peak.

Repeatability

The variability of the POSF level in a dynamic (exposure) system is determined by the airflow and feed rate through the syringe driver being set correctly and checked every 30 minutes (as the flow is pressure dependent). Statistical analysis shows that, with these checks in place, over a four hour exposure in a 3-level ADG snout only exposure system, the Coefficient of variation is typically less than 7.0 at 300 ppm. This figure includes the variability of generation as well as the repeatability of measurement.

Specificity

This Fourier Transform infrared technique monitors the absorbance of POSF in the region 1400-650 cm^{-1}, specifically between 1400 and 1000 cm^{-1}.

Linearity

Linear regression analysis of the FT-IR spectrophotometer response to standards was conducted during preliminary trials. This was used to demonstrate that the FT-IR response is linear over the concentration range. Feed rates were selected to achieve a nominal concentration of 240 to 360 ppm (see data file 010912.txt, 010912a.txt and reference sequences 010912 MIN312 and 010912a MIN312) utilising a total extract flow rate of 30 l/minute.

Least squares regression analysis with a unweighted linear regression of the predicted concentration from the nominal against FT-IR analysed concentration (240 to 360 ppm) produced a correlation coefficient of 0.999301 and relative errors less than 1.0% in the range 360 to 240 ppm. The Limit of Quantification (LOQ) for POSF will be set by the lowest acceptable nominal feed rate, however, the LOQ and Limit of Detection (LOD) are potentially as low as 12.17 and 4.01 ppm respectively (calculated statistically using the standard deviation obtained for a nominal feed rate to obtain a concentration of 240 ppm).

Uncertainty of POSF analysis

Uncertainties were said (by the Sponsor) to be less than ±5% for the AutoQuant method calculations. The POSF reference spectrum is from the 3M quantitative library and was generated at 3M using EPA guidelines. Other principal uncertainties were: ethylene path length Calibration (2%); interference from alkyl sufonyl fluorides (<4.5% v/v, but interference unknown).

APPENDIX A

(Methods of sample collection and analysis for POSF - continued)

MIN/312 - STUDY SPECIFIC SUPPLEMENT TO THE INHALATION ANALYTICAL PROCEDURE FOR POSF (PERFLUORO-OCTANE SULFONYL FLUORIDE)

This supplement details additions and amendments to the procedure to be used for the FT-IR assay of POSF obtained from air samples collected on the above study.

The assay, incorporating the additions and amendments, is suitable for the analysis of POSF, in air, at concentrations within the range of 240 to 360ppm.

Details given in this supplement supersede those in the compound specific IAP.

EFFECTIVE DATE :	**4 September 2001**

Analytical standard

Name	FX-8B, POSF, T-7661.1, Perfluoro-octane sulfonyl fluoride
Batch number	040227
Purity	>95.5% (Perfluoroalkyl sulfonylfluorides <4.5%)
Expiry date	Not Supplied
Supplier	Sponsor

Fourier Transform - Infrared Spectrophotometer

The analysis is performed using a FT-IR analyser supplied by the sponsor.

Preparation of standard solutions

Provide nominal feed rates to the chamber in the range 240 to 360 ppm.

Calibration and Quantification

Calibration of the pathlength by an ethylene standard (cylinder No. C0C9700874 (ethylene = 2020ppm ± 2%). Quantification using the 3M quantitative library generated at 3M using EPA guidelines for generating reference spectra (v).

APPENDIX B

Individual POSF concentration measurements

Exposure	Date and Time	Concentration		Room Air		Concentration - Room Air
		(ppm)	Error	(ppm)	Error	(ppm)
1	13/09/01 09:24	272.86	0.71	0.171	0.003	272.69
	13/09/01 09:54	345.50	0.88	0.245	0.003	345.26
	13/09/01 10:24	323.61	0.85	0.309	0.003	323.31
	13/09/01 10:54	315.21	0.83	0.365	0.003	314.85
	13/09/01 11:24	316.94	0.83	0.402	0.004	316.53
	13/09/01 11:54	310.42	0.84	0.413	0.004	310.01
	13/09/01 12:24	312.39	0.84	0.445	0.004	311.95
	13/09/01 12:54	316.65	0.86	0.464	0.004	316.19
	13/09/01 13:24	318.48	0.87	0.475	0.004	318.00
	13/09/01 13:54	316.82	0.87	0.488	0.004	316.33
	13/09/01 14:24	322.98	0.88	0.497	0.004	322.48
	13/09/01 14:54	320.68	0.88	0.5	0.005	320.18
	Mean	316.04	0.85	0.398	0.004	315.65
	sd	16.272	0.047	0.1066	0.0007	16.240
	CV (%)	5.1	5.5	26.8	17.6	5.1
2	14/09/01 09:25	276.10	0.72	0.166	0.003	275.93
	14/09/01 09:55	320.49	0.81	0.233	0.003	320.25
	14/09/01 10:25	321.14	0.82	0.297	0.003	320.84
	14/09/01 10:55	314.85	0.81	0.332	0.003	314.52
	14/09/01 11:25	310.00	0.81	0.357	0.003	309.65
	14/09/01 11:55	312.98	0.83	0.405	0.004	312.58
	14/09/01 12:25	317.30	0.85	0.419	0.004	316.88
	14/09/01 12:55	315.09	0.85	0.439	0.004	314.65
	14/09/01 13:25	321.14	0.87	0.448	0.004	320.69
	14/09/01 13:55	315.14	0.86	0.463	0.004	314.68
	14/09/01 14:25	319.44	0.87	0.457	0.004	318.99
	14/09/01 14:55	320.64	0.87	0.465	0.004	320.18
	Mean	313.69	0.83	0.373	0.004	313.320
	sd	12.375	0.043	0.0986	0.0005	12.3137
	CV (%)	3.9	5.1	26.4	15.0	3.9

sd standard deviation
CV Coefficient of variation (sd × 100/mean)

APPENDIX B

(Individual POSF concentration measurements - continued)

Exposure [a]	Date and Time	Concentration		Room Air		Concentration - Room Air
		(ppm)	Error	(ppm)	Error	(ppm)
3	15/09/01 07:55	[c]	0.18	0.208	0.002	
	15/09/01 07:59	[c]	0.18	0.224	0.002	
	15/09/01 08:02	[c]	0.19	0.233	0.002	
	15/09/01 08:06	[c]	0.2	0.245	0.002	
	15/09/01 08:10	[c]	0.03	0.005	0.000	
	15/09/01 08:13	0.273 [c]	0.07	0.012	0.000	
	15/09/01 08:15	[c]	0.65	0.021	0.000	
	15/09/01 08:17	318.76	0.75	0.077	0.001	318.69
	15/09/01 08:25	350.94	0.82	0.098	0.001	350.84
	15/09/01 08:55	319.95	0.78	0.18	0.002	319.77
	15/09/01 09:25	318.60	0.79	0.233	0.002	318.36
	15/09/01 09:55	323.86	0.82	0.284	0.003	323.58
	15/09/01 10:25	327.15	0.84	0.314	0.003	326.84
	15/09/01 10:55	318.38	0.84	0.345	0.003	318.04
	15/09/01 11:25	318.32	0.83	0.359	0.003	317.96
	15/09/01 11:55	321.12	0.85	0.375	0.003	320.75
	15/09/01 12:25	321.74	0.85	0.379	0.003	321.36
	15/09/01 12:58	322.46	0.86	0.404	0.004	322.05
	15/09/01 13:28	318.70	0.86	0.417	0.004	318.29
	Mean	323.33	0.82	0.289	0.003	323.04
	sd	9.101	0.034	0.117	0.0009	9.154
	CV (%)	2.8	4.2	40.5	35.1	2.8
5	17/09/01 09:07	259.59	0.68	0.189	0.003	259.40
	17/09/01 09:37	329.96	0.83	0.262	0.003	329.70
	17/09/01 10:07	333.40	0.84	0.333	0.004	333.06
	17/09/01 10:37	330.72	0.85	0.387	0.004	330.33
	17/09/01 11:07	322.77	0.81	0.303	0.003	322.46
	17/09/01 11:37	316.42	0.82	0.458	0.004	315.96
	17/09/01 12:07	313.36	0.83	0.492	0.004	312.87
	17/09/01 12:37	313.05	0.84	0.508	0.005	312.54
	17/09/01 13:07	312.63	0.85	0.528	0.005	312.11
	17/09/01 13:37	316.11	0.86	0.544	0.005	315.57
	17/09/01 14:07	315.02	0.87	0.561	0.005	314.46
	17/09/01 14:37	313.82	0.87	0.570	0.005	313.25
	Mean	314.74	0.83	0.428	0.004	314.31
	sd	18.963	0.051	0.1292	0.0008	18.931
	CV (%)	6.0	6.1	30.2	19.5	6.0

sd standard deviation

CV Coefficient of variation (sd × 100/mean)

[a] No data collected for exposure 4 due to computer problem with data capture program

[c] Problem with data capture value excluded from mean and standard deviation

PROTOCOL AND PROTOCOL AMENDMENTS

Study Number : MIN/312

CONFIDENTIAL

Huntingdon Life Sciences

PROTOCOL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Sponsor

3M Center
3M Corporate Toxicology
Building 220-2E-02
St Paul
MN 55133-3220
USA

Research Laboratory

Huntingdon Life Sciences Ltd
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Total number of pages: 20

Final Protocol

Study Number : MIN/312

Huntingdon Life Sciences

CONTACT DETAILS

Sponsor's Monitoring Scientist : John Butenhoff

Study Number : MIN/312

Huntingdon Life Sciences

PROTOCOL APPROVAL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

.....

17 August 2001

T.J. Kenny, B.Sc (Hons.).
Study Director,
Huntingdon Life Sciences Ltd.

Date

The signature of the Study Director confirms this protocol as the working document for the study. Any changes made subsequent to the date of the Study Director's signature will be documented in formal amendments.

.....

17 Aug 2001.

C.J. Hardy, B.Sc., Ph.D., M.I.Biol., C.Biol., Dip. R. C. Path. (Toxicology)
Management,
Huntingdon Life Sciences Ltd.

Date

.....

20 Aug 2001

John Butenhoff
Sponsor,
3M

Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Study Number : MIN/312

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Enquiry Number: 23923A

Number of pages for internal distribution: 17

This working document is approved for circulation and use:

.....
Study Director

14 August 2001
Date

Primary location of study

Huntingdon Research Centre
Huntingdon
Cambridgeshire
PE28 4HS

Building Number:

All procedures to be performed at the above site unless otherwise detailed below.

Location of specific tasks

Histology	:	Routinely done at Huntingdon Research Centre, Huntingdon, Cambridgeshire, PE28 4HS, but may be performed at Eye Research Centre, Eye, Suffolk, IP23 7PX for logistical reasons.
SEM X-ray analysis	:	University of Plymouth.
Test substance/metabolite analysis	:	3M.

Final Protocol

Study Number : MIN/312

Huntingdon Life Sciences

CONTENTS

Study Number : MIN/312

Huntingdon Life Sciences

1. INTRODUCTION

Management of study

Study Director	:	T.J. Kenny
Monitoring Toxicologist	:	D.W. Coombs
In the temporary absence of the Study Director, the scientific responsibilities will be taken over by the Monitoring Toxicologist; other items of routine study management should be referred to the following person in the first instance.	:	R. Davies

Objective

Assessment of systemic toxic potential in a 1 week inhalation study in CD Rats.

Good Laboratory Practice

The study will be conducted in compliance with principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

Animals (Scientific Procedures) Act 1986 compliance

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 (the Act). The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

The number of animals used will be the minimum that is consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.

Animal model	:	CD Rats, accepted by regulatory agencies, background data available.
Route	:	Inhalation.

Treatment groups and dosages

Group	:	1	2
Compound	:	Air Control	Perfluorooctanesulfonyl Fluoride (POSF)
Exposure level (mg/l)	:		
Dosage (ppm)	:	0	300

2. STUDY SCHEDULE AND STRUCTURE

2.1. Duration of treatment

Minimum period : 1 week (five days).

The treatment period may be extended, with the Sponsor's consent, to incorporate any additional observations considered necessary; documented in an amendment to protocol.

Throughout the necropsy period treatment will continue and serial observations will be recorded at appropriate intervals (Section 6). Data for any additional complete weeks before commencement of necropsies will be included in the final report.

2.2. Scheduled time plan

Animals to arrive	:	September 2001	
Treatment to commence	:	September 2001	
Terminal sacrifice to commence	:	September 2001	
Histopathology to be completed	:	October 2001	(estimated)
Draft report to be issued	:	November 2001	(estimated)

2.3. Identity of treatment groups

(to be selected from 20 animals ordered)

Group	Treatment	Exposure level (ppm)	Number of animals	
			Male	Female
1	Air Control	0	5	5
2	Perfluorooctanesulfonyl Fluoride (POSF)	300	5	5

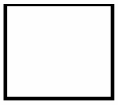
Group	Cage numbers		Animal numbers	
	Male	Female	Male	Female
1	1	3	1-5	11-15
2	2	4	6-10	16-20

3. TEST SUBSTANCE AND ATMOSPHERE GENERATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1999, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class: 

3.1. Test substance

Sponsor's identification	:	Perfluorooctanesulfonyl Fluoride (POSF) FX-8B, Lot 040277.
Storage conditions	:	At ambient temperature or in a refrigerator, unless otherwise directed by the Sponsor. Any such decision will be documented in the study data and included in the final report.
Sponsor's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis.

4. ANIMAL MANAGEMENT

4.1. Animals – supply, acclimatisation and allocation

4.1.1. Animals

Species	:	Rat.
Strain	:	Crl:CD® BR.
Age ordered	:	42 ± 2 days.
Weight range ordered	:	To be within an 11 g range for each sex.
Supplier	:	Charles River (UK) Limited.

4.1.2. Acclimatisation

Duration	:	At least 7 days before commencement of treatment.
Husbandry conditions	:	Refer to Section 4.2.

4.1.3. Allocation to treatment groups

Allocation	:	On arrival.
Method	:	Random.
Cage distribution	:	To equalise environmental influences between groups and to minimise the likelihood of cross contamination between groups.

4.1.4. Identification

Numbering	:	Unique for each animal within study.
Method	:	Tail tattoo.
Cage labels	:	Uniquely identifying the occupants.

4.1.5. Animal replacement

10 spare animals will be ordered to replace any individuals rejected during the acclimatisation period.

Replacement before treatment	:	Ill-health. Abnormalities. Bodyweight range extremes.
Replacement during treatment	:	None scheduled.

4.2. Animals – housing, diet and water supply

4.2.1. Environmental control

Rodent facility	:	Restricted entry – to minimise entry of external biological and chemical agents.
Air supply	:	Filtered, not recirculated.
Temperature	:	Maintained within the range of 19-23ºC.
Relative humidity	:	Maintained within the range of 40-70%.

Monitored continuously or daily. Excursions outside these ranges documented in the study data.

Lighting	:	12 hours light : 12 hours dark.
Alarm systems	:	Activated on ventilation failure and when temperature/humidity limits exceeded.
Electricity supply	:	Public supply with automatic stand-by generators.

4.2.2. Animal accommodation

Animals per cage	:	Five of the same sex, unless reduced by mortality or isolation.
Cage material	:	Polycarbonate or stainless steel.
Cage flooring	:	Stainless steel grid.

The cages will be suspended above absorbent paper. The latter will be changed at appropriate intervals each week; cages, cage-trays, food hoppers and water bottles will be changed at appropriate intervals. Precise details of caging will be included in the final report.

4.2.3. Diet and water supply

Copies of all certificates of analysis are stored in the archives.

Diet supply

Diet name	:	Rat and Mouse No. 1 Maintenance Diet. Fish meal is not present in this diet.
Diet type	:	Pelleted diet.
Availability	:	Non-restricted.
Certification	:	Before delivery each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

This diet contains no added antibiotic or other chemotherapeutic or prophylactic agent.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene or polycarbonate bottles with sipper tubes.
Certification	:	Certificates of analysis are routinely received from the supplier.

4.2.4. Contaminants assay

It is the Sponsor's responsibility to advise Huntingdon Life Sciences of any specific contaminants likely to prejudice the outcome of the study. Analyses for such contaminants may be performed if requested by the Sponsor. (PFOS, a metabolite of POSF may be present in fish meal used in some animal diets).

5. INHALATION PROCEDURES

5.1. Pre-study characterisation

Before commencement of treatment the system will be characterised at the target exposure vapour concentrations without animals in order to:

- demonstrate reproducibility of vapour concentration.
- demonstrate homogeneity of vapour concentration and distribution between levels in the chamber.

5.2. Test atmosphere generation

Equipment	:	All glass vaporiser.
Method	:	The test substance (liquid) will be metered to the vaporiser (through which dried air is passed) from an infusion pump. The vapour/air mixture produced passes directly into the exposure chamber.
Concentrations	:	Different concentrations of the test substance will be produced using different liquid feed rates controlled by the infusion pump and using syringes of an appropriate volume.

5.3. Test atmosphere administration

Route	:	Inhalation –snout only exposure.
Exposure system	:	ADG exposure chamber, modular construction in aluminium alloy comprising a base unit, a variable number of sections each having 20 exposure ports, and a top section incorporating a central aerosol inlet with a tangential air inlet. A separate chamber will be used for each group. During exposure, the rats will be held in restraining tubes with their snouts protruding from the ends of the tubes into the exposure chambers. The restraining tubes will be attached to chamber level 2. Animal exposure ports not in use will be closed with blanking plugs. Each exposure system will be housed in a separate extract cabinet to avoid possible cross-contamination between groups.
Controls (Group 1)	:	Air only.
Sham dosing	:	The animals on study will be acclimated to the method of restraint, normally over a 5 day period immediately preceding the first test substance exposure.
Duration of daily exposure	:	6 hours, for 5 consecutive days.
Airflow	:	30 l/minute.

5.4. Test atmosphere monitoring

Equipment	:	Fourier-transformed infrared spectroscopy (FTIR). Supplied by Sponsor.

5.4.1. Test atmosphere sampling

Sampling	:	Samples of the test atmosphere will be drawn from each test chamber through the FTIR instrument.

5.4.2. Test atmosphere analysis

Concentration	:	FTIR (at frequent intervals to demonstrate adequate control of temporal variation).
Chemical analysis: methodology	:	An FTIR analyser, with suitable methodology will be supplied by the Sponsor and this will be validated at Huntingdon Life Sciences. This methodology will be followed in principle, although specific conditions may be modified at the discretion of the Head of Section, Aerosol Technology and Analysis.

6. SERIAL OBSERVATIONS

The precise times of all examinations will be included in the final report.

6.1. Clinical observations

Animals and their cages	:	Inspected at least twice daily for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	:	Nature and severity. Date and time of onset. Duration and progress of the observed condition.
Physical examination	:	Once each week for all animals.

In addition, detailed observations will be made daily, on days of exposure according to the following frequency:

1. Pre-exposure observation.
2. Observation severely restricted due to tube restraint.
3. As each animal is returned to its home cage.
4. As late as possible in the working day.

The above schedule will be amended, as necessary, in the light of signs observed.

6.2. Mortality

Debilitated animals	:	Observed carefully, may be isolated to prevent cannibalism.
Premature sacrifice	:	Animals may be killed on humane grounds or if considered *in extremis.*
Animals found dead, killed *in extremis* or on humane grounds	:	A necropsy is performed as soon as possible. Animals found outside the normal workday will be preserved in a refrigerator (approximately 4°C) provided for this purpose.

6.3. Bodyweight

Bodyweight recording	:	Week –1. Day that treatment commences. Daily. At necropsy.

More frequent weighing may be performed to aid the monitoring of the condition of animals displaying ill-health. These data will be retained in the archives.

6.4. Food consumption

Food consumption recording	:	Week –1. Daily.
Food supplied	:	Daily.
Food spilled	:	Recorded at cage cleaning.
Food remaining	:	Recorded daily.

6.5. Water consumption

Water consumption recording	:	Week -1. Daily.
Water supplied	:	Daily.
Water remaining	:	Recorded daily.

7. NECROPSY AND HISTOLOGY

7.1. Pre-terminal urine sampling

Individual urine samples will be collected within 2 hours following lights on in the animal holding room. The urine samples will be immediately assayed for pH using a microelectrode, centrifuged to obtain the sediment and the prepared SEM stubs despatched to the Principal Investigator at Roy Moate University of Plymouth for calculi and crystal analysis by SEM X-ray element identification.

If fresh void urine is not obtained from a proportion of the animals within the time allowed, then a sample will be removed from the urinary bladder at necropsy.

7.2. Test substance/metabolite analyses

Samples of blood (for serum) will be taken at necropsy by cardiac/aorta puncture, and run into tubes allowed, to clot at room temperature (up to 4 ml), and the serum separated and frozen prior to despatch to the Sponsor.

The frozen samples will be despatched to the Sponsor's Principal Investigator for analysis.

Principal Investigator	:	Lisa Clemen.
Address	:	3M Environmental Laboratory 935 Bush Avenue, Building 2-3E-09 St. Paul, Minnesota 55133-3331, USA
Tel No.	:	651-778-6018 (0) 651-778-6176 (F).

Any further processing and analysis of the samples and data collation will be the responsibility of the Sponsor. A copy of the Sponsor's report will be included as an addendum to the study report. All raw data relevant to the analyses of test substance and metabolites in plasma will be retained by the Sponsor.

7.3. Method of kill

Method	:	Intra-peritoneal sodium pentobarbitone. Followed by exsanguination.
Sequence	:	To allow satisfactory inter-group comparison.

7.4. Macroscopic Pathology

(Table 1)

Complete	:	All animals.
Checks	:	Retained tissues.
Photography	:	Unusual or suspected treatment-related findings; at the discretion of the necropsy supervisor or Study Director.

7.5. Organ weights

(Table 1)

Data collection	:	For bilateral organs, left and right organs will be weighed together unless otherwise specified on the Pathology Procedures Table. Organ weights are not routinely recorded for animals killed or dying prematurely.
Data presentation	:	Absolute. Adjusted for terminal bodyweight.

7.6. Fixation

(Table 1)

Standard	:	Zinc Formalin.
Others	:	Eyes: In Davidson's fluid. Urinary bladder: Bouins. Inflate lungs with fixative and slightly distend the urinary bladder with fixative.
Fresh frozen liver	:	At terminal kill and after organ weighing, a sample of liver will be removed from all animals, immediately frozen by immersion in liquid nitrogen and stored at -70ºC prior to despatch to the Sponsor.

7.7. Histology

(Table 1 and Section 8.1)

Processing	:	All animals killed or dying prematurely. All terminal animals. Urinary bladder: section sagittally, using one half for SEM X-ray microprobe analysis, and the other half for light microscopy and cell proliferation index. Kidney pelvis: sections to be present for light microscopy.
Routine staining	:	4-5 μm sections stained with haematoxylin and eosin.
Special staining	:	None.

7.8. PCNA staining for cell proliferation (Table 1)

Sections of urinary bladder will be taken from all animals for PCNA immunostaining in order to assess the degree of cell proliferation present. A total of 3,000 cells will be counted from 3 separate sections (1,000 cells/section) in order to determine the cell proliferation index.
Positive PCNA staining cells will be counted, and the examining pathologist will assess the sections and count S-phase positive cells, if practicable. In addition sections of the duodenum from each animal will be taken and stained to act as positive controls for the immunostaining methodology.

Study Number : MIN/312

Huntingdon Life Sciences

TABLE 1-Pathology procedures

	Necropsy		Histology	Pathology	PCNA
Tissue and regions to be examined	**Weigh**	**Fix**		**Light microscopy**	
Abnormalities		*			
Adrenals	*	*			
Aorta – thoracic		*			
Brain		*			
Caecum		*			
Colon		*			
Duodenum		*			*
Epididymides		*			
Eyes		*			
Femur (longitudinal section through joint)		*			
Harderian glands		*			
Head		a)			
Heart	*	*			
Ileum		*			
Jejunum		*			
Kidneys	*	*			
Lachrymal glands		*			
Larynx (2 levels)		*			
Liver	*	*			
Lungs (section from all lobes including bronchi)	*	*			
Lymph nodes – mandibular		*			
- mesenteric		*			
- tracheobronchial		*			
Mammary area – caudal		*			
Nasal turbinates (3 levels)		*			
Oesophagus		*			
Optic nerves		*			
Ovaries		*			
Pancreas		*			
Pituitary		*			
Prostate		*			
Rectum		*			
Salivary glands (submandibular/sublingual)		*			
Sciatic nerves		*			
Seminal vesicles		*			
Skeletal muscle – thigh		*			
Skin		*			
Spinal cord (transverse and longitudinal sections at the cervical, lumbar and thoracic levels)		*			
Spleen		*			
Sternum		*			
Stomach		*			
Testes		*			
Thymus		*			
Thyroid with parathyroids		*			
Tongue		*			
Trachea (2 levels)		*			
Urinary bladder (with kidney pelvis)		*	*	*	*
Uterus with cervix		*			
Vagina		*			

a) Including nasal cavity, paranasal sinuses and nasopharynx.
* Organs weighed, samples fixed or sections examined microscopically.

8. PATHOLOGY

8.1. Light microscopy

Category	Animals	Tissues
Premature deaths	All from all groups.	All specified in Table 1.
Terminal sacrifice	All animals.	All specified in Table 1.

Peer Review : Carried out by a reviewing pathologist to internationally accepted standards.

8.2. Extension of initial examination

At the discretion of the pathologist, further processing and staining techniques may be used to evaluate individual lesions. Details of these techniques will be documented and retained in the archives.

Light microscopy may be extended, following consultation with the Sponsor, as follows:

Any such requirement will be documented in an amendment to the protocol.

SEM of urinary bladder epithelium for assessment of possible necrosis.

9. DATA TREATMENT

9.1. Statistical analysis

Data-types

The following data types will be analysed at each timepoint separately:-

bodyweight, food consumption and water consumption, over appropriate study periods.
organ weights, both absolute and adjusted for terminal bodyweight where appropriate.
pathological findings, for the number of animals with and without each finding.

Methods

For categorical data, the proportion of animals will be analysed using Fisher's Exact test for each treated group versus the control.

For continuous data, Bartlett's test will first be applied to test the homogeneity of variance between the groups. Using tests dependent on the outcome of Bartlett's test, treated groups will then be compared with the control group, incorporating adjustment for multiple comparisons where necessary.

10. REPORTING

Study progress	:	Periodic verbal and written updates on study progress will be provided by the Study Director. Routine synopsis reports will be sent after 1 week and at termination of the in-life phase.
Draft final report	:	For review by the Sponsor.
Authorised final report	:	After approval from the Sponsor.

Routinely reports are supplied on A4 paper. The following numbers of reports are supplied.

Type of report	Printing	Number of copies	
		Bound	Unbound
Draft report	Double-sided	0	2
Authorised final	Double-sided	1	0
	Single-sided	0	1
Photographic report (if any)	Single-sided	1	0

Any additions or corrections to an authorised final report will be documented as a formal addendum/amendment to the final report.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

11. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

11.1. Quality Assurance

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Authorised protocol and any amendments.
Study based inspections	:	Critical phases of this study will be inspected.
Report Audit	:	The draft report and study data will be audited after issue of the draft report to the Sponsor.
Test substance/metabolite assay	:	This phase of the study and associated reports will be conducted and produced according to local SOP's and subject to the Sponsor's own QA procedures.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

11.2. Archiving

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

Study Number : **MIN/312**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 4

Number of pages for internal distribution: 4

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 24 August 2001
(Study Director) pp

3 September 2001

For the Sponsor

Approved by: John Z. Butenhoff Date: 27 August 2001

Study Number : **MIN/312**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments : To include building number where study is to take place.
To update the timeplan.
To include hazard class.
To clarify necropsy table as to which tissues to process.

Amendments

Primary location of study

Huntingdon Research Centre
Huntingdon
Cambridgeshire
PE28 4HS

Building Number: Y14

2.2. Scheduled time plan

~~Animals to arrive~~	:	5 ~~September~~ 2001	
Treatment to commence	:	13 September 2001	
Terminal sacrifice to commence	:	18 September 2001	
Histopathology to be completed	:	~~October~~ November 2001	(estimated)
Draft report to be issued	:	November 2001	(estimated)

Study Number : **MIN/312**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

3. TEST SUBSTANCE AND ATMOSPHERE GENERATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1999, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class: 2

Study Number : **MIN/312**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

TABLE 1-Pathology procedures

	Necropsy		Histology	Pathology	PCNA
Tissue and regions to be examined	**Weigh**	**Fix**		**Light microscopy**	
Abnormalities		*			
Adrenals	*	*			
Aorta – thoracic		*			
Brain		*			
Caecum		*			
Colon		*			
Duodenum		*			*
Epididymides		*			
Eyes		*			
Femur (longitudinal section through joint)		*			
Harderian glands		*			
Head		a)			
Heart	*	*			
Ileum		*			
Jejunum		*			
Kidneys (with pelvic region)	*	*	*	*	*
Lachrymal glands		*			
Larynx (2 levels)		*			
Liver	*	*			
Lungs (section from all lobes including bronchi)	*	*			
Lymph nodes – mandibular		*			
- mesenteric		*			
- tracheobronchial		*			
Mammary area – caudal		*			
Nasal turbinates (3 levels)		*			
Oesophagus		*			
Optic nerves		*			
Ovaries		*			
Pancreas		*			
Pituitary		*			
Prostate		*			
Rectum		*			
Salivary glands (submandibular/sublingual)		*			
Sciatic nerves		*			
Seminal vesicles		*			
Skeletal muscle – thigh		*			
Skin		*			
Spinal cord (transverse and longitudinal sections at the cervical, lumbar and thoracic levels)		*			
Spleen		*			
Sternum		*			
Stomach		*			
Testes		*			
Thymus		*			
Thyroid with parathyroids		*			
Tongue		*			
Trachea (2 levels)		*			
Urinary bladder		*	*	*	*
Uterus with cervix		*			
Vagina		*			

a) Including nasal cavity, paranasal sinuses and nasopharynx.
* Organs weighed, samples fixed or sections examined microscopically.

Study Number : **MIN/312**
Protocol Amendment Number : 2

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 4

Number of pages for internal distribution: 4

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 11 September 2001
(Study Director)

For the Sponsor

Approved by: John L. Rutenkoff Date: 21 September 2001

Study Number : **MIN/312**
Protocol Amendment Number : 2

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments :
Clarification of name and address of Principal Investigator for SEM X-ray analyses
Change to fixative following discussion with Sponsor.
Addition/clarification of QA and Archiving procedures.

Amendments

7. NECROPSY AND HISTOLOGY

7.1. Pre-terminal urine sampling

Individual urine samples will be collected within 2 hours following lights on in the animal holding room. The urine samples will be immediately assayed for pH using a microelectrode, centrifuged to obtain the sediment and the prepared SEM stubs despatched to the Principal Investigator ~~at~~ Roy Moate, at University of Plymouth, Drake Circus, Plymouth, PL4 8AA, for calculi and crystal analysis by SEM X-ray element identification.

If fresh void urine is not obtained from a proportion of the animals within the time allowed, then a sample will be removed from the urinary bladder at necropsy.

A copy of the Principal Investigator's report will be included as an addendum to the study report. All raw data relevant to the analyses will be returned to Huntingdon Life Sciences for archiving.

7.6. Fixation

(Table 1)

Standard	:	~~Zinc Formalin~~. 10% neutral buffered formalin
Others	:	Eyes: In Davidson's fluid. Urinary bladder: Bouins. Inflate lungs with fixative (10% NBF) and slightly distend the urinary bladder with fixative (Bouin's). Testes and epididymides: Initially in Bouin's fluid
Fresh frozen liver	:	At terminal kill and after organ weighing, a sample of liver will be removed from all animals, immediately frozen by immersion in liquid nitrogen and stored at -70°C prior to despatch to the Sponsor.

11. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

11.1. Quality Assurance

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Authorised protocol and any amendments.
Study based inspections	:	Critical phases of this study will be inspected.
Report Audit	:	The draft report and study data will be audited after issue of the draft report to the Sponsor.
Test substance/metabolite assay	:	This phase of the study and associated reports will be conducted and produced according to local SOP's and subject to the Sponsor's own QA procedures.
SEM X-ray calculi and crystal analysis	:	The facility undertaking the analysis will be inspected by and the data and the Principal Investigator's report will be audited by Huntingdon Life Sciences Quality Assurance Department

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

11.2. Archiving

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Raw data, samples and specimens arising from the test substance/metabolite assay conducted by the Sponsor will be archived by the Sponsor.

Raw data, samples and specimens arising from the SEM X-ray calculi and crystal analysis will be archived by Huntingdon Life Sciences.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

Study Number : **MIN/312**
Protocol Amendment Number : 3

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 3

Number of pages for internal distribution: 3

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 18 September 2002
(Study Director)

For the Sponsor

Approved by: John T. Butterhoff Date: 27 September 2002

Study Number : **MIN/312**
Protocol Amendment Number : 3

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments :
The Sponsor has changed the Principal Investigator

Amendments

Study Number : **MIN/312**
Protocol Amendment Number : 3

Huntingdon Life Sciences

7.2. Test substance/metabolite analyses

Samples of blood (for serum) will be taken at necropsy by cardiac/aorta puncture, and run into tubes allowed, to clot at room temperature (up to 4 ml), and the serum separated and frozen prior to despatch to the Sponsor.

The frozen samples will be despatched to the Sponsor's Principal Investigator for analysis.

Principal Investigator	:	Lisa Clemen.
Address	:	3M Environmental Laboratory 935 Bush Avenue, Building 2-3E-09 St. Paul, Minnesota 55133-3331, USA
Tel No.	:	651-778-6018 (0) 651-778-6176 (F).

Subsequent to this amendment the duties of Principal Investigator will be transferred to:

Principal Investigator	:	Dr Richard Grazzini
Address	:	Exygen Research 3058 Research Drive State College PA 16801 USA
Tel No.	:	001 814 231 8032
Fax No.	:	001 814 231 1580

Any further processing and analysis of the samples and data collation will be the responsibility of the Sponsor. A copy of the Sponsor's report will be included as an addendum to the study report. All raw data relevant to the analyses of test substance and metabolites in plasma will be retained by the Sponsor.

Study Number : **MIN/312**
Protocol Amendment Number : 4

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 3

Number of pages for internal distribution: 3

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 5 September 2003
(Study Director)

For the Sponsor

Approved by: John Z. Butenhoff Date: 11 September, 2003

Study Number : **MIN/312**
Protocol Amendment Number : 4

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments :
Assignment of liver samples for test substance/metabolite analyses
Addition of analytical method details.

Amendments

7.2. Test substance/metabolite analyses

Samples of blood (for serum) will be taken at necropsy by cardiac/aorta puncture, and run into tubes allowed, to clot at room temperature (up to 4 ml), and the serum separated and

Samples of liver will be collected at necropsy in accordance Section 7.6 of the Protocol and frozen prior to despatch to the Sponsor.

The frozen samples will be despatched to the Sponsor's Principal Investigator for analysis.

Principal Investigator	:	Lisa Clemen.
Address	:	3M Environmental Laboratory 935 Bush Avenue, Building 2-3E-09 St. Paul, Minnesota 55133-3331, USA
Tel No.	:	651-778-6018 (0) 651-778-6176 (F).

Study Number : **MIN/312**
Protocol Amendment Number : 4

Huntingdon Life Sciences

Subsequent to ~~this~~ Protocol Amendment 3 the duties of Principal Investigator will be transferred to:

Principal Investigator	:	Dr Richard Grazzini
Address	:	Exygen Research 3058 Research Drive State College PA 16801 USA
Tel No.	:	001 814 231 8032
Fax No.	:	001 814 231 1580

All samples will be analysed for POSF, PFOS and PFOA.

Samples will be analysed for POSF according to Exygen method ExM-023-071A in its current revision.

Samples will be analysed for PFOS and PFOA according to Exygen method ExM-023-071 in its current revision.

Any further processing and analysis of the samples and data collation will be the responsibility of the Sponsor. A copy of the Sponsor's report will be included as an addendum to the study report. All raw data relevant to the analyses of test substance and metabolites in plasma will be retained by the Sponsor.

ANALYTICAL PHASE REPORT

STUDY TITLE

Perfluorooctanesulfonyl Fluoride (POSF; T-7661.1) Preliminary Toxicity Study by Inhalation Administration to CD Rats for 1 Week

DATA REQUIREMENTS

Analytical Method Requirements

STUDY DIRECTOR

Terry J. Kenny
Huntington Life Sciences, Ltd.

STUDY COMPLETED ON

March 10, 2004

PERFORMING LABORATORY / TESTING FACILITY

Exygen Research
3058 Research Drive
State College, PA 16801
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STUDY SPONSOR

3M Corporate Toxicology
Building 220-2E-02
St. Paul, MN 55133-3220

PROJECT

Protocol Number: MIN/312
Exygen Study Number: 023-080

Total Pages: 165

Exygen Study No.: 023-080

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Exygen Study Number 023-080, the analytical phase of the study entitled "Perfluorooctanesulfonyl Fluoride (POSF; T-7661.1) Preliminary Toxicity Study by Inhalation Administration to CD Rats for 1 Week," conducted for 3M Corporate Toxicology, was performed in compliance with OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17 by Exygen Research.

Richard A. Grazzini
Principal Investigator
Exygen Research

10-MAR-04
Date

Terry J. Kenny
Study Director
Huntingdon Life Sciences Ltd

25 March 2004
Date

John Butenhoff
Sponsor Representative
3M Corporate Toxicology

Date

Exygen Study No.: 023-080

QUALITY ASSURANCE STATEMENT

Exygen Research's Quality Assurance Unit reviewed Exygen Study Number 023-080, the analytical phase of the study entitled, "Perfluorooctanesulfonyl Fluoride (POSF; T-7661.1) Preliminary Toxicity Study by Inhalation Administration to CD Rats for 1 Week". All reviewed phases were inspected for conduct according to Exygen Research's Standard Operating Procedures, the Study Protocol, and all applicable Good Laboratory Practice Standards. All findings were reported to the Study Director and to management.

Phase	Date Inspected	Date Reported to Exygen PI and Management	Date Reported to Study Director and Test Facility Management
1. Protocol Review	09/23/02	11/13/02 11/21/02	12/09/02
2. Extraction, Fortification	09/23/02	11/13/02 11/14/02	12/09/02
3. Extraction, Fortification	09/24/02	11/13/02 11/21/02	12/09/02
4. Raw Data Review	11/04,05/02	11/13/02 11/14/02	12/09/02
5. Raw Data Review	11/25,26/02	12/03/02 12/18/02	01/20/03
6. Draft Analytical Report Review	11/07,10/03	11/17/03	03/10/04
7. Final Analytical Report Review	03/08/04	03/08/04 03/10/04	03/10/04

Miwa Nabetani
Quality Assurance Auditor

March 10, 04
Date

CERTIFICATION OF AUTHENTICITY

This report, for Exygen Study Number 023-080, is a true and complete representation of the raw data for the study.

Submitted by: Exygen Research
3058 Research Drive
State College, PA 16801
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Principal Investigator, Exygen:

Richard A. Grazzini
President
Exygen Research

10-MAR-04
Date

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John M. Flaherty
Vice President
Exygen Research

3/10/04
Date

Study Director, Huntingdon Life Sciences, Ltd:

Terry J. Kenney
Huntingdon Life Sciences, Ltd

25 March 2004
Date

Sponsor Representative, 3M:

John Butenhoff
3M Corporate Toxicology

Date

STUDY IDENTIFICATION

Perfluorooctanesulfonyl Fluoride (POSF; T-7661.1) Preliminary Toxicity Study by Inhalation Administration to CD Rats for 1 Week

PROTOCOL NUMBER:	MIN/312
EXYGEN STUDY NUMBER:	023-080
TYPE OF STUDY:	Analytical
SAMPLE MATRIX:	Rat Liver and Serum
TEST SUBSTANCES:	Perfluorooctanesulfonyl Fluoride (POSF), Perfluorooctanesulfonate (PFOS) and Pentadecafluorooctanoic Acid (PFOA)
SPONSOR:	3M Corporate Toxicology Building 220-2E-02 St. Paul, MN 55133-3220
STUDY DIRECTOR:	Terry J. Kenny Huntingdon Life Sciences Ltd Woolley Road Alconbury Huntingdon Cambridgeshire, England PE28 4HS
SPONSOR REPRESENTATIVE:	John Butenhoff 3M Corporate Toxicology Building 220-2E-02 St. Paul, MN 55133-3220
PERFORMING LABORATORY:	Exygen Research 3058 Research Drive State College, PA 16801
ANALYTICAL PHASE TIMETABLE:	Study Initiation Date: 08/17/01 Experimental Start Date: 09/20/02 Experimental Termination Date: 09/28/02 Study Completion Date: 03/10/04

PROJECT PERSONNEL

The Study Director for this project was Terry J. Kenny at Huntingdon Life Sciences Ltd. The following personnel from Exygen Research were associated with various phases of the study:

Name	Title
Richard A. Grazzini	President
Emily Decker	Scientist
Paul Connolly	Technical Lead-LC/MS
Karen Risha	Scientist
Lawrence Ord	Sample Custodian
Rickey Kelley	Sample Custodian
Chas Simons	Technical Lead-GC/MS
Carisa Kelley	Technician
Joe Gallagher	Scientist
Chris Pfleegor	Scientist
Xiaoming Zhu	Technician

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1.0 SUMMARY

Exygen Resarch extracted and analyzed rat liver and serum samples for the determination of perfluorooctanesulfonate (PFOS) and pentadecafluorooctanoic acid (PFOA) according to Exygen Method ExM-023-071 (**Appendix B**) and for the determination of perfluorooctanesulfonyl fluoride (POSF) according to Exygen Method ExM-023-071A (**Appendix C**).

The limit of quantitation for PFOS and PFOA in rat liver was 10 ng/g and 10 ng/mL in rat serum. The limit of quantitation for POSF in rat liver was 2.5 µg/g and 2.5 µg/mL for rat serum. The LOQ for each matrix was determined in a method validation studies performed at Exygen (Exygen Studies: 023-074 and 023-075). However, the LOQ for PFOA and PFOS in each matrix was determined using a 1 mL sample aliquot for serum and urine and 1.0 g sample weight for liver. Due to limited sample size, only 0.1 mL was used for serum and 0.1 g for liver, which increased the LOQ for each matrix by a factor of 10. The LOQ for POSF in serum was determined using 0.1 mL sample aliquot and in some cases only 0.05 mL was used which increased the LOQ for POSF in serum by a factor of 2.

PFOS in the rat liver samples ranged from non-detected levels to 67,600 ng/g. PFOA in the rat liver samples ranged from non-detected levels to 6110 ng/g. There was no POSF detected in any of the rat liver samples. PFOS in the rat serum samples ranged from non-detected levels to 13,900 ng/mL. PFOA in the rat serum samples ranged from non-detected levels to 8850 ng/mL. There was no POSF detected in any of the rat serum samples.

The average percent recoveries ± standard deviations for PFOS in rat liver and serum samples were 91% ± 6% and 109% ± 19%, respectively. The average percent recoveries ± standard deviations for PFOA in rat liver and serum samples were 134% ± 4% and 112% ± 7%, respectively. The average percent recoveries ± standard deviations for POSF in rat liver and serum samples were 85% ± 6% and 72% ± 19%, respectively.

2.0 OBJECTIVE

The objective of this phase was to determine levels of perfluorooctanesulfonyl fluoride (POSF), perfluorooctanesulfonate (PFOS), and pentadecafluorooctanoic acid (PFOA) in specimens of rat liver and serum samples according to Protocol MIN/312 (**Appendix A**).

3.0 INTRODUCTION

This report details the results of the analysis for the determination of PFOS and PFOA in rat liver and serum samples, using the analytical method entitled, "Method of Analysis for

the Determination of Perfluorohexanesulfonate (PFHS), Perfluorooctanesulfonate (PFOS) and Pentadecafluorooctanoic Acid (PFOA) in Rat Liver, Serum and Urine" and also the results for the determination of POSF in rat liver and serum samples, using the analytical method entitled, "Method of Analysis for the Determination of Perfluorooctanesulfonyl Fluoride (POSF) in Rat Urine, Serum, and Liver by GC/MS."

The study was initiated on August 17, 2001, when the study director signed protocol number MIN/312. The analytical experimental start date was September 20, 2002, and the analytical experimental termination date was September 28, 2002.

4.0 TEST SYSTEM

The control rat liver and serum used for the matrix blanks and matrix fortifications were received frozen on dry ice on June 12, 2002 and August 7, 2002 from Pel-Freez Biologicals, Rogers, Arkansas.

Twenty rat liver and twenty rat serum samples were received frozen on dry ice at Exygen from 3M Environmental Laboratory on August 1, 2002 and logged in by Exygen personnel and placed in frozen storage.

Sample log-in and chain of custody information can be found in the raw data package associated with this study. Storage records will be kept at Exygen Research and a true copy of the storage records can be found in the raw data package associated with this study.

5.0 REFERENCE MATERIAL

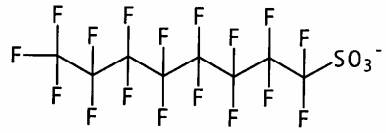
The analytical standard PFOA was received at Exygen on July 3, 2002 from Sigma-Aldrich and stored refrigerated. The analytical standard PFOS was received at Exygen on May 15, 2000 from 3M Environmental Technology and Services and stored frozen. The analytical standard POSF was received at Exygen on June 6, 2002 from Sigma-Aldrich and stored ambient.

The available information for the reference material is listed below.

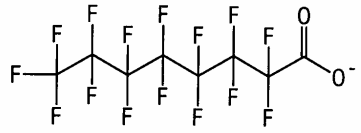
Compound	Exygen Inventory No..	TCR No.	Purity (%)	Expiration Date
PFOA	SP1328	NA	99.9	07/03/03
PFOS	SP249	SD018	86.9	08/31/06
POSF	SP1187	15721HQ	98.6	06/06/03

The molecular structures of test substances are given below:

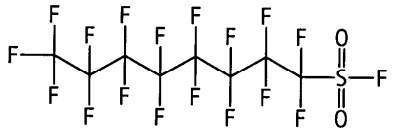
Name: **PFOS**
Chemical Name: Perfluorooctane sulfonate
Molecular Weight: 499, as shown



Name: **PFOA**
Chemical Name: Perfluorooctanoic acid
Molecular Weight: 413, as shown



Name: **POSF**
Chemical Name: Perfluorooctanesulfonyl fluoride
Molecular Weight: 502, as shown



6.0 DESCRIPTION OF ANALYTICAL METHOD

The analytical methods ExM-023-071 and ExM-023-071A were used for this study. The methods were versioned after the analysis of the samples were completed to allow for different sample sizes and to make minor typographical corrections. The revised methods are included with this report.

6.1.1 PFOS and PFOA Extraction Procedure

A 100 µL aliquot of the serum and 0.1 g of the liver were used for the extraction procedure. After fortification of appropriate samples, the serum samples were brought up

to 20 mL with Type I Water and the liver samples were brought up to 10 mL with Type I Water. The serum samples were vortexed for ~ 1 minute and the liver samples were homogenized with a tissuemizer for ~ 1 minute. An aliquot of one milliliter was transferred from each sample and 5 mL of acetonitrile was added and the samples were shaken for ~ 20 minutes. The samples were centrifuged and the supernatant was decanted onto a conditioned SPE column. Then the samples were eluted with 2 mL of methanol. Each sample was analyzed by LC/MS/MS electrospray.

6.1.2 POSF Extraction Procedure

A 100 µL aliquot of the serum and 0.1 g of the liver were used for the extraction procedure. After fortification of appropriate samples, the samples were heated to 60°C-80°C for ~ 3-4 minutes and then analyzed by GC/MS.

6.2 Preparation of Standards and Fortification Solutions

A stock standard solution of PFOA was prepared on July 8, 2002 and a stock standard solution of PFOS was prepared on August 2, 2002 as specified in Exygen method ExM-023-071. The stock standard solutions were prepared at a concentration of 100 µg/mL by dissolving 10 mg of each standard (corrected for purity and salt content) in methanol. From this solution, a 1.0 µg/mL fortification standard solution was prepared by taking 1 mL of each stock and bringing the volume up to 100 mL with methanol.

A 0.1 µg/mL fortification standard was prepared by taking 10 mL of the 1.0 µg/mL mixed fortification standard and bringing the volume up to 100 mL with methanol.

A set of standards containing PFOA and PFOS was prepared dilution of the 0.1 µg/mL and various calibration solutions in the following manner:

Initial Conc. (µg/mL)[1]	Volume (mL)	Diluted to (mL)	Final Conc. (µg/mL)
0.1	5	100	0.005
0.1	2	100	0.002
0.1	1	100	0.001
0.005	10	100	0.0005
0.002	10	100	0.0002
0.001	10	100	0.0001

[1] of PFOA and PFOS

A stock standard solution of POSF was prepared on June 11, 2002 as specified in Exygen method ExM-023-071A. The stock standard solution was prepared at a concentration of 1990 µg/mL by dissolving ~ 0.2 g of the standard (corrected for purity) in methanol. From this solution, a 1000 µg/mL calibration standard solution was prepared by taking ~ 25 mL of the stock and bringing the volume up to 50 mL with methanol.

A set of standards containing POSF was prepared by dilution of the 1000 µg/mL:

Initial Conc. (µg/mL)[1]	Volume (mL)	Diluted to (mL)	Final Conc. (µg/mL)
1000	5	10	500
1000	2.5	10	250
1000	1	10	100
1000	0.5	10	50
1000	0.25	10	25
1000	0.1	10	10

[1] of POSF

The stock standard solutions and all fortification and calibration standard solutions were stored in a refrigerator (4° ± 2°C) when not in use. Documentation of standard preparation can be found in the raw data associated with this report.

6.3 Chromatography

Quantification of PFOA and PFOS was accomplished by LC/MS/MS electrospray. The retention time of PFOA was ~ 8.6 and ~ 8.8 min for PFOS. Peaks were detected in the control liver sample corresponding to the analyte retention time of PFOS, but the amounts detected were only significant enough to alter several fortification recoveries and the rest were less than the lowest calibration standard (0.0001 µg/mL).

Quantification of POSF was accomplished by GC/MS. The retention time of POSF was ~ 3.5 min. Peaks were not detected in the control liver sample corresponding to the analyte retention time of POSF.

6.4 Instrument Sensitivity

The smallest standard amount injected during the chromatographic run had a concentration of 0.0001 µg/mL of PFOA and PFOS and 25 µg/mL for POSF.

6.5.1 Description of LC/MS/MS Instrument and Operating Conditions

Instrument: PE SCIEX API 365 Biomolecular Mass Analyzer

Interface: SCIEX TurboIon Spray Liquid Introduction Interface

Computer: Dell OptiPlex GX 110

Software: PE SciexAnalyst version 1.1
Windows NT, version 4

HPLC: Hewlett Packard (HP) Series 1100
HP Quat Pump
HP Vacuum Degasser
HP Autosampler
HP Column Oven

HPLC Column:Genesis C_8 (Jones Chromatography), 2.1 mm x 50 mm, 4µ
Column Temp.: 35° C
Injection Vol.: 15 µL
Mobile Phase (A): 2 mM Ammonium Acetate in Water
Mobile Phase (B): Methanol
Flow Rate: 0.3 mL/min.

Time	% A	% B
0	90	10
2.0	90	10
5.0	10	90
9.0	10	90
9.5	0	100
14.0	0	100
14.5	90	10
20.0	90	10

Ions monitored:

Analyte	Mode	Transition Monitored	Approximate Retention Time (min)
PFOA	negative	413 → 369	8.6
PFOS	negative	499 → 99	8.8

Tune File Parameters

Controls	Set
IS-Iospray	-4200.0
DP-Declustering Potential	-51.0
FP-Focusing Potential	-230.0
EP-Entrance Potential	10.0
CE-Collision Energy	-70
CXP-Collision Cell Exit Potential	-5
DF-Deflector	300.0
CEM-Channel Electron Multiplier	2800.0

Gas Flows	Set
Nebulizer Gas	12
Curtain Gas	13
Collision Gas	4
TIS Temperature	350°C

6.5.2 Description of GC/MS Instrument and Operating Conditions

Instrument:	Hewlett-Packard model 6890 Series Gas Chromatograph/model 5973 mass selective detector
Column:	Restek RTX-502.2, 60 m x 0.25 mm ID, 1.4 μm df
Oven Temperature:	Hold at 40°C for 4 min., ramp 20°C/min. to 100°C, hold for 0 min.
Injector Temperature:	220°C
Transfer Line Temperature:	220°C
Carrier Gas:	Helium
GC Head Pressure:	5 psi for 0.25 min., ramp 80 psi/min. to 60 psi, hold 0.5 min., ramp –80 psi/min. to 30 psi, hold at 30 psi for remainder of the analytical run.
Injection Mode:	Splitless
Injection Liner:	4 mm ID straight glass
Injection Purge Delay:	2 min.
Purge Flow to Split Vent:	10 mL/min.
Injection Volume:	1 mL
Electron Multiplier Voltage:	From ATUNE + 200V
Scan Mode:	SIM
Monitored Ions:	m/z's 69 (quantitation ion); 131, 219 (qualifier ions)
Dwell Time:	75 ms
Integrator:	Hewlett-Packard ChemStation software
Retention Times:	~3.5 minutes
Total run time:	~7 minutes

6.6.1 PFOA and PFOS Quantitation and Example Calculation

Fifteen microliters of sample or calibration standard were injected into the LC/MS/MS. The peak area was measured and the standard curve was generated (using 1/x fit weighted linear regression) by Analyst software using six concentrations of standards. The concentration was determined from the equations below.

Equation 1 calculated the amount of analyte found (in ng/mL, based on peak area) using the standard curve (linear regression parameters) generated by the Analyst software program. Then Equation 2 calculated the amount of analyte found in ng/g for liver and ng/mL for serum.

Equation 1:

$$\text{Analyte found (ng/mL)} = \frac{(\text{Peak area} - \text{intercept})}{\text{slope}} \times \text{AF}$$

Where: AF = Aliquot Factor

Equation 2:
Analyte found (ppb, ng/g for liver and ng/mL for serum) =

$$\frac{\text{anal. found (ng/mL)} \times \text{FV (mL)} \times \text{DF}}{\text{sample vol. (mL) or sample weight (g)}}$$

Where: FV = Final Volume
DF = Dilution Factor

For samples fortified with known amounts of PFOA and PFOS prior to extraction, Equation 3 calculated the percent recovery.

Equation 3:
Recovery (%) =

$$\frac{(\text{anal. found (ppb)} - \text{avg. anal. in ctrl (ppb)}) \times 100\%}{\text{amount added (ppb)}}$$

An example of a calculation using an actual sample follows (for PFOS only):
Rat liver sample Exygen ID 0201684 Spk A (Set: 092602A), fortified at 10 ng/g where:

peak area	=	841
intercept	=	18.8663
slope	=	1012.44
dilution factor	=	1
ppb added (fort level)	=	10
avg. amt in controls	=	7.16 ppb
final volume	=	2 mL
aliquot factor	=	10
sample weight	=	1.0 g

From equation 1:

$$\text{Analyte found (ng/mL)} = \frac{[841 - 18.8663] \times 10}{1012.44}$$

$$= 8.1 \text{ ng/mL}$$

From equation 2:

$$\text{Analyte found (ppb)} = \frac{(8.1 \text{ ng/mL} \times 2 \text{ mL} \times 1)}{(1.0 \text{ g})}$$

$$= 16.2 \text{ ppb}$$

From equation 3:

$$\% \text{ Recovery} = \frac{(16.2 \text{ ppb} - 7.16 \text{ ppb})}{10 \text{ ppb}} \times 100\%$$

$$= 90\%$$

6.6.2 POSF Quantitation and Example Calculation

One milliliter of headspace of the sample or calibration standard was injected into the GC/MS. The peak area was measured and the standard curve was generated (using 1/x fit weighted linear regression) by HP Chemstation software using five concentrations of standards. The concentration was determined from the equations below.

Equation 1 calculated the amount of analyte found (in µg/mL, based on peak area) using the standard curve (linear regression parameters) generated by the HP Chemstation software program. Then Equation 2 calculated the amount of analyte found in µg/g for liver and µg/mL for serum.

Equation 1:

$$\text{Analyte found (µg/mL)} = \frac{(\text{Peak area} - \text{intercept}) \times \text{DF}}{\text{slope}}$$

Where: DF = Dilution Factor

Equation 2:

Analyte found (ppm, µg/g for liver and µg/mL for serum) =

$$\frac{\text{anal. found (ppm)} \times \text{standard volume added (mL)}}{\text{SV (mL) or SW (g)}}$$

Where: SW = Sample Weight
SV = Sample Volume

For samples fortified with known amounts of POSF prior to analysis, Equation 3 calculated the percent recovery.

Equation 3:

Recovery (%) =

$$\frac{(\text{anal. found (ppm)} - \text{avg. anal. in ctrl (ppm)}) \times 100\%}{\text{amount added (ppm)}}$$

An example of a calculation using an actual sample follows:

Rat liver sample Exygen ID 0202877 Spk A (Set: 092002G8), fortified at 5 µg/g where:

peak area	=	276118
intercept	=	4400
slope	=	3230
dilution factor	=	1
ppm added (fort level)	=	5
standard volume added (mL)	=	0.005
sample weight (g)	=	0.10403

From equation 1:

Analyte found (µg/mL) = $\frac{[276118 - 4400] \times 1}{3230}$

= 84.12 µg/mL

From equation 2:

Analyte found (ppm) = $\frac{84.12\ (\mu g/mL) \times 0.005\ (mL)}{0.10403\ (g)}$

= 4.04 µg/g

From equation 3:

% Recovery = $\frac{(4.04\ ppm)}{5\ ppm} \times 100\%$

= 81%

Note: This example calculation was done using rounded numbers, and therefore may be slightly different from the values shown in the RAW DATA.

7.0 EXPERIMENTAL DESIGN

Each set of samples (liver or serum) consisted of one matrix blank, two matrix blanks fortified at known concentrations, and ~ 20 samples. Each set of samples (liver or serum) for GC/MS analysis consisted of one matrix blank, one matrix blanks fortified at known concentrations, two field samples fortified at a known concentration and ~ 10 samples. Each sample was extracted using the appropriate method and then analyzed in duplicate for PFOA and PFOS and extracted in duplicate for POSF.

8.0 RESULTS

The PFOS found in the control rat liver and serum samples are listed in **Tables I-II**. The PFOA found in the control rat liver and serum samples are listed in **Tables III-IV**. The POSF found in the control rat liver and serum samples are listed in **Tables V-VI**. Peaks were detected in the control liver sample corresponding to the analyte retention time of PFOS, but the amounts detected were only significant enough to alter several fortification recoveries.

Individual recoveries for PFOS in the rat liver and serum samples are detailed in **Tables VII-VIII**. The average percent recoveries ± standard deviations for PFOS in rat liver and serum samples were 91% ± 6% and 109% ± 19%, respectively. Individual recoveries for PFOA in the rat liver and serum samples are detailed in **Tables IX-X**. The average percent recoveries ± standard deviations for PFOA in rat liver and serum samples were

134% ± 4% and 112% ± 7%, respectively. Individual recoveries for POSF in the rat liver and serum samples are detailed in **Tables XI-XII**. The average percent recoveries ± standard deviations for POSF in rat liver and serum samples were 85% ± 6% and 72% ± 19%, respectively.

PFOS in the rat liver samples ranged from non-detected levels to 67,600 ng/g. Individual results are listed in **Table XIII**. PFOA in the rat liver samples ranged from non-detected levels to 6110 ng/g. Individual results are listed in **Table XIV**. There was no POSF detected in any of the rat liver samples. Individual results are listed in **Table XV**. PFOS in the rat serum samples ranged from non-detected levels to 13,900 ng/mL. Individual results are listed in **Table XVI**. PFOA in the rat serum samples ranged from non-detected levels to 8850 ng/mL. Individual results are listed in **Table XVII**. There was no POSF detected in any of the rat serum samples. Individual results are listed in **Table XVIII**.

9.0 CONCLUSIONS

The rat liver and serum samples were successfully extracted and analyzed for PFOS, PFOA and POSF according to the appropriate analytical method.

10.0 RETENTION OF DATA AND SAMPLES

When the final analytical report is complete, all original paper data generated by Exygen Research will be shipped to the sponsor. This does not include facility-specific raw data such as instrument logs, however exact copies of temperature logs will be submitted. Exact copies of all raw data, as well as a signed copy of the final analytical report and all original facility-specific raw data, will be retained in the Exygen Research archives for the period of time specified in OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17. Retained samples of reference substances are archived by the sponsor.

TABLES

Table I. Summary of PFOS in Control Rat Liver Sample

Sponsor ID	Exygen ID	Set Number	PFOS Found (ng/g)
Lot #14124	0201684 Control A	092602A	7.23
Lot #14124*	0201684 Control A	092602A	7.09

Table II. Summary of PFOS in Control Rat Serum Sample

Sponsor ID	Exygen ID	Set Number	PFOS Found (ng/mL)
Lot #07024	0201682 Control A	092002A	ND
Lot #07024*	0201682 Control A	092002A	ND

Table III. Summary of PFOA in Control Rat Liver Sample

Sponsor ID	Exygen ID	Set Number	PFOA Found (ng/g)
Lot #14124	0201684 Control A	092602A	ND
Lot #14124*	0201684 Control A	092602A	ND

Table IV. Summary of PFOA in Control Rat Serum Sample

Sponsor ID	Exygen ID	Set Number	PFOA Found (ng/mL)
Lot #07024	0201682 Control A	092002A	ND
Lot #07024*	0201682 Control A	092002A	ND

* Duplicate Injection

ND = Not Detected (Area less than the lowest concentration of the calibration standards (0.0001 µg/mL))

Table V. Summary of POSF in Rat Liver Control Sample

Sponsor ID	Exygen ID	Set Number	POSF Found (µg/g)
Lot #21824	0202877 Ctrl	092002G8	ND
Lot #21824	0202877 Ctrl	092302G8	ND

Table VI. Summary of POSF in Rat Serum Control Sample

Sponsor ID	Exygen ID	Set Number	POSF Found (µg/mL)
Lot #07024	0201682 Ctrl	092402G8	ND
Lot #07024	0201682 Ctrl	093002A	ND

ND = Not Detected (Area less than the lowest concentration of the calibration standards (25 µg/mL))

Table VII. Summary of PFOS Fortification Recoveries in Rat Liver

Sponsor ID	Exygen ID	Set Number	Amt Added (ng/g)	% Recovery
Lot #14124	0201684 Spk A	092602A	10	90
Lot #14124	0201684 Spk A*	092602A	10	87
Lot #14124	0201684 Spk B	092602A	50	92
Lot #14124	0201684 Spk B*	092602A	50	84
E01-1643-33846	0202800 Spk C	092602A	5000	98
E01-1643-33846	0202800 Spk C*	092602A	5000	97
			AVERAGE:	**91**
			STANDARD DEVIATION:	**6**
			RELATIVE STANDARD DEVIATION:	**6**

Table VIII. Summary of PFOS Fortification Recoveries in Rat Serum

Sponsor ID	Exygen ID	Set Number	Amt Added (ng/mL)	% Recovery
Lot #07024	0201682 Spk A	092002A	10	127
Lot #07024	0201682 Spk A*	092002A	10	116
Lot #07024	0201682 Spk B	092002A	50	127
Lot #07024	0201682 Spk B*	092002A	50	109
E01-1643-33826	0202780 Spk C	092002A	5000	85
E01-1643-33826	0202780 Spk C*	092002A	5000	87
			AVERAGE:	**109**
			STANDARD DEVIATION:	**19**
			RELATIVE STANDARD DEVIATION:	**17**

Table IX. Summary of PFOA Fortification Recoveries in Rat Liver

Sponsor ID	Exygen ID	Set Number	Amt Added (ng/g)	% Recovery
Lot #14124	0201684 Spk A	092602A	10	130
Lot #14124	0201684 Spk A*	092602A	10	130
Lot #14124	0201684 Spk B	092602A	50	134
Lot #14124	0201684 Spk B*	092602A	50	135
E01-1643-33846	0202800 Spk C	092602A	5000	140
E01-1643-33846	0202800 Spk C*	092602A	5000	135
			AVERAGE:	**134**
			STANDARD DEVIATION:	**4**
			RELATIVE STANDARD DEVIATION:	**3**

* Duplicate Injection

Table X. Summary of PFOA Fortification Recoveries in Rat Serum

Sponsor ID	Exygen ID	Set Number	Amt Added (ng/mL)	% Recovery
Lot #07024	0201682 Spk A	092002A	10	103
Lot #07024	0201682 Spk A*	092002A	10	107
Lot #07024	0201682 Spk B	092002A	50	120
Lot #07024	0201682 Spk B*	092002A	50	121
E01-1643-33826	0202780 Spk C	092002A	5000	108
E01-1643-33826	0202780 Spk C*	092002A	5000	114
			AVERAGE:	**112**
			STANDARD DEVIATION:	**7**
			RELATIVE STANDARD DEVIATION:	**7**

Table XI. Summary of POSF Fortification Recoveries in Rat Liver

Sponsor ID	Exygen ID	Set Number	Amt Added (µg/g)	% Recovery
Lot #21824	0202877 Spk A	092002G8	5	81
E01-1643-33854	0202808 Spk B	092002G8	5	78
E01-1643-33854	0202808 Spk C	092002G8	5	79
Lot #21824	0202877 Spk A	092302G8	5	91
E01-1643-33856	0202810 Spk B	092302G8	5	93
E01-1643-33856	0202810 Spk C	092302G8	5	87
			AVERAGE:	**85**
			STANDARD DEVIATION:	**6**
			RELATIVE STANDARD DEVIATION:	**8**

Table XII. Summary of POSF Fortification Recoveries in Rat Serum

Sponsor ID	Exygen ID	Set Number	Amt Added (µg/mL)	% Recovery
Lot #07024	0201682 Spk A	092402G8	5	86
E01-1643-33827	0202781 Spk B	092402G8	5	92
E01-1643-33827	0202781 Spk C	092402G8	5	89
Lot #07024	0201682 Spk A	093002A	5	66
E01-1643-33841	0202795 Spk B	093002A	5	51
E01-1643-33841	0202795 Spk C	093002A	5	49
			AVERAGE:	**72**
			STANDARD DEVIATION:	**19**
			RELATIVE STANDARD DEVIATION:	**27**

Table XIII. Summary of PFOS Residues in Rat Liver Samples

Sponsor ID	Exygen ID	Set Number	PFOS Found (ng/g)
E01-1643-33846	0202800	092602A	ND
E01-1643-33846*	0202800	092602A	ND
E01-1643-33847	0202801	092602A	ND
E01-1643-33847*	0202801	092602A	ND
E01-1643-33848	0202802	092602A	ND
E01-1643-33848*	0202802	092602A	ND
E01-1643-33849	0202803	092602A	ND
E01-1643-33849*	0202803	092602A	ND
E01-1643-33850	0202804	092602A	ND
E01-1643-33850*	0202804	092602A	ND
E01-1643-33851	0202805	092602A	33600
E01-1643-33851*	0202805	092602A	34000
E01-1643-33852	0202806	092602A	28200
E01-1643-33852*	0202806	092602A	28900
E01-1643-33853	0202807	092602A	44100
E01-1643-33853*	0202807	092602A	47000
E01-1643-33854	0202808	092602A	60600
E01-1643-33854*	0202808	092602A	52800
E01-1643-33855	0202809	092602A	51800
E01-1643-33855*	0202809	092602A	52200
E01-1643-33856	0202810	092602A	84.2
E01-1643-33856*	0202810	092602A	65.0
E01-1643-33857	0202811	092602A	38.0
E01-1643-33857*	0202811	092602A	48.4
E01-1643-33858	0202812	092602A	40.1
E01-1643-33858*	0202812	092602A	39.1
E01-1643-33859	0202813	092602A	44.7
E01-1643-33859*	0202813	092602A	44.5
E01-1643-33860	0202814	092602A	31.2
E01-1643-33860*	0202814	092602A	31.8
E01-1643-33861	0202815	092602A	56700
E01-1643-33861*	0202815	092602A	61700
E01-1643-33862	0202816	092602A	56200
E01-1643-33862*	0202816	092602A	43700
E01-1643-33863	0202817	092602A	65600
E01-1643-33863*	0202817	092602A	67600
E01-1643-33864	0202818	092602A	53100
E01-1643-33864*	0202818	092602A	60100
E01-1643-33865	0202819	092602A	44200
E01-1643-33865*	0202819	092602A	44000

* Duplicate Injection

ND = Not Detected (Area less than lowest calibration standard of 0.0001 μg/mL)

Table XIV. Summary of PFOA Residues in Rat Liver Samples

Sponsor ID	Exygen ID	Set Number	PFOA Found (ng/g)
E01-1643-33846	0202800	092602A	ND
E01-1643-33846*	0202800	092602A	ND
E01-1643-33847	0202801	092602A	ND
E01-1643-33847*	0202801	092602A	ND
E01-1643-33848	0202802	092602A	ND
E01-1643-33848*	0202802	092602A	ND
E01-1643-33849	0202803	092602A	ND
E01-1643-33849*	0202803	092602A	ND
E01-1643-33850	0202804	092602A	ND
E01-1643-33850*	0202804	092602A	ND
E01-1643-33851	0202805	092602A	3400
E01-1643-33851*	0202805	092602A	3280
E01-1643-33852	0202806	092602A	3080
E01-1643-33852*	0202806	092602A	3100
E01-1643-33853	0202807	092602A	4680
E01-1643-33853*	0202807	092602A	4590
E01-1643-33854	0202808	092602A	5930
E01-1643-33854*	0202808	092602A	6110
E01-1643-33855	0202809	092602A	4120
E01-1643-33855*	0202809	092602A	4070
E01-1643-33856	0202810	092602A	ND
E01-1643-33856*	0202810	092602A	ND
E01-1643-33857	0202811	092602A	ND
E01-1643-33857*	0202811	092602A	ND
E01-1643-33858	0202812	092602A	ND
E01-1643-33858*	0202812	092602A	ND
E01-1643-33859	0202813	092602A	ND
E01-1643-33859*	0202813	092602A	ND
E01-1643-33860	0202814	092602A	ND
E01-1643-33860*	0202814	092602A	ND
E01-1643-33861	0202815	092602A	54.9
E01-1643-33861*	0202815	092602A	57.6
E01-1643-33862	0202816	092602A	31.5
E01-1643-33862*	0202816	092602A	35.7
E01-1643-33863	0202817	092602A	41.6
E01-1643-33863*	0202817	092602A	42.6
E01-1643-33864	0202818	092602A	78.2
E01-1643-33864*	0202818	092602A	75.7
E01-1643-33865	0202819	092602A	43.7
E01-1643-33865*	0202819	092602A	41.4

* Duplicate Injection
ND = Not Detected (Area less than lowest calibration standard of 0.0001 µg/mL)

Table XV. Summary of POSF Residues in Rat Liver Samples

Sponsor ID	Exygen ID	Set Number	POSF Found (µg/g)
E01-1643-33846	0202800	092002G8	ND
E01-1643-33846*	0202800	092002G8	ND
E01-1643-33847	0202801	092002G8	ND
E01-1643-33847*	0202801	092002G8	ND
E01-1643-33848	0202802	092002G8	ND
E01-1643-33848*	0202802	092002G8	ND
E01-1643-33849	0202803	092002G8	ND
E01-1643-33849*	0202803	092002G8	ND
E01-1643-33850	0202804	092002G8	ND
E01-1643-33850*	0202804	092002G8	ND
E01-1643-33851	0202805	092002G8	ND
E01-1643-33851*	0202805	092002G8	ND
E01-1643-33852	0202806	092002G8	ND
E01-1643-33852*	0202806	092002G8	ND
E01-1643-33853	0202807	092002G8	ND
E01-1643-33853*	0202807	092002G8	ND
E01-1643-33854	0202808	092002G8	ND
E01-1643-33854*	0202808	092002G8	ND
E01-1643-33855	0202809	092002G8	ND
E01-1643-33855*	0202809	092002G8	ND
E01-1643-33856	0202810	092302G8	ND
E01-1643-33856*	0202810	092302G8	ND
E01-1643-33857	0202811	092302G8	ND
E01-1643-33857*	0202811	092302G8	ND
E01-1643-33858	0202812	092302G8	ND
E01-1643-33858*	0202812	092302G8	ND
E01-1643-33859	0202813	092302G8	ND
E01-1643-33859*	0202813	092302G8	ND
E01-1643-33860	0202814	092302G8	ND
E01-1643-33860*	0202814	092302G8	ND
E01-1643-33861	0202815	092302G8	ND
E01-1643-33861*	0202815	092302G8	ND
E01-1643-33862	0202816	092302G8	ND
E01-1643-33862*	0202816	092302G8	ND
E01-1643-33863	0202817	092302G8	ND
E01-1643-33863*	0202817	092302G8	ND
E01-1643-33864	0202818	092302G8	ND
E01-1643-33864*	0202818	092302G8	ND
E01-1643-33865	0202819	092302G8	ND
E01-1643-33865*	0202819	092302G8	ND

* Duplicate Aliquot

ND = Not Detected (Area less than lowest calibration standard of 25 µg/mL)

Table XVI. Summary of PFOS Residues in Rat Serum Samples

Sponsor ID	Exygen ID	Set Number	PFOS Found (ng/mL)
E01-1643-33826	0202780	092002A	ND
E01-1643-33826*	0202780	092002A	ND
E01-1643-33827	0202781	092002A	ND
E01-1643-33827*	0202781	092002A	ND
E01-1643-33828	0202782	092002A	ND
E01-1643-33828*	0202782	092002A	ND
E01-1643-33829	0202783	092002A	ND
E01-1643-33829*	0202783	092002A	ND
E01-1643-33830	0202784	092002A	ND
E01-1643-33830*	0202784	092002A	ND
E01-1643-33831	0202785	092002AR	13200
E01-1643-33831*	0202785	092002AR	12700
E01-1643-33832	0202786	092002AR	8870
E01-1643-33832*	0202786	092002AR	8610
E01-1643-33833	0202787	092002AR	9100
E01-1643-33833*	0202787	092002AR	9750
E01-1643-33834	0202788	092002AR	8480
E01-1643-33834*	0202788	092002AR	8240
E01-1643-33835	0202789	092002AR	9860
E01-1643-33835*	0202789	092002AR	9900
E01-1643-33836	0202790	092002A	ND
E01-1643-33836*	0202790	092002A	ND
E01-1643-33837	0202791	092002A	ND
E01-1643-33837*	0202791	092002A	ND
E01-1643-33838	0202792	092002A	ND
E01-1643-33838*	0202792	092002A	ND
E01-1643-33839	0202793	092002A	ND
E01-1643-33839*	0202793	092002A	ND
E01-1643-33840	0202794	092002A	ND
E01-1643-33840*	0202794	092002A	ND
E01-1643-33841	0202795	092002AR	11300
E01-1643-33841*	0202795	092002AR	10600
E01-1643-33842	0202796	092002AR	13800
E01-1643-33842*	0202796	092002AR	13000
E01-1643-33843	0202797	092002AR	10300
E01-1643-33843*	0202797	092002AR	10800
E01-1643-33844	0202798	092002AR	13900
E01-1643-33844*	0202798	092002AR	12600
E01-1643-33845	0202799	092002AR	10800
E01-1643-33845*	0202799	092002AR	9610

* Duplicate Injection
ND = Not Detected (Area less than lowest calibration standard of 0.0001 µg/mL)

Table XVII. Summary of PFOA Residues in Rat Serum Samples

Sponsor ID	Exygen ID	Set Number	PFOA Found (ng/mL)
E01-1643-33826	0202780	092002A	ND
E01-1643-33826*	0202780	092002A	ND
E01-1643-33827	0202781	092002A	ND
E01-1643-33827*	0202781	092002A	ND
E01-1643-33828	0202782	092002A	ND
E01-1643-33828*	0202782	092002A	ND
E01-1643-33829	0202783	092002A	ND
E01-1643-33829*	0202783	092002A	ND
E01-1643-33830	0202784	092002A	ND
E01-1643-33830*	0202784	092002A	ND
E01-1643-33831	0202785	092002AR	8760
E01-1643-33831*	0202785	092002AR	8850
E01-1643-33832	0202786	092002AR	5960
E01-1643-33832*	0202786	092002AR	6020
E01-1643-33833	0202787	092002AR	7750
E01-1643-33833*	0202787	092002AR	7760
E01-1643-33834	0202788	092002AR	7170
E01-1643-33834*	0202788	092002AR	7140
E01-1643-33835	0202789	092002AR	5970
E01-1643-33835*	0202789	092002AR	5990
E01-1643-33836	0202790	092002A	ND
E01-1643-33836*	0202790	092002A	ND
E01-1643-33837	0202791	092002A	ND
E01-1643-33837*	0202791	092002A	ND
E01-1643-33838	0202792	092002A	ND
E01-1643-33838*	0202792	092002A	ND
E01-1643-33839	0202793	092002A	ND
E01-1643-33839*	0202793	092002A	ND
E01-1643-33840	0202794	092002A	ND
E01-1643-33840*	0202794	092002A	ND
E01-1643-33841	0202795	092002A	81.5
E01-1643-33841*	0202795	092002A	88.9
E01-1643-33842	0202796	092002A	39.9
E01-1643-33842*	0202796	092002A	40.9
E01-1643-33843	0202797	092002A	53.8
E01-1643-33843*	0202797	092002A	50.5
E01-1643-33844	0202798	092002A	88.7
E01-1643-33844*	0202798	092002A	99.9
E01-1643-33845	0202799	092002A	58.2
E01-1643-33845*	0202799	092002A	58.7

* Duplicate Injection
ND = Not Detected (Area less than lowest calibration standard of 0.0001 µg/mL)

Table XVIII. Summary of POSF Residues in Rat Serum Samples

Sponsor ID	Exygen ID	Set Number	POSF Found (µg/mL)
E01-1643-33826	0202780	092402G8	ND
E01-1643-33826*	0202780	092402G8	ND
E01-1643-33827	0202781	092402G8	ND
E01-1643-33827*	0202781	092402G8	ND
E01-1643-33828	0202782	092402G8	ND
E01-1643-33829	0202783	092402G8	ND
E01-1643-33829*	0202783	092402G8	ND
E01-1643-33830	0202784	092402G8	ND
E01-1643-33831	0202785	092402G8	ND
E01-1643-33831*	0202785	092402G8	ND
E01-1643-33832	0202786	092402G8	ND
E01-1643-33832*	0202786	092402G8	ND
E01-1643-33833	0202787	092402G8	ND
E01-1643-33833*	0202787	092402G8	ND
E01-1643-33834	0202788	092402G8	ND
E01-1643-33835	0202789	092402G8	ND
E01-1643-33835*	0202789	092402G8	ND
E01-1643-33836	0202790	093002A	ND
E01-1643-33836*	0202790	093002A	ND
E01-1643-33837	0202791	093002A	ND
E01-1643-33837*	0202791	093002A	ND
E01-1643-33838	0202792	093002A	ND
E01-1643-33838*	0202792	093002A	ND
E01-1643-33839	0202793	093002A	ND
E01-1643-33839*	0202793	093002A	ND
E01-1643-33840	0202794	093002A	ND
E01-1643-33840*	0202794	093002A	ND
E01-1643-33841	0202795	093002A	ND
E01-1643-33841*	0202795	093002A	ND
E01-1643-33842	0202796	093002A	ND
E01-1643-33842*	0202796	093002A	ND
E01-1643-33843	0202797	093002A	ND
E01-1643-33843*	0202797	093002A	ND
E01-1643-33844	0202798	093002A	ND
E01-1643-33844*	0202798	093002A	ND
E01-1643-33845	0202799	093002A	ND
E01-1643-33845*	0202799	093002A	ND

* Duplicate Aliquot

ND = Not Detected (Area less than lowest calibration standard of 25 µg/mL)

FIGURES

Figure 1. Typical Calibration Curve for PFOS

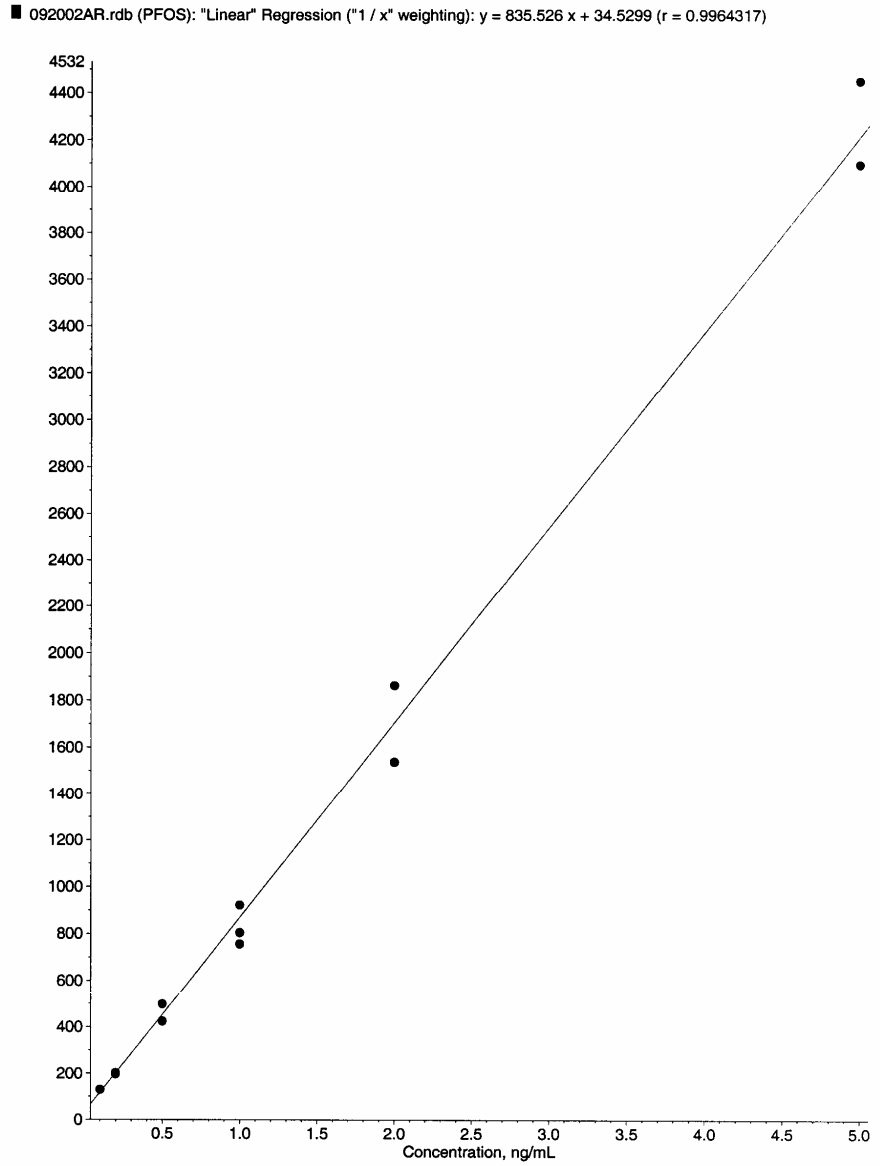


Figure 2. Typical Calibration Curve for PFOA

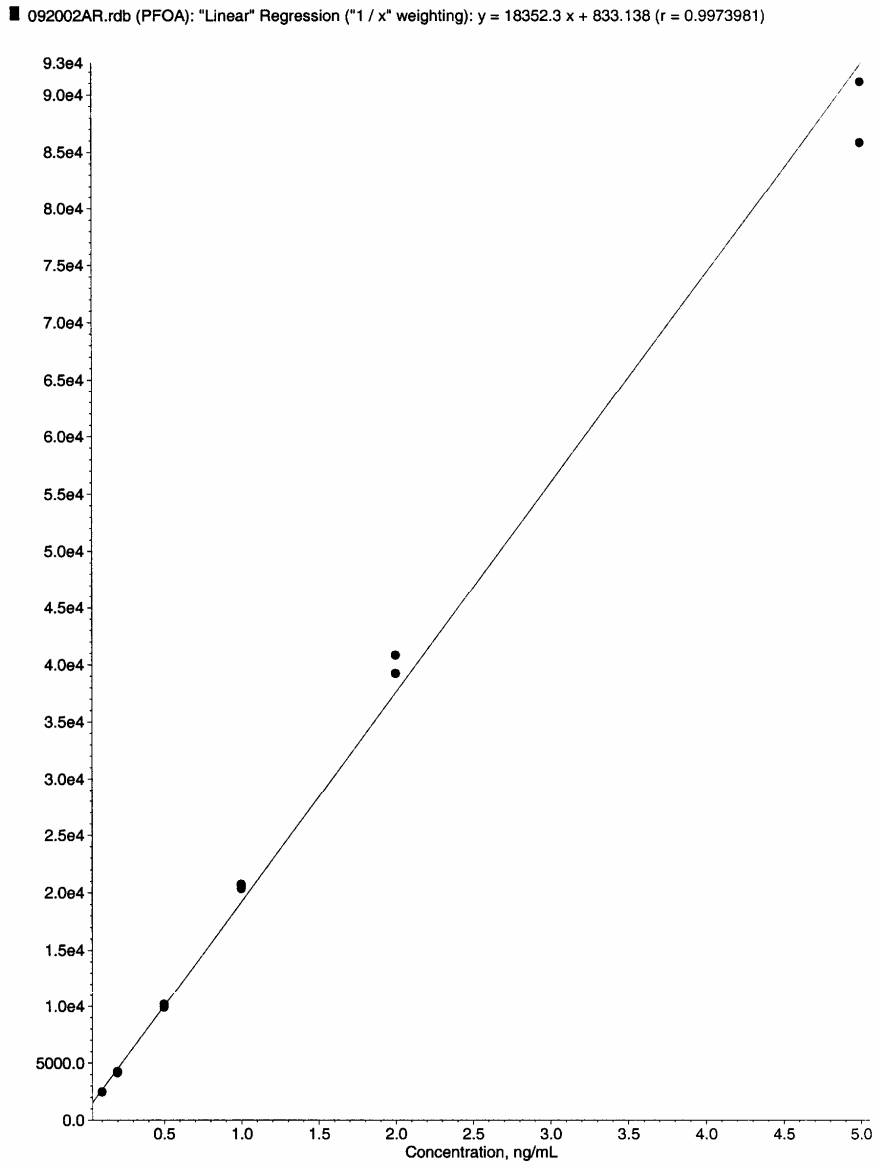


Figure 3. Typical Calibration Curve for POSF

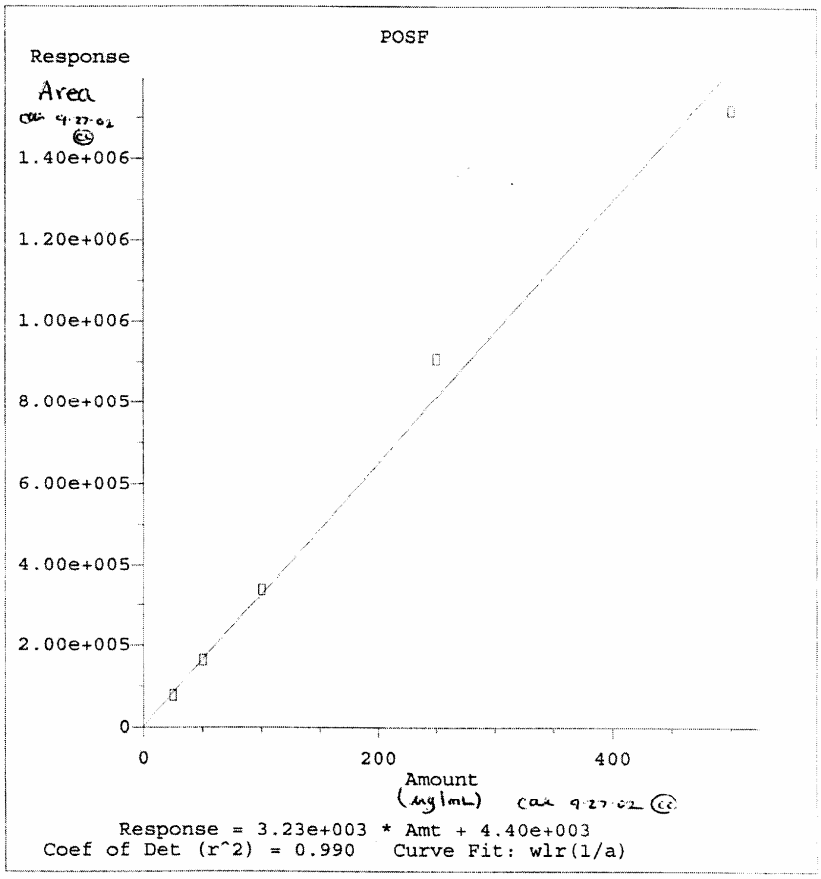


Figure 4. Chromatogram Representing a 0.1 ng/mL standard for PFOS and PFOA

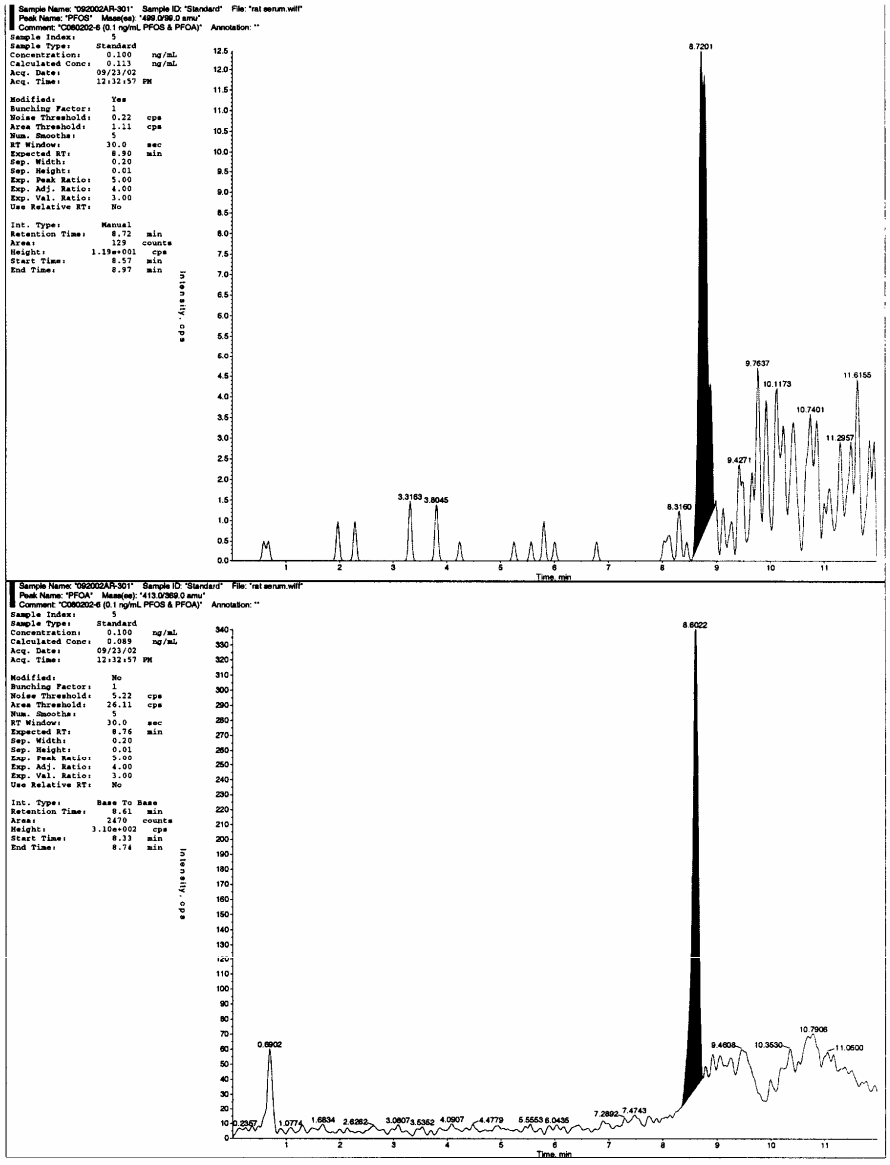


Figure 5. Chromatogram Representing a 25 µg/mL standard for POSF

```
File        : L:\023-080\RAWDATA\092002G8\HS200202.D
Operator    : cp
Acquired    : 20 Sep 2002   1:02 pm using AcqMethod PAL502
Instrument :   GC/MS 8
Sample Name: POSF Std.  5µL of 25µg/mL, 1mL headspace
Misc Info   : C092002-6, Study#023-080
Vial Number: 2
```

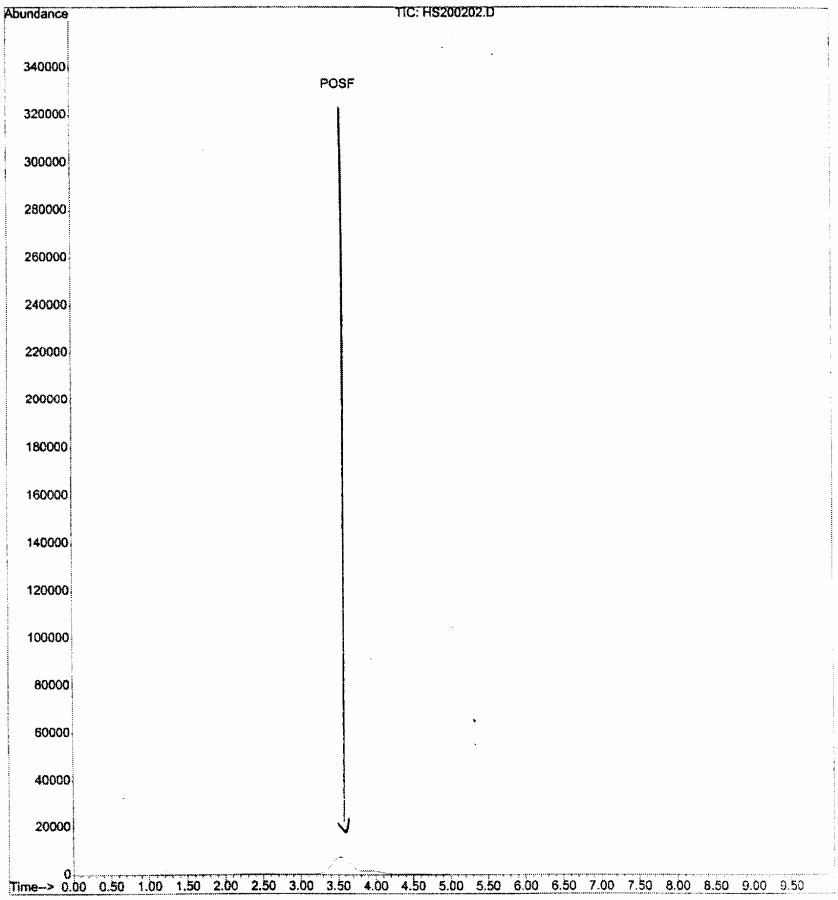


Figure 6. Chromatogram Representing a Rat Liver Control Sample for PFOS and PFOA (Exygen ID 0201684 Control A, Set: 092602A)

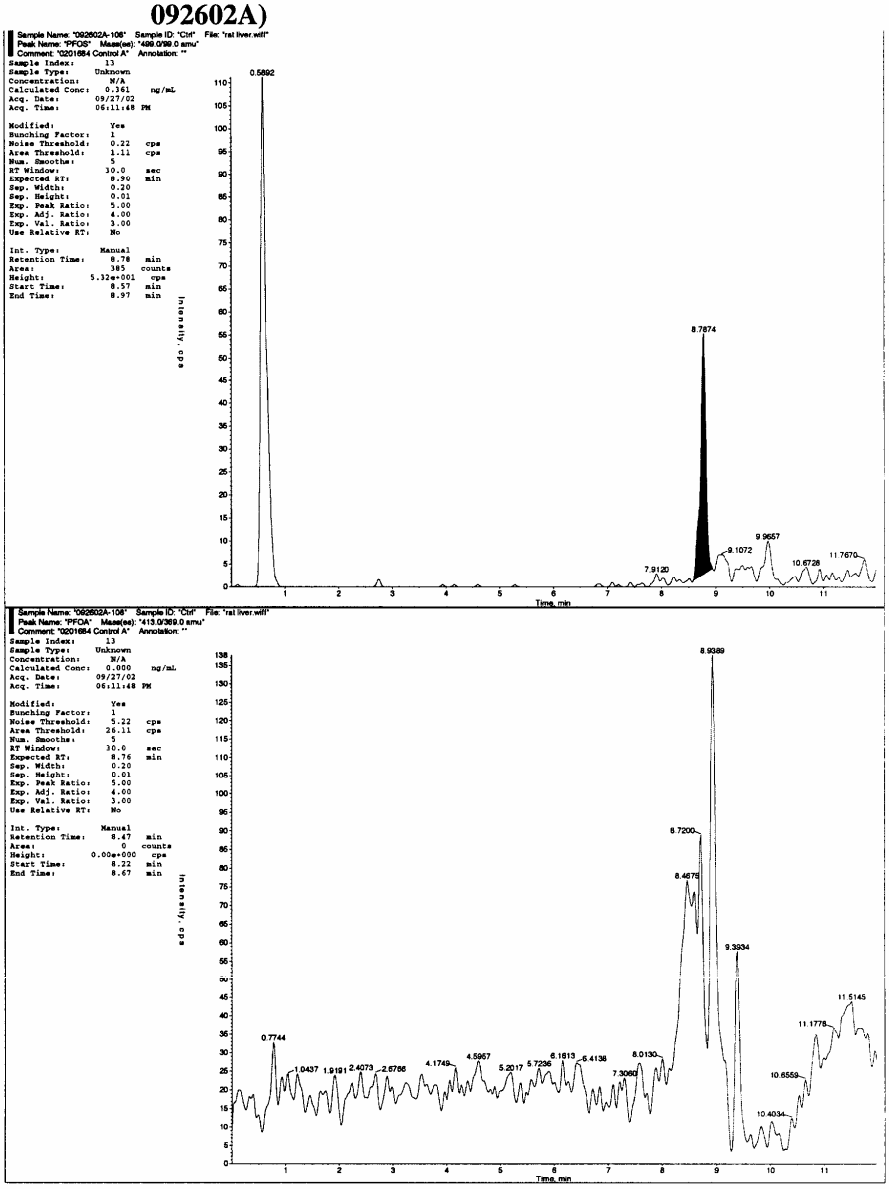


Figure 7. Chromatogram Representing Control Rat Liver Sample for POSF (Exygen ID: 0202877, Set 092002G8)

```
File        : L:\023-080\RAWDATA\092002G8\HS200208.D
Operator    : cp
Acquired    : 20 Sep 2002    2:19 pm using AcqMethod PAL502
Instrument :
  GC/MS 8
Sample Name: Control, 1mL headspace
Misc Info   : Study#023-080
Vial Number: 8
```

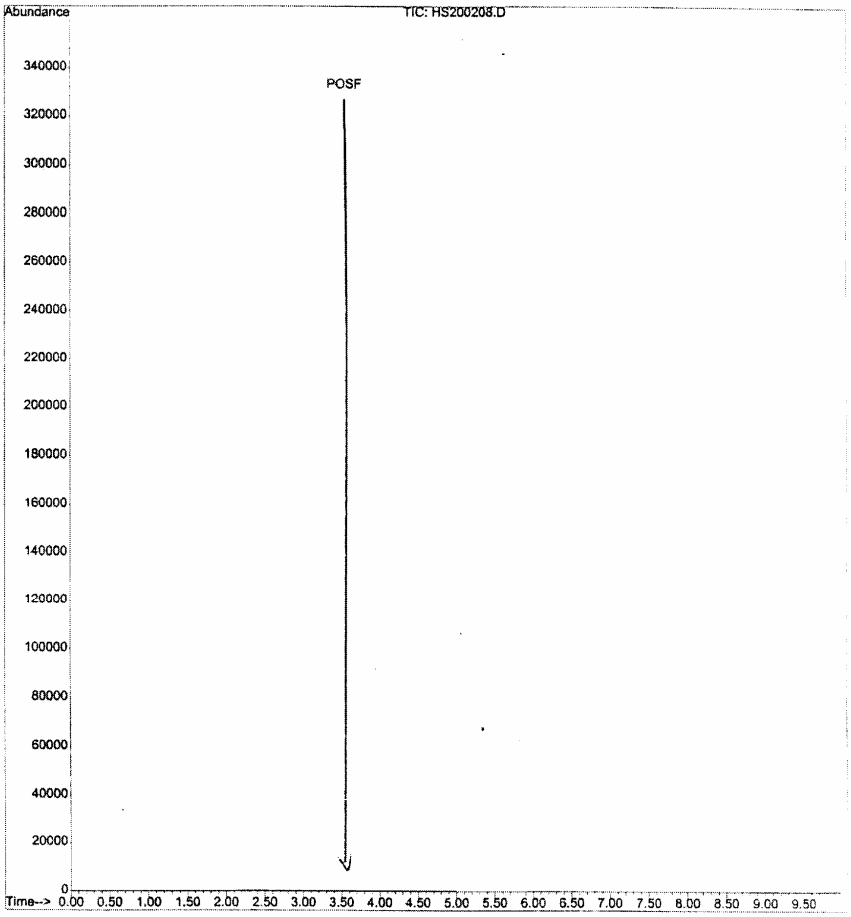


Figure 8. Chromatogram Representing Control Rat Serum for PFOS and PFOA, (Exygen ID: 0201682 Control A, Set: 092002A)

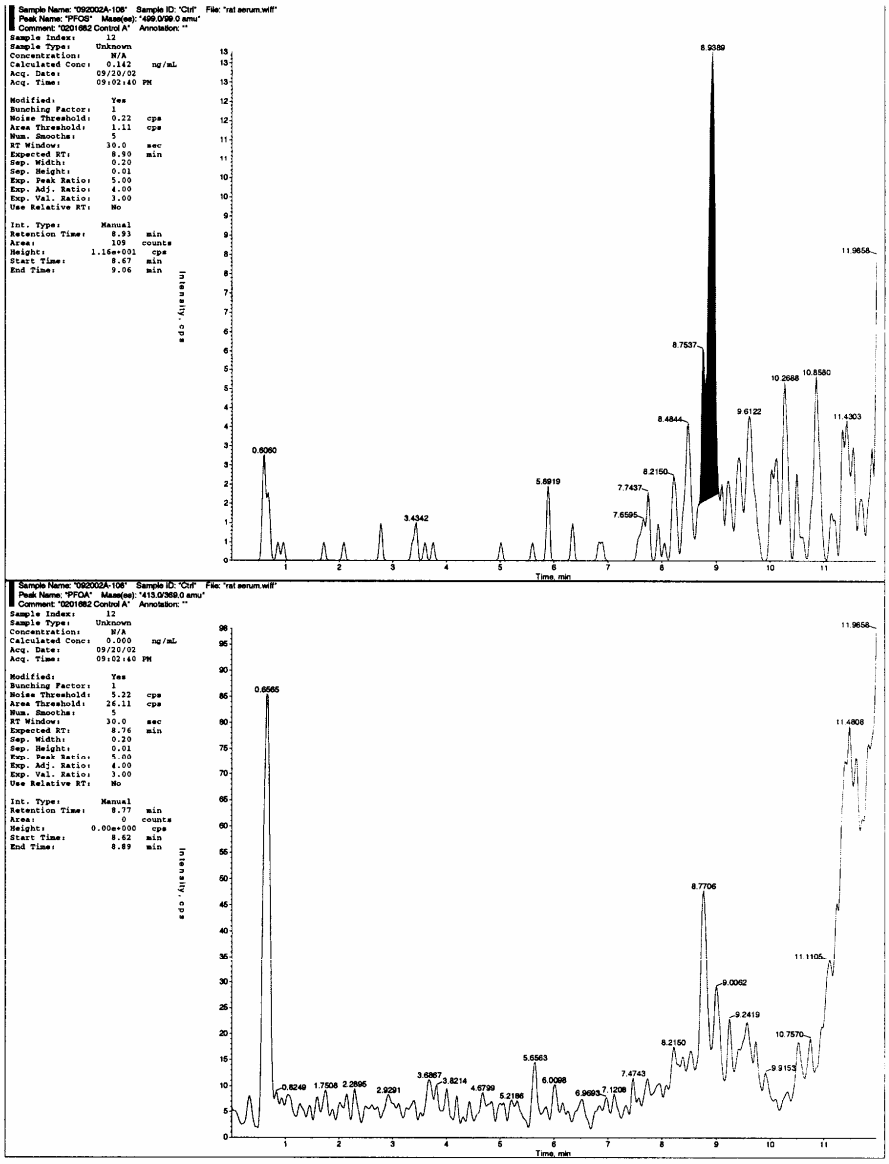


Figure 9. Chromatogram Representing Control Rat Serum for POSF (Exygen ID: 0201682, Set: 092402G8)

```
File        : L:\023-080\RAWDATA\092402G8\HS240207.D
Operator    : CP
Acquired    : 24 Sep 2002   7:36 am using AcqMethod PAL502
Instrument :   GC/MS 8
Sample Name: Control, 1mL headspace
Misc Info   : Study#023-080
Vial Number: 8
```

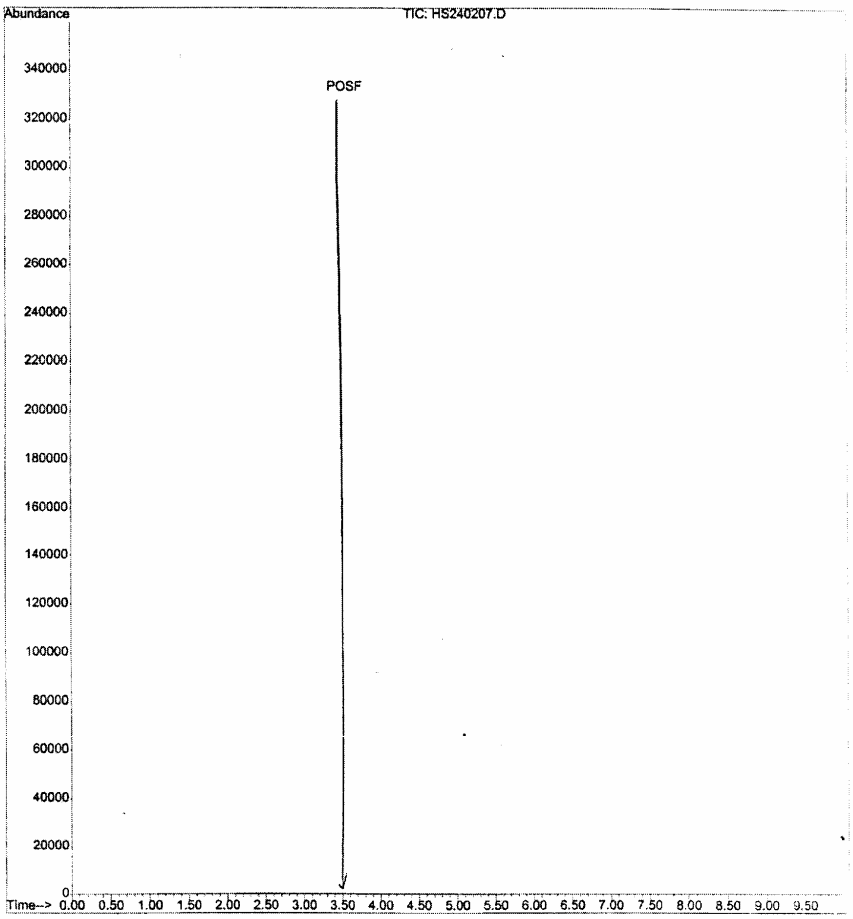


Figure 10. Chromatogram Representing Control Rat Liver fortified with 10 ng/g of PFOS and PFOA (Exygen ID: 0201684 Spk A, Set: 092602A)

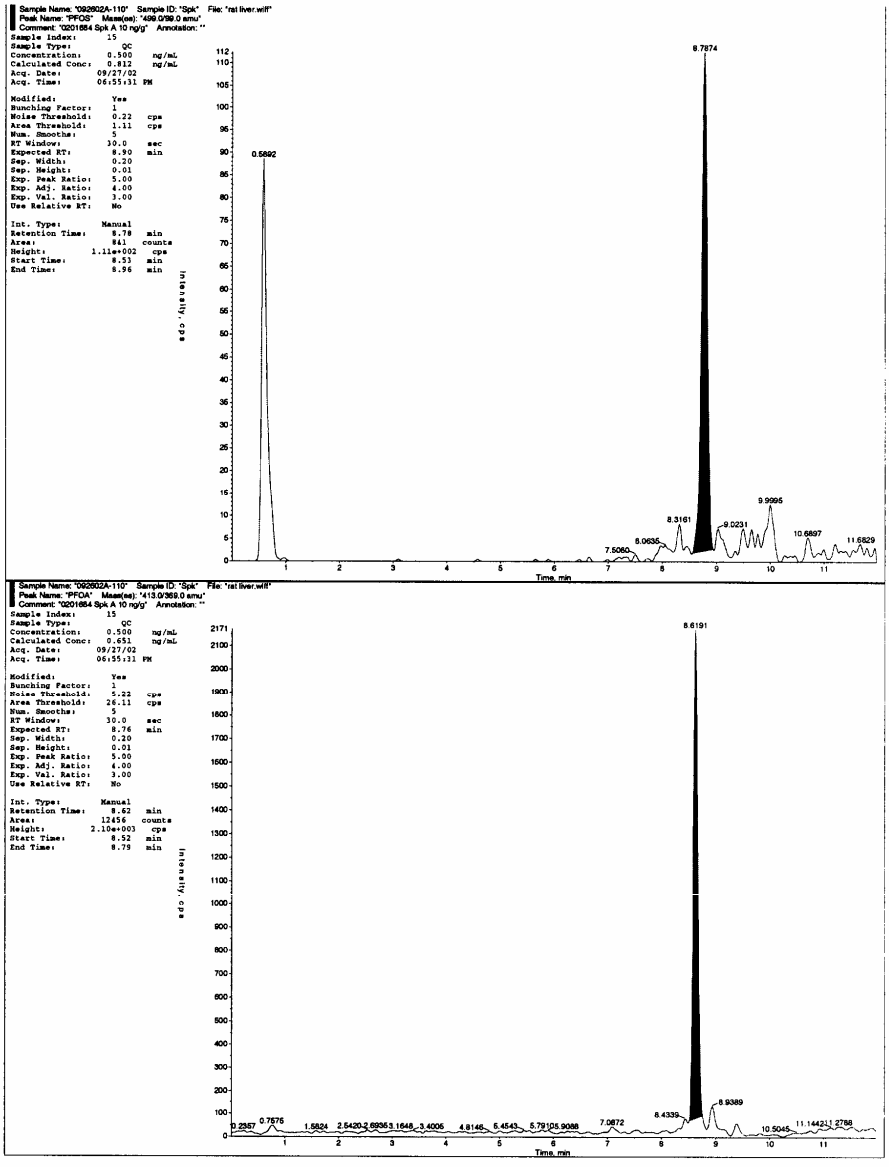


Figure 11. Chromatogram Representing Control Rat Liver fortified at 5 µg/g with POSF (Exygen ID: 0202877 Spk A, Set: 092002G8)

```
File        : L:\023-080\RAWDATA\092002G8\HS200209.D
Operator    : cp
Acquired    : 20 Sep 2002   2:31 pm using AcqMethod PAL502
Instrument :   GC/MS 8
Sample Name: Spike A, 5µL of 100µg/mL, 1mL headspace
Misc Info   : Control Fortification, Study#023-080
Vial Number: 9
```

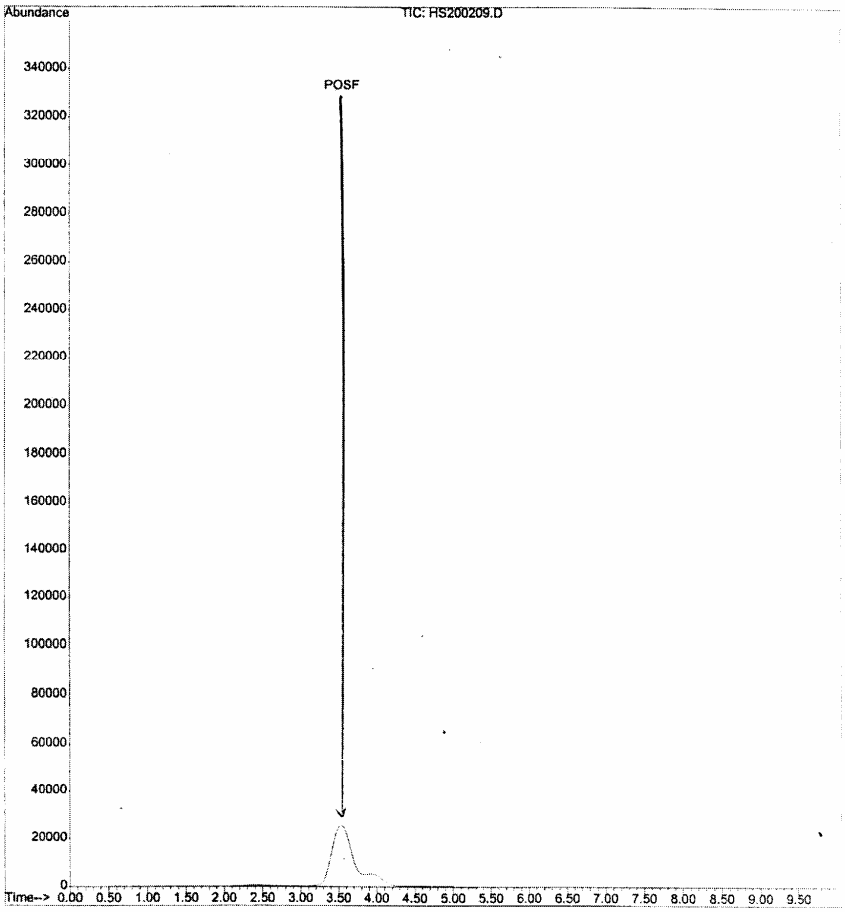


Figure 12. Chromatogram Representing Control Rat Serum fortified with 10 ng/mL of PFOS and PFOA (Exygen ID: 0201682 Spk A, Set: 092002A)

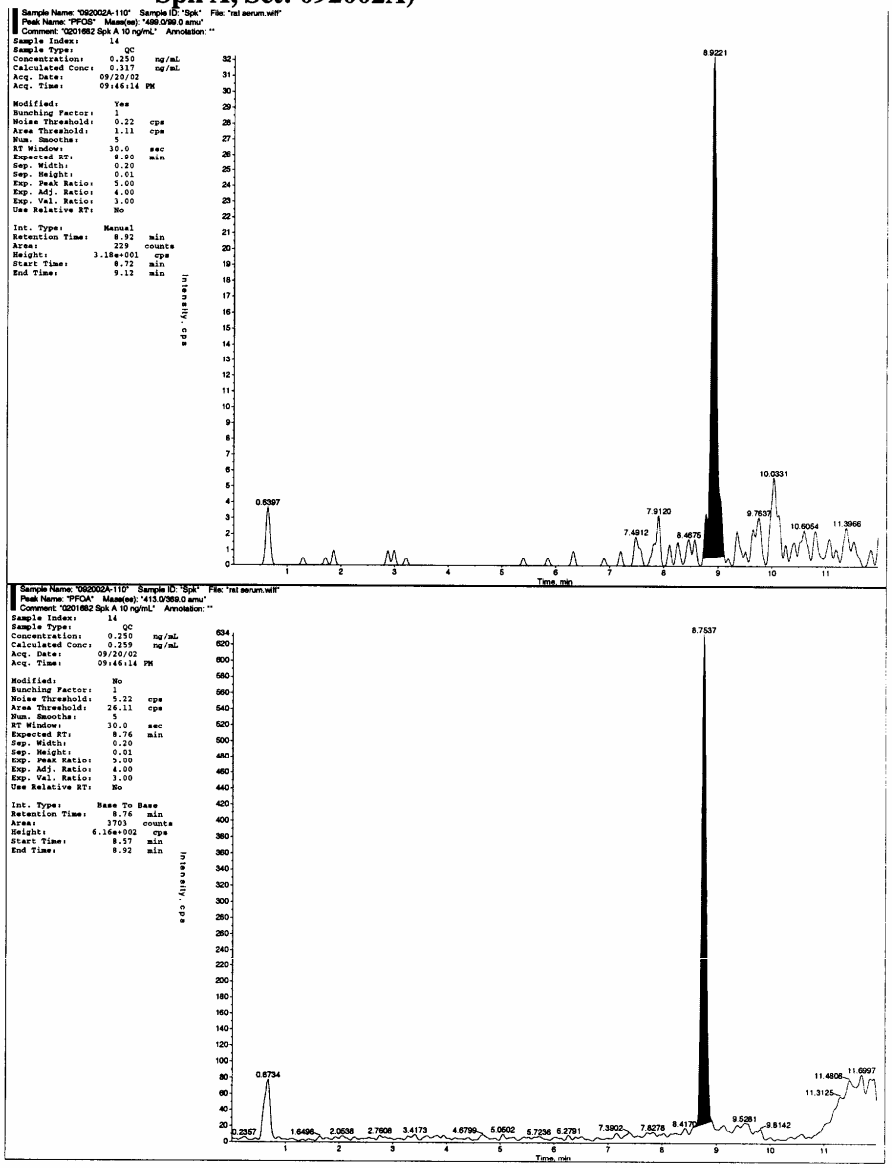


Figure 13. Chromatogram Representing Control Rat Serum fortified with 5 µg/mL of POSF (Exygen ID: 0201682 Spk A, Set: 092402G8)

```
File        : L:\023-080\RAWDATA\092402G8\HS240208.D
Operator    : CP
Acquired    : 24 Sep 2002   7:49 am using AcqMethod PAL502
Instrument :   GC/MS 8
Sample Name: Spike A, 5µL of 100µg/mL, 1mL headspace
Misc Info   : Control Fortification, Study#023-080
Vial Number: 9
```

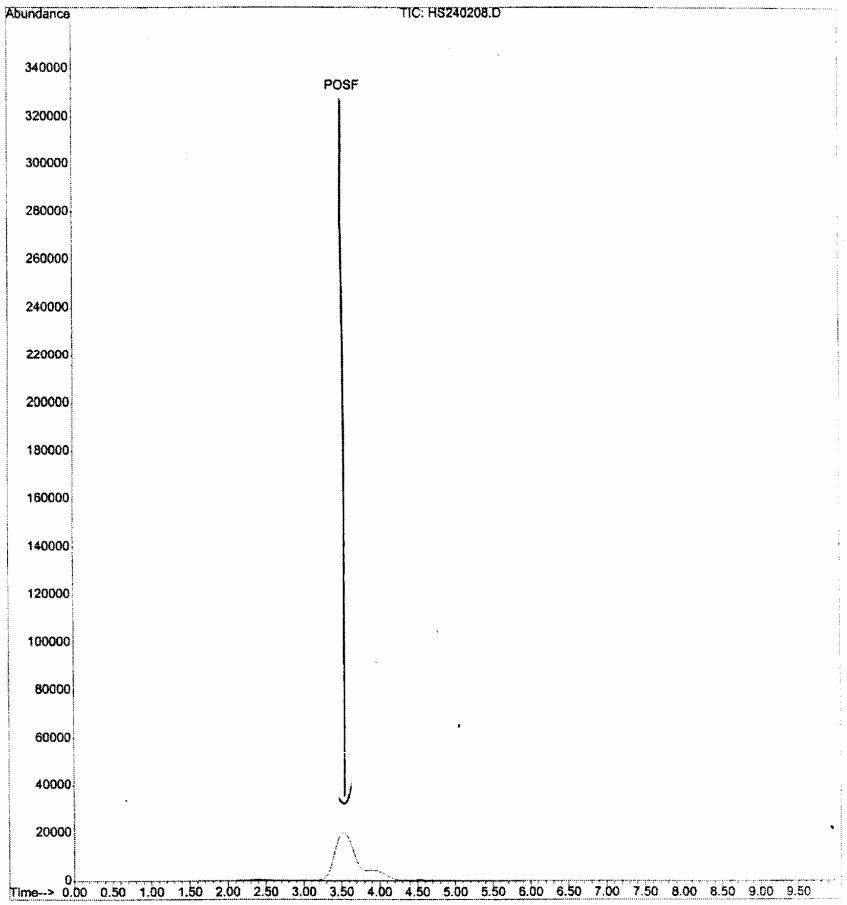


Figure 14. Chromatogram Representing Rat Liver Sample for PFOS and PFOA (Exygen ID: 0202805, Sponsor ID: E01-1643-33851 Set: 092602A)

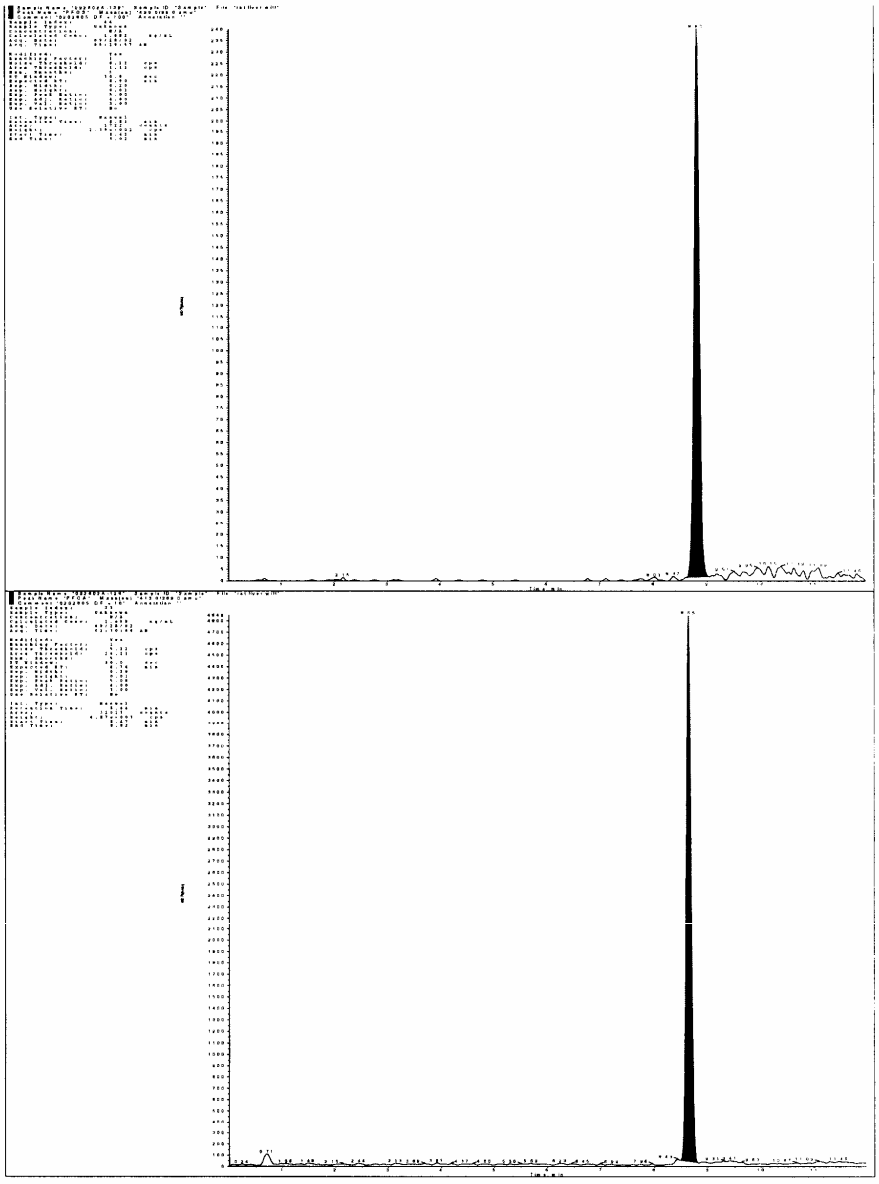


Figure 15. Chromatogram Representing Rat Liver Sample for POSF (Exygen ID: 0202800, Sponsor ID: E01-1643-33846 Set: 092002G8)

```
File        : L:\023-080\RAWDATA\092002G8\HS200212.D
Operator    : cp
Acquired    : 20 Sep 2002   3:10 pm using AcqMethod PAL502
Instrument :   GC/MS 8
Sample Name: Sample, #0202800, 1mL headspace
Misc Info   : Study#023-080
Vial Number: 12
```

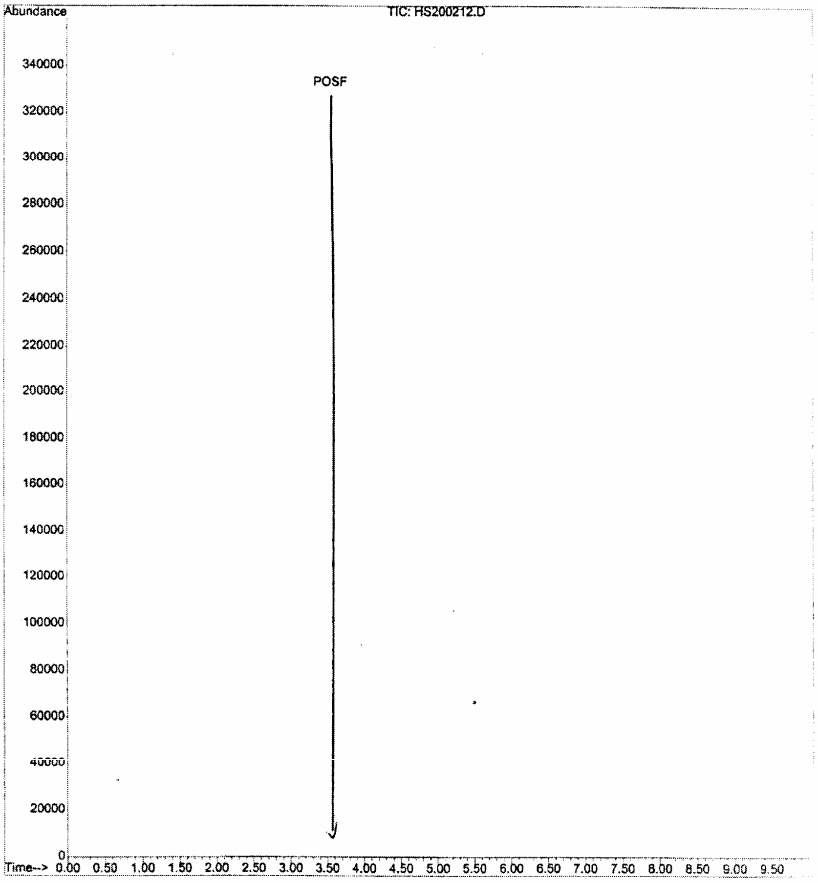


Figure 16. Chromatogram Representing Rat Serum Sample for PFOS and PFOA (Exygen ID: 0202785, Sponsor ID: E01-1643-33831 Set: 092002AR)

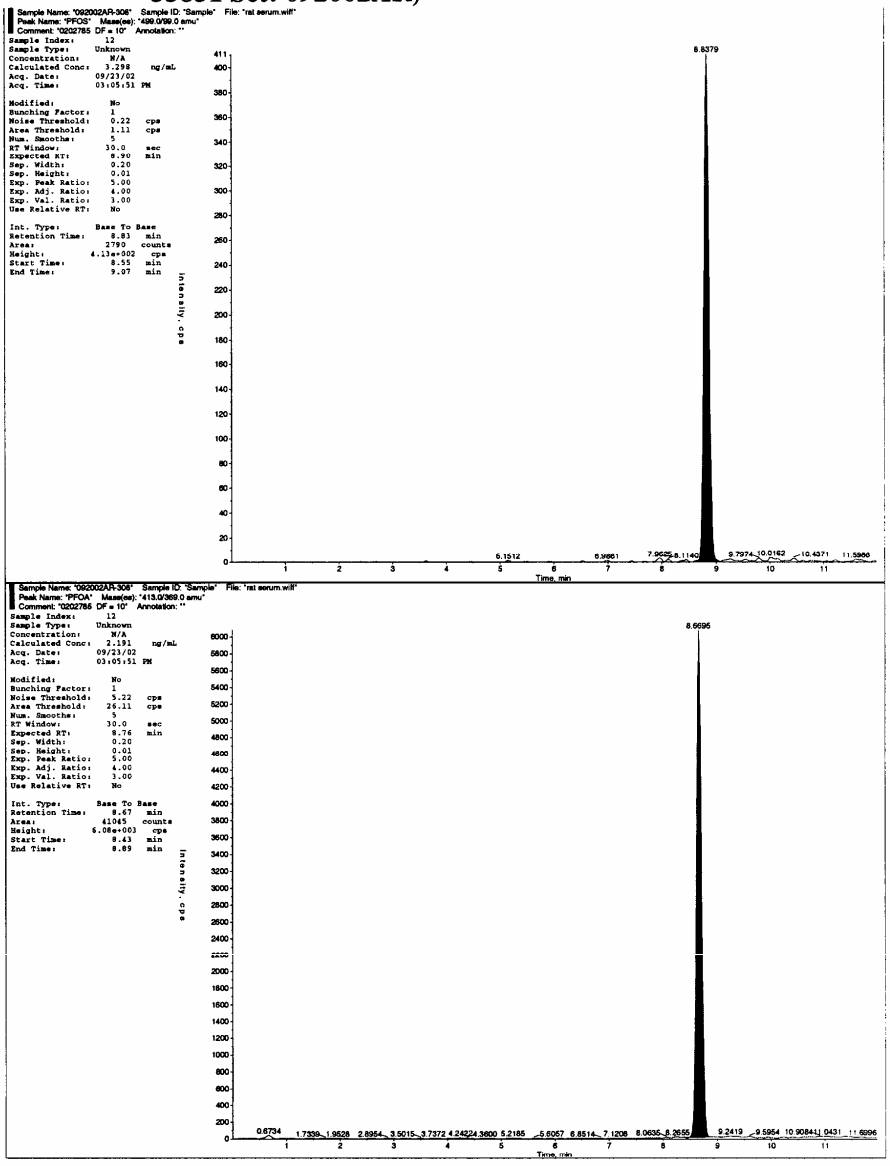
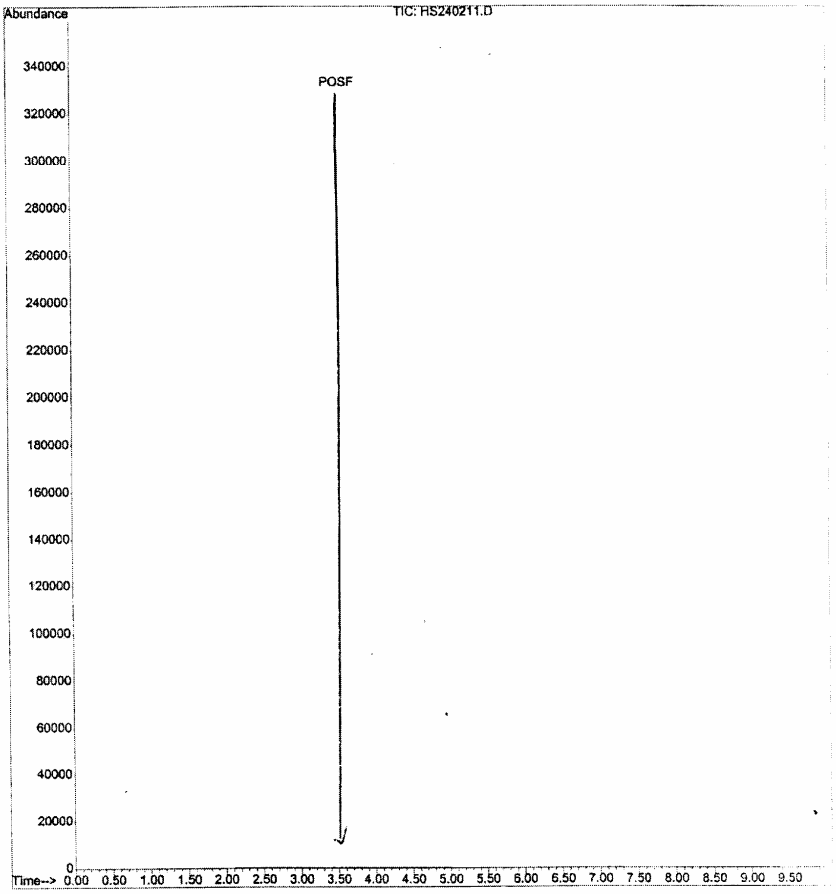


Figure 17. Chromatogram Representing Rat Serum Sample for POSF (Exygen ID: 0202780, Sponsor ID: E01-1643-33826 Set: 092402G8)

```
File        : L:\023-080\RAWDATA\092402G8\HS240211.D
Operator    : CP
Acquired    : 24 Sep 2002   8:27 am using AcqMethod PAL502
Instrument :   GC/MS 8
Sample Name: Sample, #0202780, 1mL headspace
Misc Info   : Study#023-080
Vial Number: 12
```



APPENDIX A

Study Protocol
MIN/312
(Exygen Study No. 023-080)
and
Amendments and Deviations

T-7661.1

Study Number : MIN/312

CONFIDENTIAL

Huntingdon Life Sciences

PROTOCOL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Sponsor

3M Center
3M Corporate Toxicology
Building 220-2E-02
St Paul
MN 55133-3220
USA

Research Laboratory

Huntingdon Life Sciences Ltd
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Total number of pages: 20

Final Protocol

Study Number : MIN/312

Huntingdon Life Sciences

CONTACT DETAILS

Sponsor's Monitoring Scientist : John Butenhoff

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Huntingdon Life Sciences

PROTOCOL APPROVAL

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

T.J. Kenny, B.Sc (Hons.).
Study Director,
Huntingdon Life Sciences Ltd.

17 August 2001
Date

The signature of the Study Director confirms this protocol as the working document for the study. Any changes made subsequent to the date of the Study Director's signature will be documented in formal amendments.

C.J. Hardy, B.Sc., Ph.D., M.I.Biol., C.Biol., Dip. R. C. Path. (Toxicology)
Management,
Huntingdon Life Sciences Ltd.

17 Aug 2001
Date

John Butenhoff
Sponsor,
3M

20 Aug 2001
Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Final Protocol

Study Number : **MIN/312**

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Enquiry Number: 23923A

Number of pages for internal distribution: 17

This working document is approved for circulation and use:

Study Director

14 August 2009
Date

Primary location of study

Huntingdon Research Centre
Huntingdon
Cambridgeshire
PE28 4HS

Building Number:

All procedures to be performed at the above site unless otherwise detailed below.

Location of specific tasks

Histology	:	Routinely done at Huntingdon Research Centre, Huntingdon, Cambridgeshire, PE28 4HS, but may be performed at Eye Research Centre, Eye, Suffolk, IP23 7PX for logistical reasons.
SEM X-ray analysis	:	University of Plymouth.
Test substance/metabolite analysis	:	3M.

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CONTENTS

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1. INTRODUCTION

Management of study

Study Director	:	T.J. Kenny
Monitoring Toxicologist	:	D.W. Coombs
In the temporary absence of the Study Director, the scientific responsibilities will be taken over by the Monitoring Toxicologist; other items of routine study management should be referred to the following person in the first instance.	:	R. Davies

Objective

Assessment of systemic toxic potential in a 1 week inhalation study in CD Rats.

Good Laboratory Practice

The study will be conducted in compliance with principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

Animals (Scientific Procedures) Act 1986 compliance

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 (the Act). The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

The number of animals used will be the minimum that is consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.

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Animal model	:	CD Rats, accepted by regulatory agencies, background data available.	
Route	:	Inhalation.	
Treatment groups and dosages			
Group	:	1	2
Compound	:	Air Control	Perfluorooctanesulfonyl Fluoride (POSF)
Exposure level (mg/l)	:		
Dosage (ppm)	:	0	300

2. STUDY SCHEDULE AND STRUCTURE

2.1. Duration of treatment

Minimum period : 1 week (five days).

The treatment period may be extended, with the Sponsor's consent, to incorporate any additional observations considered necessary; documented in an amendment to protocol.

Throughout the necropsy period treatment will continue and serial observations will be recorded at appropriate intervals (Section 6). Data for any additional complete weeks before commencement of necropsies will be included in the final report.

2.2. Scheduled time plan

Animals to arrive	:	September 2001	
Treatment to commence	:	September 2001	
Terminal sacrifice to commence	:	September 2001	
Histopathology to be completed	:	October 2001	(estimated)
Draft report to be issued	:	November 2001	(estimated)

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2.3. Identity of treatment groups

(to be selected from 20 animals ordered)

Group	Treatment	Exposure level (ppm)	Number of animals	
			Male	Female
1	Air Control	0	5	5
2	Perfluorooctanesulfonyl Fluoride (POSF)	300	5	5

Group	Cage numbers		Animal numbers	
	Male	Female	Male	Female
1	1	3	1-5	11-15
2	2	4	6-10	16-20

3. TEST SUBSTANCE AND ATMOSPHERE GENERATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1999, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class: [ ]

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3.1. Test substance

Sponsor's identification	:	Perfluorooctanesulfonyl Fluoride (POSF) FX-8B, Lot 040277.
Storage conditions	:	At ambient temperature or in a refrigerator, unless otherwise directed by the Sponsor. Any such decision will be documented in the study data and included in the final report.
Sponsor's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis.

4. ANIMAL MANAGEMENT

4.1. Animals – supply, acclimatisation and allocation

4.1.1. Animals

Species	:	Rat.
Strain	:	Crl:CD® BR.
Age ordered	:	42 ± 2 days.
Weight range ordered	:	To be within an 11 g range for each sex.
Supplier	:	Charles River (UK) Limited.

4.1.2. Acclimatisation

Duration	:	At least 7 days before commencement of treatment.
Husbandry conditions	:	Refer to Section 4.2.

4.1.3. Allocation to treatment groups

Allocation	:	On arrival.
Method	:	Random.
Cage distribution	:	To equalise environmental influences between groups and to minimise the likelihood of cross contamination between groups.

4.1.4. Identification

Numbering	:	Unique for each animal within study.
Method	:	Tail tattoo.
Cage labels	:	Uniquely identifying the occupants.

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4.1.5. Animal replacement

10 spare animals will be ordered to replace any individuals rejected during the acclimatisation period.

Replacement before treatment	:	Ill-health. Abnormalities. Bodyweight range extremes.
Replacement during treatment	:	None scheduled.

4.2. Animals – housing, diet and water supply

4.2.1. Environmental control

Rodent facility	:	Restricted entry – to minimise entry of external biological and chemical agents.
Air supply	:	Filtered, not recirculated.
Temperature	:	Maintained within the range of 19-23°C.
Relative humidity	:	Maintained within the range of 40-70%.

Monitored continuously or daily. Excursions outside these ranges documented in the study data.

Lighting	:	12 hours light : 12 hours dark.
Alarm systems	:	Activated on ventilation failure and when temperature/humidity limits exceeded.
Electricity supply	:	Public supply with automatic stand-by generators.

4.2.2. Animal accommodation

Animals per cage	:	Five of the same sex, unless reduced by mortality or isolation.
Cage material	:	Polycarbonate or stainless steel.
Cage flooring	:	Stainless steel grid.

The cages will be suspended above absorbent paper. The latter will be changed at appropriate intervals each week; cages, cage-trays, food hoppers and water bottles will be changed at appropriate intervals. Precise details of caging will be included in the final report.

4.2.3. Diet and water supply

Copies of all certificates of analysis are stored in the archives.

Diet supply

Diet name	:	Rat and Mouse No. 1 Maintenance Diet. Fish meal is not present in this diet.
Diet type	:	Pelleted diet.
Availability	:	Non-restricted.
Certification	:	Before delivery each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

This diet contains no added antibiotic or other chemotherapeutic or prophylactic agent.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene or polycarbonate bottles with sipper tubes.
Certification	:	Certificates of analysis are routinely received from the supplier.

4.2.4. Contaminants assay

It is the Sponsor's responsibility to advise Huntingdon Life Sciences of any specific contaminants likely to prejudice the outcome of the study. Analyses for such contaminants may be performed if requested by the Sponsor. (PFOS, a metabolite of POSF may be present in fish meal used in some animal diets).

5. INHALATION PROCEDURES

5.1. Pre-study characterisation

Before commencement of treatment the system will be characterised at the target exposure vapour concentrations without animals in order to:

- demonstrate reproducibility of vapour concentration.
- demonstrate homogeneity of vapour concentration and distribution between levels in the chamber.

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5.2. Test atmosphere generation

Equipment	:	All glass vaporiser.
Method	:	The test substance (liquid) will be metered to the vaporiser (through which dried air is passed) from an infusion pump. The vapour/air mixture produced passes directly into the exposure chamber.
Concentrations	:	Different concentrations of the test substance will be produced using different liquid feed rates controlled by the infusion pump and using syringes of an appropriate volume.

5.3. Test atmosphere administration

Route	:	Inhalation –snout only exposure.
Exposure system	:	ADG exposure chamber, modular construction in aluminium alloy comprising a base unit, a variable number of sections each having 20 exposure ports, and a top section incorporating a central aerosol inlet with a tangential air inlet. A separate chamber will be used for each group. During exposure, the rats will be held in restraining tubes with their snouts protruding from the ends of the tubes into the exposure chambers. The restraining tubes will be attached to chamber level 2. Animal exposure ports not in use will be closed with blanking plugs. Each exposure system will be housed in a separate extract cabinet to avoid possible cross-contamination between groups.
Controls (Group 1)	:	Air only.
Sham dosing	:	The animals on study will be acclimated to the method of restraint, normally over a 5 day period immediately preceding the first test substance exposure.
Duration of daily exposure	:	6 hours, for 5 consecutive days.
Airflow	:	30 l/minute.

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5.4. Test atmosphere monitoring

Equipment	:	Fourier-transformed infrared spectroscopy (FTIR). Supplied by Sponsor.

5.4.1. Test atmosphere sampling

Sampling	:	Samples of the test atmosphere will be drawn from each test chamber through the FTIR instrument.

5.4.2. Test atmosphere analysis

Concentration	:	FTIR (at frequent intervals to demonstrate adequate control of temporal variation).
Chemical analysis: methodology	:	An FTIR analyser, with suitable methodology will be supplied by the Sponsor and this will be validated at Huntingdon Life Sciences. This methodology will be followed in principle, although specific conditions may be modified at the discretion of the Head of Section, Aerosol Technology and Analysis.

6. SERIAL OBSERVATIONS

The precise times of all examinations will be included in the final report.

6.1. Clinical observations

Animals and their cages	:	Inspected at least twice daily for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	:	Nature and severity. Date and time of onset. Duration and progress of the observed condition.
Physical examination	:	Once each week for all animals.

In addition, detailed observations will be made daily, on days of exposure according to the following frequency:

1. Pre-exposure observation.
2. Observation severely restricted due to tube restraint.
3. As each animal is returned to its home cage.
4. As late as possible in the working day.

The above schedule will be amended, as necessary, in the light of signs observed.

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6.2. Mortality

Debilitated animals	:	Observed carefully, may be isolated to prevent cannibalism.
Premature sacrifice	:	Animals may be killed on humane grounds or if considered *in extremis*.
Animals found dead, killed *in extremis* or on humane grounds	:	A necropsy is performed as soon as possible. Animals found outside the normal workday will be preserved in a refrigerator (approximately 4°C) provided for this purpose.

6.3. Bodyweight

Bodyweight recording	:	Week –1. Day that treatment commences. Daily. At necropsy.

More frequent weighing may be performed to aid the monitoring of the condition of animals displaying ill-health. These data will be retained in the archives.

6.4. Food consumption

Food consumption recording	:	Week –1. Daily.
Food supplied	:	Daily.
Food spilled	:	Recorded at cage cleaning.
Food remaining	:	Recorded daily.

6.5. Water consumption

Water consumption recording	:	Week -1. Daily.
Water supplied	:	Daily.
Water remaining	:	Recorded daily.

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7. NECROPSY AND HISTOLOGY

7.1. Pre-terminal urine sampling

Individual urine samples will be collected within 2 hours following lights on in the animal holding room. The urine samples will be immediately assayed for pH using a microelectrode, centrifuged to obtain the sediment and the prepared SEM stubs despatched to the Principal Investigator at Roy Moate University of Plymouth for calculi and crystal analysis by SEM X-ray element identification.

If fresh void urine is not obtained from a proportion of the animals within the time allowed, then a sample will be removed from the urinary bladder at necropsy.

7.2. Test substance/metabolite analyses

Samples of blood (for serum) will be taken at necropsy by cardiac/aorta puncture, and run into tubes allowed, to clot at room temperature (up to 4 ml), and the serum separated and frozen prior to despatch to the Sponsor.

The frozen samples will be despatched to the Sponsor's Principal Investigator for analysis.

Principal Investigator	:	Lisa Clemen.
Address	:	3M Environmental Laboratory 935 Bush Avenue, Building 2-3E-09 St. Paul, Minnesota 55133-3331, USA
Tel No.	:	651-778-6018 (0) 651-778-6176 (F).

Any further processing and analysis of the samples and data collation will be the responsibility of the Sponsor. A copy of the Sponsor's report will be included as an addendum to the study report. All raw data relevant to the analyses of test substance and metabolites in plasma will be retained by the Sponsor.

7.3. Method of kill

Method	:	Intra-peritoneal sodium pentobarbitone. Followed by exsanguination.
Sequence	:	To allow satisfactory inter-group comparison.

7.4. Macroscopic Pathology

(Table 1)

Complete	:	All animals.
Checks	:	Retained tissues.
Photography	:	Unusual or suspected treatment-related findings; at the discretion of the necropsy supervisor or Study Director.

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7.5. Organ weights

(Table 1)

Data collection	:	For bilateral organs, left and right organs will be weighed together unless otherwise specified on the Pathology Procedures Table. Organ weights are not routinely recorded for animals killed or dying prematurely.
Data presentation	:	Absolute. Adjusted for terminal bodyweight.

7.6. Fixation

(Table 1)

Standard	:	Zinc Formalin.
Others	:	Eyes: In Davidson's fluid. Urinary bladder: Bouins. Inflate lungs with fixative and slightly distend the urinary bladder with fixative.
Fresh frozen liver	:	At terminal kill and after organ weighing, a sample of liver will be removed from all animals, immediately frozen by immersion in liquid nitrogen and stored at -70°C prior to despatch to the Sponsor.

7.7. Histology

(Table 1 and Section 8.1)

Processing	:	All animals killed or dying prematurely. All terminal animals. Urinary bladder: section sagittally, using one half for SEM X-ray microprobe analysis, and the other half for light microscopy and cell proliferation index. Kidney pelvis: sections to be present for light microscopy.
Routine staining	:	4-5 µm sections stained with haematoxylin and eosin.
Special staining	:	None.

7.8. PCNA staining for cell proliferation (Table 1)

Sections of urinary bladder will be taken from all animals for PCNA immunostaining in order to assess the degree of cell proliferation present. A total of 3,000 cells will be counted from 3 separate sections (1,000 cells/section) in order to determine the cell proliferation index. Positive PCNA staining cells will be counted, and the examining pathologist will assess the sections and count S-phase positive cells, if practicable. In addition sections of the duodenum from each animal will be taken and stained to act as positive controls for the immunostaining methodology.

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TABLE 1-Pathology procedures

	Necropsy		Histology	Pathology	PCNA
Tissue and regions to be examined	Weigh	Fix		Light microscopy	
Abnormalities		*			
Adrenals	*	*			
Aorta – thoracic		*			
Brain		*			
Caecum		*			
Colon		*			
Duodenum		*			*
Epididymides		*			
Eyes		*			
Femur (longitudinal section through joint)		*			
Harderian glands		*			
Head		a)			
Heart	*	*			
Ileum		*			
Jejunum		*			
Kidneys	*	*			
Lachrymal glands		*			
Larynx (2 levels)		*			
Liver	*	*			
Lungs (section from all lobes including bronchi)	*	*			
Lymph nodes – mandibular		*			
- mesenteric		*			
- tracheobronchial		*			
Mammary area – caudal		*			
Nasal turbinates (3 levels)		*			
Oesophagus		*			
Optic nerves		*			
Ovaries		*			
Pancreas		*			
Pituitary		*			
Prostate		*			
Rectum		*			
Salivary glands (submandibular/sublingual)		*			
Sciatic nerves		*			
Seminal vesicles		*			
Skeletal muscle – thigh		*			
Skin		*			
Spinal cord (transverse and longitudinal sections at the cervical, lumbar and thoracic levels)		*			
Spleen		*			
Sternum		*			
Stomach		*			
Testes		*			
Thymus		*			
Thyroid with parathyroids		*			
Tongue		*			
Trachea (2 levels)		*			
Urinary bladder (with kidney pelvis)		*	*	*	*
Uterus with cervix		*			
Vagina		*			

a) Including nasal cavity, paranasal sinuses and nasopharynx.
* Organs weighed, samples fixed or sections examined microscopically.

Final Protocol

8. PATHOLOGY

8.1. Light microscopy

Category	Animals	Tissues
Premature deaths	All from all groups.	All specified in Table 1.
Terminal sacrifice	All animals.	All specified in Table 1.

Peer Review : Carried out by a reviewing pathologist to internationally accepted standards.

8.2. Extension of initial examination

At the discretion of the pathologist, further processing and staining techniques may be used to evaluate individual lesions. Details of these techniques will be documented and retained in the archives.

Light microscopy may be extended, following consultation with the Sponsor, as follows:

Any such requirement will be documented in an amendment to the protocol.

SEM of urinary bladder epithelium for assessment of possible necrosis.

9. DATA TREATMENT

9.1. Statistical analysis

Data-types

The following data types will be analysed at each timepoint separately:-

bodyweight, food consumption and water consumption, over appropriate study periods.
organ weights, both absolute and adjusted for terminal bodyweight where appropriate.
pathological findings, for the number of animals with and without each finding.

Methods

For categorical data, the proportion of animals will be analysed using Fisher's Exact test for each treated group versus the control.

For continuous data, Bartlett's test will first be applied to test the homogeneity of variance between the groups. Using tests dependent on the outcome of Bartlett's test, treated groups will then be compared with the control group, incorporating adjustment for multiple comparisons where necessary.

Study Number : **MIN/312**

Huntingdon Life Sciences

10. REPORTING

Study progress	:	Periodic verbal and written updates on study progress will be provided by the Study Director. Routine synopsis reports will be sent after 1 week and at termination of the in-life phase.
Draft final report	:	For review by the Sponsor.
Authorised final report	:	After approval from the Sponsor.

Routinely reports are supplied on A4 paper. The following numbers of reports are supplied.

Type of report	Printing	Number of copies	
		Bound	Unbound
Draft report	Double-sided	0	2
Authorised final	Double-sided	1	0
	Single-sided	0	1
Photographic report (if any)	Single-sided	1	0

Any additions or corrections to an authorised final report will be documented as a formal addendum/amendment to the final report.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

11. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

11.1. Quality Assurance

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Authorised protocol and any amendments.
Study based inspections	:	Critical phases of this study will be inspected.
Report Audit	:	The draft report and study data will be audited after issue of the draft report to the Sponsor.
Test substance/metabolite assay	:	This phase of the study and associated reports will be conducted and produced according to local SOP's and subject to the Sponsor's own QA procedures.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

Final Protocol

11.2. Archiving

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

Study Number : MIN/312
Protocol Amendment Number : 1

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 4

Number of pages for internal distribution: 4

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 24 August 2001
(Study Director)

For the Sponsor

Approved by: _____ Date: 27 August 2001

Study Number : **MIN/312**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments : To include building number where study is to take place.
To update the timeplan.
To include hazard class.
To clarify necropsy table as to which tissues to process.

Amendments

Primary location of study

Huntingdon Research Centre
Huntingdon
Cambridgeshire
PE28 4HS

Building Number: Y14

2.2. Scheduled time plan

Animals to arrive	:	5 September 2001	
Treatment to commence	:	13 September 2001	
Terminal sacrifice to commence	:	18 September 2001	
Histopathology to be completed	:	~~October~~ November 2001	(estimated)
Draft report to be issued	:	November 2001	(estimated)

Study Number : **MIN/312**
Protocol Amendment Number : **1**

Huntingdon Life Sciences

3. TEST SUBSTANCE AND ATMOSPHERE GENERATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1999, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class: 2

Study Number : MIN/312
Protocol Amendment Number : 1

Huntingdon Life Sciences

TABLE 1-Pathology procedures

	Necropsy		Histology	Pathology	PCNA
Tissue and regions to be examined	**Weigh**	**Fix**		**Light microscopy**	
Abnormalities		*			
Adrenals	*	*			
Aorta – thoracic		*			
Brain		*			
Caecum		*			
Colon		*			
Duodenum		*			*
Epididymides		*			
Eyes		*			
Femur (longitudinal section through joint)		*			
Harderian glands		*			
Head		a)			
Heart	*	*			
Ileum		*			
Jejunum		*			
Kidneys (with pelvic region)	*	*	*	*	*
Lachrymal glands		*			
Larynx (2 levels)		*			
Liver	*	*			
Lungs (section from all lobes including bronchi)	*	*			
Lymph nodes – mandibular		*			
- mesenteric		*			
- tracheobronchial		*			
Mammary area – caudal		*			
Nasal turbinates (3 levels)		*			
Oesophagus		*			
Optic nerves		*			
Ovaries		*			
Pancreas		*			
Pituitary		*			
Prostate		*			
Rectum		*			
Salivary glands (submandibular/sublingual)		*			
Sciatic nerves		*			
Seminal vesicles		*			
Skeletal muscle – thigh		*			
Skin		*			
Spinal cord (transverse and longitudinal sections at the cervical, lumbar and thoracic levels)		*			
Spleen		*			
Sternum		*			
Stomach		*			
Testes		*			
Thymus		*			
Thyroid with parathyroids		*			
Tongue		*			
Trachea (2 levels)		*			
Urinary bladder		*	*	*	*
Uterus with cervix		*			
Vagina		*			

a) Including nasal cavity, paranasal sinuses and nasopharynx.
* Organs weighed, samples fixed or sections examined microscopically.

Study Number : MIN/312
Protocol Amendment Number : 2

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 4

Number of pages for internal distribution: 4

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 11 September 2001
(Study Director)

For the Sponsor

Approved by: _____ Date: 21 September 2001

Study Number : MIN/312
Protocol Amendment Number : 2

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments :
Clarification of name and address of Principal Investigator for SEM X-ray analyses
Change to fixative following discussion with Sponsor.
Addition/clarification of QA and Archiving procedures.

Amendments

7. NECROPSY AND HISTOLOGY

7.1. Pre-terminal urine sampling

Individual urine samples will be collected within 2 hours following lights on in the animal holding room. The urine samples will be immediately assayed for pH using a microelectrode, centrifuged to obtain the sediment and the prepared SEM stubs despatched to the Principal Investigator at Roy Moate, at University of Plymouth, Drake Circus, Plymouth, PL4 8AA, for calculi and crystal analysis by SEM X-ray element identification.

If fresh void urine is not obtained from a proportion of the animals within the time allowed, then a sample will be removed from the urinary bladder at necropsy.

A copy of the Principal Investigator's report will be included as an addendum to the study report. All raw data relevant to the analyses will be returned to Huntingdon Life Sciences for archiving.

Study Number : **MIN/312**
Protocol Amendment Number : 2

Huntingdon Life Sciences

7.6. Fixation

(Table 1)

Standard	:	~~Zinc Formalin.~~ 10% neutral buffered formalin
Others	:	Eyes: In Davidson's fluid. Urinary bladder: Bouins. Inflate lungs with fixative (10% NBF) and slightly distend the urinary bladder with fixative (Bouin's). Testes and epididymides: Initially in Bouin's fluid
Fresh frozen liver	:	At terminal kill and after organ weighing, a sample of liver will be removed from all animals, immediately frozen by immersion in liquid nitrogen and stored at -70°C prior to despatch to the Sponsor.

11. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

11.1. Quality Assurance

The following will be inspected or audited in relation to this study.

Protocol Audit	:	Authorised protocol and any amendments.
Study based inspections	:	Critical phases of this study will be inspected.
Report Audit	:	The draft report and study data will be audited after issue of the draft report to the Sponsor.
Test substance/metabolite assay	:	This phase of the study and associated reports will be conducted and produced according to local SOP's and subject to the Sponsor's own QA procedures.
SEM X-ray calculi and crystal analysis	:	The facility undertaking the analysis will be inspected by and the data and the Principal Investigator's report will be audited by Huntingdon Life Sciences Quality Assurance Department

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

Study Number : **MIN/312**
Protocol Amendment Number : 2

Huntingdon Life Sciences

11.2. Archiving

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Raw data, samples and specimens arising from the test substance/metabolite assay conducted by the Sponsor will be archived by the Sponsor.

Raw data, samples and specimens arising from the SEM X-ray calculi and crystal analysis will be archived by Huntingdon Life Sciences.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

SEP. 19. 2002 10:12AM MEDICAL 220 2E 02 NO. 8718 P. 1
-SEP. 02(THU) 11:58 INHALATION TOX FAX:01480892288 P. 001

Post-it® Fax Note 7671	Date 9/19/02 # of pages ▶ 7
To JOHN FLAHERTY	From JOHN BUTENHOFF
Co./Dept. EXYGEN	Co.
Phone #	Phone #
Fax #	Fax #

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Working for a better future

FAX HEADER SHEET

TOTAL NUMBER OF PAGES INCLUDING THIS PAGE: 7

If this transmission is unsatisfactory please contact us on Tel: +44 (0) 1480 892224

ATTENTION OF:	**Dr R Grazzini/Karen Risha**	FROM:	**Terry Kenny**
COMPANY:	**Exygen Research,**	DATE:	**189 September 2002**
FAX NUMBER:	**001 814 272 1039**	CC:	**J Butenhoff (Fax 001 651 733 1773)**
MESSAGE:			

Re: Perfluorooctanesulfonyl fluoride (POSF; T-7661.4); Toxicity study by inhalation administration to CD rats for 13 Weeks followed by a 4-Week recovery period (Study number MIN/313)

Dear Dr Grazzini,

Please find attached a copy of protocol amendment 3 for the above study together with a copy of protocol amendment 3 for the preliminary study MIN/312.

Best regards

Terry Kenny

Direct dial: +44 1480 892070
Local facsimile machine: +44 1480 893258

Woolley Road, Alconbury, Huntingdon, Cambridgeshire PE28 4HS, England. Tel: +44 (0) 1480 892000 Fax: +44 (0) 1480 890693
Huntingdon Life Sciences Limited. Registered in England No. [illegible]

Study Number : MIN/312
Protocol Amendment Number : 3

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 3

Number of pages for internal distribution: 3

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 18 September 2002
(Study Director)

For the Sponsor

Approved by: _____ Date: _____

Study Number : MIN/312
Protocol Amendment Number : 3

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments
The Sponsor has changed the Principal Investigator

Amendments

Study Number : MIN/312
Protocol Amendment Number : 3

Huntingdon Life Sciences

7.2. Test substance/metabolite analyses

Samples of blood (for serum) will be taken at necropsy by cardiac/aorta puncture, and run into tubes allowed, to clot at room temperature (up to 4 ml), and the serum separated and frozen prior to despatch to the Sponsor.

The frozen samples will be despatched to the Sponsor's Principal Investigator for analysis.

Principal Investigator	:	Lisa Clemen.
Address	:	3M Environmental Laboratory 935 Bush Avenue, Building 2-3E-09 St. Paul, Minnesota 55133-3331, USA
Tel No.	:	651-778-6018 (0) 651-778-6176 (F).

Subsequent to this amendment the duties of Principal Investigator will be transferred to:

Principal Investigator	:	Dr Richard Grazzini
Address	:	Exygen Research 3058 Research Drive State College PA 16801 USA
Tel No.	:	001 814 231 8032
Fax No.	:	001 814 231 1580

Any further processing and analysis of the samples and data collation will be the responsibility of the Sponsor. A copy of the Sponsor's report will be included as an addendum to the study report. All raw data relevant to the analyses of test substance and metabolites in plasma will be retained by the Sponsor.

Page 3

Study Number : **MIN/312**
Protocol Amendment Number : 4

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Total number of pages: 3

Number of pages for internal distribution: 3

Study Director : T.J. Kenny, B.Sc (Hons.).

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 5 September 2003
(Study Director)

For the Sponsor

Approved by: John J. Butenhoff Date: 11 September 2003

Page 1

Study Number : MIN/312
Protocol Amendment Number : 4

Huntingdon Life Sciences

PERFLUOROOCTANESULFONYL FLUORIDE (POSF; T-7661.1)

PRELIMINARY TOXICITY STUDY BY

INHALATION ADMINISTRATION TO

CD RATS FOR 1 WEEK

Reasons for amendments :
Assignment of liver samples for test substance/metabolite analyses
Addition of analytical method details.

Amendments

7.2. Test substance/metabolite analyses

Samples of blood (for serum) will be taken at necropsy by cardiac/aorta puncture, and run into tubes allowed, to clot at room temperature (up to 4 ml), and the serum separated and

Samples of liver will be collected at necropsy in accordance Section 7.6 of the Protocol and frozen prior to despatch to the Sponsor.

The frozen samples will be despatched to the Sponsor's Principal Investigator for analysis.

Principal Investigator	:	Lisa Clemen.
Address	:	3M Environmental Laboratory 935 Bush Avenue, Building 2-3E-09 St. Paul, Minnesota 55133-3331, USA
Tel No.	:	651-778-6018 (O) 651-778-6176 (F).

Study Number : **MIN/312**
Protocol Amendment Number : 4

Huntingdon Life Sciences

Subsequent to ~~this~~ Protocol Amendment 3 the duties of Principal Investigator will be transferred to:

Principal Investigator	:	Dr Richard Grazzini
Address	:	Exygen Research 3058 Research Drive State College PA 16801 USA
Tel No.	:	001 814 231 8032
Fax No.	:	001 814 231 1580

All samples will be analysed for POSF, PFOS and PFOA.

Samples will be analysed for POSF according to Exygen method ExM-023-071A in its current revision.

Samples will be analysed for PFOS and PFOA according to Exygen method ExM-023-071 in its current revision.

Any further processing and analysis of the samples and data collation will be the responsibility of the Sponsor. A copy of the Sponsor's report will be included as an addendum to the study report. All raw data relevant to the analyses of test substance and metabolites in plasma will be retained by the Sponsor.

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Page 1 of 1

PROTOCOL DEVIATION

Deviation Number: 1
Date of Occurrence: 09/27-28/02

Exygen Study Number: 023-080 Protocol Number: MIN/312

DESCRIPTION OF DEVIATION

Amendment #4 ExM-023-071 Revision 1 Section 4.5.3.e - accepted recoveries of 134%, 135%, 140% and 135% for fortifications for PFOA in Set 092602A.

ACTIONS TAKEN
for example - deviation issued, SOP revision, etc.

Deviation issued.

Recorded By: [signature] Date: 7/24/03

IMPACT ON STUDY

No negative impact on study.

[signature] Principal Investigator Signature — Date: 24-FEB-04

[signature] Study Director Signature — Date: 2 Feb 2004

[signature] Management Signature — Date: 26-FEB-2004

(Sponsor) — Date

Exygen QAU Review [signature] 11/17/03

U:\Forms\PROTOCOL_DEVIATION.doc 9/25/2002/5

3058 Research Drive
State College, PA 16801, USA
T: 800.281.3219
F: 814.272.1019
exygen.com

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PROTOCOL DEVIATION

Deviation Number: 2
Date of Occurrence: 09/24/02, 09/30/02

Exygen Study Number: 023-080 Protocol Number: MIN/312

DESCRIPTION OF DEVIATION

Amendment #4 ExM-023-071A Revision 2 Section 4.4.2 - used 0.05 mL for serum sample Exygen ID 0202782 in Set 092402G8.

Amendment #4 ExM-023-071A Revision 2 Section 4.5.4.f - accepted recoveries of 51% and 49% for fortifications for POSF in Set 093002A.

ACTIONS TAKEN
for example - deviation issued, SOP revision, etc.

Deviation issued.

Recorded By: Julie R Decker Date: 11/14/03

IMPACT ON STUDY

No negative impact on study.

Principal Investigator Signature — Date: 24-Feb-04

Study Director Signature — Date: 26 Feb 2004

Management Signature — Date: 26-Feb-2004

(Sponsor) — Date:

Exygen QAU Review — 11/19/03

U:\Forms\PROTOCOL_DEVIATION.doc 9/25/2002/5

3058 Research Drive
State College, PA 16801, USA
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F: 814.272.1019
exygen.com

APPENDIX B

Analytical Method:

Method of Analysis for the Determination of Perfluorohexanesulfonate (PFHS), Perfluorooctanesulfonate (PFOS) and Pentadecafluorooctanoic Acid (PFOA) in Rat Liver, Serum and Urine (Exygen Method No. ExM-023-071)

TITLE

Method of Analysis for the Determination of Perfluorohexanesulfonate (PFHS), Perfluorooctanesulfonate (PFOS) and Pentadecafluorooctanoic Acid (PFOA) in Rat Liver, Serum and Urine Revision 1

AUTHORS

John Flaherty, Karen Risha, and Emily Decker

DATE ISSUED

November 20, 2002

SPONSOR

3M Medical Department Corporate Toxicology
3M Center, Building 220-2E-02
St. Paul, MN 55144-1000

PERFORMING LABORATORY

Exygen Research
3058 Research Drive
State College, PA 16801

METHOD NUMBER

ExM-023-071 Revision 1

TOTAL NUMBER OF PAGES

43

MANAGEMENT APPROVAL

John M Flaherty 11/30/02

John Flaherty Date
Laboratory Manager
Exygen Research

John L. Butenhoff 11/21/02

John L. Butenhoff Date
Sponsor Representative
3M Medical Department Corporate Toxicology

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1. SUMMARY

This report details a method of analysis for residues of Perfluorohexanesulfonate (PFHS), Perfluorooctanesulfonate (PFOS) and Pentadecafluorooctanoic Acid (PFOA) in Rat Liver, Serum and Urine.

Residues of PFHS, PFOS and PFOA are extracted from each matrix with acetonitrile. The acetonitrile extract is added to water and loaded onto a conditioned C18 solid phase extraction (SPE) cartridge. Analyte residues are eluted with 2 mL of methanol. Quantification of PFHS, PFOS and PFOA is accomplished by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis using multiple reaction monitoring (MRM).

The proposed limit of quantitation (LOQ; the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) for this method is 10 ng/g (parts-per-billion) each for PFHS, PFOS and PFOA.

The theoretical limit of detection (LOD) will be based on the signal to noise ratio and will be at least greater than 3 times the level of noise, based on the instrumentation system used. For all analytes, the lowest analytical standard corresponds to 0.1 ng/mL.

This method was developed using rat liver, serum and urine. Typical percent recoveries ± standard deviations (at 10 and 50 ng/g) are shown below:

Fortification Level (ng/g)	PFHS Recovery in Rat Liver	Fortification Level (ng/mL)	PFHS Recovery in Rat Serum
10	115% ± 9.9% (n=3)	10	108% ± 4.7% (n=3)
50	98% ± 3.5% (n=3)	50	111% ± 9.6% (n=3)

Fortification Level (ng/g)	PFOS Recovery in Rat Liver	Fortification Level (ng/mL)	PFOS Recovery in Rat Serum	PFOS Recovery in Rat Urine
10	96% ± 8.5% (n=3)	10	88% ± 9.8% (n=3)	93% ± 4.7% (n=3)
50	88% ± 1.5% (n=3)	50	120% ± 2.1% (n=3)	79% ± 1.2% (n=3)

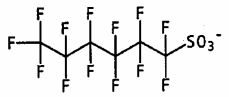
Fortification Level (ng/g)	PFOA Recovery in Rat Liver	Fortification Level (ng/mL)	PFOA Recovery in Rat Serum	PFOA Recovery in Rat Urine
10	98% ± 3.1% (n=3)	10	117% ± 1.5% (n=3)	89% ± 2.5% (n=3)
50	94% ± 2.5% (n=3)	50	111% ± 4.0% (n=3)	87% ± 2.1% (n=3)

Representative calibration curves are shown in **Figures 1-3**. Representative chromatograms are shown in **Figures 4 to 39**.

2. EXPERIMENTAL COMPOUNDS

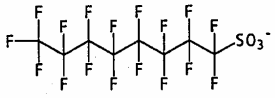
The structures for PFHS, PFOS and PFOA are given below.

PFHS
Chemical Name = Perfluorohexanesulfonate
Molecular weight = 399, as shown



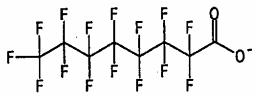
PFHS is supplied as the potassium salt ($C_6F_{13}SO_3^-K^+$), molecular weight = 438

PFOS
Chemical Name = Perfluorooctanesulfonate
Molecular weight = 499, as shown



PFOS is supplied as the potassium salt ($C_8F_{17}SO_3^-K^+$) molecular weight = 538

PFOA
Chemical Name = Pentadecafluorooctanoic Acid
Molecular weight = 413, as shown



3. CHEMICALS AND SUPPLIES

3.1. CHEMICALS

Chemical	Grade	Source	Catalog No.
Methanol (MeOH)	HPLC	EM Science	MX0475-1
Ammonium Acetate	Reagent	JT Baker	0596-01
Water	Type I	Exygen	NA
Acetonitrile	HPLC	EM Science	AX0145-1

Type I water = electrical resistivity, minimum of 16.67 MΩ/cm at 25 °C, from a Labconco Waterpro™ workstation.

3.2. STANDARDS

Standard	TCR Number	Purity (%)	Source
Perfluorohexanesulfonate (PFHS)	SE-036	99.99 all isomers, 84.36 straight chain	3M
Perfluorooctanesulfonate (PFOS)	SD-018	86.9	3M
Pentadecafluorooctanoic Acid (PFOA)	Lot No: 08316DO	96	Aldrich Chem

3.3. EQUIPMENT AND SUPPLIES

Equipment	Supplier
Balance, analytical (display at least 0.0001 g)	Mettler
125-mL LDPE narrow mouth bottles	Nalgene
Disposable glass micropipets (50-100 & 100-200 µL)	Drummond (VWR)
Tissumizer	Tekmar
Wrist action shaker	Burrell Scientific
Sorvall RC 5C plus Centrifuge	Dupont
50 mL disposable polypropylene centrifuge tubes	VWR
15 mL disposable polypropylene centrifuge tubes	VWR
Visiprep vacuum manifold	Supelco
Sep Pak Vac 6 cc (1g) tC18 cartridges (part # WAT 036795	Waters
2-mL clear HPLC vial Kit (cat # 5181-3400)	Hewlett-Packard
Class A pipets and volumetric flasks	various suppliers
Standard lab equipment (graduated cylinders, disposable tubes etc.)	various suppliers
Stand-alone drop-in guard cartridge holder (part #844017-400)	Keystone Scientific
Hypercarb drop-in guard column (4 mm) (part # 844017-400)	Keystone Scientific
HPLC Pump (LC-10AD)	Shimadzu
LC/MS/MS and HPLC systems	As described in section 4.5.

Notes:

1. In order to avoid contamination, the use of disposable labware (containers, tubes, pipettes, etc.) is highly recommended.
2. Teflon or Teflon-lined containers or equipment should not be used.
3. It may be necessary to check the solvents (acetonitrile, methanol) for the presence of contaminants (especially PFOA) by LC/MS/MS before use. Certain lot numbers have been found to be unsuitable for use.
4. Use disposable micropipettes or pipettes to aliquot standard solutions when preparing standards and samples for extraction.
5. Equivalent materials may be substituted for those specified in this method.

3.4. SOLUTIONS

(1) 2 mM ammonium acetate in water is prepared by weighing 0.154 g of ammonium acetate and dissolving in 1 L of water.

(2) Hypercarb filtered type I water is prepared by filtering type I water through a Hypercarb guard column using a HPLC pump at ~2-3 mL/min. Before use, wash the guard cartridge with ~25 mL of HPLC grade acetonitrile, then ~ 25 mL of type I water, then begin collecting the filtered type I water eluate for use in the extraction. Repeat the wash after filtering ~2L of water.

Note: The aforementioned example is provided for guidance, alternative volumes may be prepared as long as the appropriate ratios of the solvent to solute are maintained.

3.5. PREPARATION OF STANDARD SOLUTIONS

Analytical standards are used for three purposes:

1. Calibration Standards – These standards are prepared in methanol and are used to calibrate the response of the detector used in the analysis.
2. Laboratory Control Spikes – These fortifications are prepared at concentrations corresponding to the LOQ and 5x LOQ and are used to determine analytical recovery. Laboratory control spikes are prepared in control matrix.
3. Matrix Spikes – These fortifications are prepared by spiking into the field samples at a known concentration. Matrix spikes are used to evaluate the effect of the sample matrix on analytical recovery and are prepared at the client's request.

The analyst may vary the absolute volumes of the standards as long as the correct proportions of solute to solvent are maintained.

3.5.1. Stock solution

Prepare individual stock solutions at 100 µg/mL for PFHS, PFOS and PFOA by weighing out 10 mg of each analytical standard (corrected for purity and if necessary, salt content) and dilute to 100 mL with methanol in separate 100-mL volumetric flasks. The stock solutions (in 125-mL LDPE bottles) are to be stored in a refrigerator at 2°C to 6°C and are stable for a maximum period of one year from the date of preparation.

3.5.2. Fortification Solutions

a. Prepare a mixed fortification standard at 1.0 µg/mL (1000 ng/mL) of PFHS, PFOS and PFOA by adding 1.0 mL of each of the 100 µg/mL stock solutions into a 100-mL volumetric flask and bring up to volume with methanol.
b. Prepare a mixed fortification standard at 0.1 µg/mL (100 ng/mL) of PFHS, PFOS and PFOA by diluting 10.0 mL of the 1.0 µg/mL mixed fortification solution to 100 mL with methanol in a volumetric flask.

Example: one hundred microliters of the 0.1 µg/mL solution spiked into 1 g of liver or 1 mL of serum/urine is equivalent to a 10 ppb (10 ng/mL or ng/g) fortification.

Store all fortification standard solutions in a refrigerator (in 125-mL LDPE bottles) at 2°C to 6°C for a maximum period of one year from the date of preparation. Note also that additional concentrations may be prepared if necessary.

3.5.3. Calibration Standards

LC/MS/MS calibration standards containing PFHS, PFOS and PFOA are prepared at 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 ng/mL in methanol via dilution of the 0.1 µg/mL mixed fortification solution (section 3.5.2.b).

The following is a typical example; additional concentrations may be prepared as needed.

Initial Conc. (ng/mL)	Volume (mL)	Diluted to (mL)	Final Conc. (ng/mL)
100	5.0	100	5.0
100	2.0	100	2.0
100	1.0	100	1.0
5.0	10.0	100	0.5
2.0	10.0	100	0.2
1.0	10.0	100	0.1

The standards may be used for a period of one year (in 125-mL LDPE bottles) when stored refrigerated (at 2°C to 6°C).

4. METHOD

4.1. Flow Diagram

The flow diagram of the method is given below, followed by a detailed description of each step.

Method Flow Diagram

Weigh appropriate amount of liver or measure appropriate volume of serum or urine (fortify samples designated as matrix spikes and laboratory control spikes)
↓
Add water to liver for a final volume of 10 mL, add water to serum and urine for a final volume of 20 mL, homogenize
↓
Remove 1 mL and add 5 mL of ACN, shake
↓
Centrifuge
↓
Decant supernatant into 35 mL of water
↓
Load onto conditioned SPE
↓
Elute with 2 mL methanol
↓
LC/MS/MS Analysis

4.2. Sample Processing

For liver samples, place frozen samples in a food processor and homogenize with dry ice. Then place samples in containers and leave open in frozen storage overnight to allow for CO_2 sublimation. Seal and place the samples in frozen storage below −10°C until time of extraction. Alternately, if there is an insufficient amount of sample (~ less than 5 g), then no processing is necessary and the sample can be used as supplied. No sample processing is needed for serum and urine samples. However, frozen serum and urine samples must be allowed to completely thaw to room temperature before use.

4.3. Batch Set up

a. A batch of samples should not contain more than 20 field samples.

b. Each batch of samples analyzed must include at least one control (method blank using control matrix) and two matrix controls fortified at known concentrations (typically 10 and 50 ng/g for liver or ng/mL for serum and urine) to verify procedural recovery for the batch.

c. At least one field sample in each batch must also be separately fortified at a known concentration and carried through the procedure to verify recovery. Additional samples in the batch may also be fortified if desired.

d. All samples require duplicate injections.

4.4. Sample Extraction

4.4.1. Liver Extraction

a. Weigh 1 g of liver sample into a 50 mL disposable centrifuge tube and fortify, if appropriate. Note that alternate weights of liver may be measured depending on the sample size available for use.
b. Add water to the sample for a final volume of 10 mL. Cap tightly.
c. Homogenize sample using a tissuemizer for ~1 minute.
d. Transfer 1 mL of the sample using a disposable pipette into 15 mL disposable centrifuge tubes. Add 5 mL of ACN and shake for ~20 minutes on a wrist action shaker.
e. Centrifuge tubes at ~3000 rpm for ~ 5 minutes. Carefully decant supernatant into a 50 mL disposable centrifuge tube and add 35 mL of water.
f. Load the sample onto a conditioned SPE column (for conditioning details, see section 4.4.3.). Discard the eluate. Any analyte residues will be trapped on the SPE column at this point.
g. Elute with 2 mL of methanol. Collect 2 mL of elute into a graduated 15 mL centrifuge tube.
h. Analyze samples using electrospray LC/MS/MS.

4.4.2. Serum and Urine Extraction

a. Measure 1 mL of serum or urine sample into a 50 mL disposable centrifuge tube and fortify, if appropriate. Note that alternate volumes of serum and urine may be measured depending on sample size available for use.

b. Add water to sample for a final volume of 20 mL. Cap tightly and vortex for ~1 minute. Then continue with steps d-h in section 4.4.1.

4.4.3. SPE Column Conditioning

Place the unconditioned SPE columns on the vacuum manifold. Condition the SPE columns by passing ~ 10 mL of methanol through the column followed by ~ 5 mL of water. The washes may be pulled through the SPE column using vacuum at a flow rate of ~1 drop/sec or may be allowed to pass through the column unaided. Discard all washes. Do not allow the column to dry.

4.5. QUANTITATION

4.5.1. LC/MS/MS System and Operating Conditions

Mass Spec: Micromass Quattro Ultima (Micromass)
Interface: Electrospray (Micromass)
Harvard infusion pump (Harvard Instruments), for tuning
Computer: COMPAQ Professional Workstation AP200
Software: Windows NT, Masslynx 3.3

HPLC: Hewlett Packard (HP) Series 1100
HP Quat Pump
HP Vacuum Degasser
HP Autosampler
HP Column Oven

Note: A 4 x 10 mm hypercarb drop in guard cartridge is attached on-line after the purge valve and before the sample injector port to trap any residue contaminants that may be in the mobile phase and/or HPLC system.

HPLC Column: Genesis C_8 (Jones Chromatography), 2.1 mm x 50 mm, 4µ
Column Temperature: 35° C
Injection Volume: 15 µL
Mobile Phase (A): 2 mM Ammonium Acetate in Type I water
Mobile Phase (B): Methanol

Time	% A	% B	Flow Rate (mL/min)
0.0	90	10	0.3
2.0	90	10	0.3
5.0	10	90	0.3
9.0	10	90	0.3
9.5	0	100	0.3
14.0	0	100	0.3
14.5	90	10	0.3
20.0	90	10	0.3

It may be necessary to adjust the HPLC gradient in order to optimize instrument performance. Columns with different dimensions (e.g. 2.1 x 30) and also columns from different manufacturers (Keystone Betasil C_{18} etc.) could be used, provided equivalent chromatography is obtained.

Ions monitored:

Analyte	Mode	Transition Monitored	Approximate Retention Time
PFHS	Negative	399 → 80	~8.2 min.
PFOS	Negative	499 → 99	~8.8 min
PFOA	Negative	413 → 369	~8.6 min

The retention times may vary, on a day to day basis, depending on the batch of mobile phase etc. Drift in retention times (up to ± 4 %) is acceptable within an analytical run, as long as the drift continues through the entire analysis and the standards are included at the beginning and end of the analytical run.

Note: An alternative LC/MS/MS system may be used once demonstrated to be equivalent.

The mass spectrometer is tuned for each analyte by infusing a ~ 1.0 µg/mL standard solution (at 10 µL/min, using an infusion pump) via a "T" into a stream of mobile phase containing 50% methanol and 50% 2mM ammonium acetate in water at 0.2 mL/min flow rate. Each analyte is initially tuned for the parent ion and then tuned for the product ion. Once the instrument is tuned, the optimized parameters are saved as a tune file. This tune file is then used during routine analysis.

4.5.2. Calibration Curve Procedures

a. Inject the same aliquot (between 10 to 50 µL) of each calibration standard (ranging from the lowest level standard to the highest level prepared), into the LC/MS/MS.

b. Use weighted linear standard curves for quantitation. Linear standard curves are generated for each analyte by linear regression using 1/x weighting of peak area versus calibration standard concentration using Masslynx (or equivalent) software system. Any calibration standard found to be a statistical outlier by using an appropriate outlier test, may be excluded from the calculation of the calibration curve. However, the total number of calibration standards that may be excluded must not exceed 20% of the total number of standards injected.

c. The correlation coefficient (R) for calibration curves generated must be ≥0.9925 (R^2 ≥0.985). If calibration results fall outside these limits, then appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be reanalyzed.

Typical calibration curves for PFHS, PFOS and PFOA can be found in Figures 1-3.

4.5.3 Sample Analysis

a. Inject the same aliquot (between 10 to 50 µL) of each standard, sample, recovery, control, etc. into the LC/MS/MS system.

b. Standards corresponding to at least five or more concentration levels (starting with the LOQ level or below) must be included in an analytical set.

c. An entire set of calibration standards should be injected at the beginning of a set followed by calibration standards interspersed approximately every 5-10 samples (to account for a second set of extracted standards). As an alternative, an entire set of calibration standards may be included at the beginning and at the end of a sample set. In either case, calibration standards must be the first and last injection in a sample set.

d. The concentration of each sample/fortification/control is determined from the standard curve, based on the peak area of each analyte. The standard responses should bracket responses of the residue found in each sample set. Results may be quantitated up to 10% outside the curve by extrapolation. If necessary, dilute the samples to give a response within the standard curve range.

e. Fortification recoveries falling within 70 to 130% are considered acceptable.

f. Samples must be stored refrigerated between 2°C to 6°C until analysis.

g. Samples in which either no peaks are detected or peaks less than the lowest concentration of the calibration standards are detected at the corresponding analyte retention time will be reported as ND (not detected). Samples in which peaks are detected at the corresponding analyte retention time that are less than the LOQ and greater than or equal to the lowest concentration of the calibration standards will be reported as NQ (not quantifiable).

The analysis performed during the method development included fortifications at 10 and 50 ng/g of PFHS in rat liver, 10 and 50 ng/mL of PFHS in serum, 10 and 50 ng/g of PFOS and PFOA in rat liver and 10 and 50 ng/mL of PFOS and PFOA in serum and urine. Typical chromatograms can be found in Figures 4-39.

4.6. Acceptance Criteria

The following criteria must be met to ensure the presence of PFHS, PFOS and PFOA:

1. The chromatogram must show a peak of a daughter ion at 80 amu from a parent of 399 amu for PFHS, a daughter ion at 99 amu from a parent of 499 amu for PFOS, and a daughter ion at 369 amu from a parent of 413 amu for PFOA.

2. Method blanks must not contain analyte at levels greater than the LOQ. If a blank contains the analyte at levels greater than 10 ng/mL, then a new blank sample must be obtained and the entire set must be re-extracted.

3. Recoveries of control spikes and matrix spikes (if any) must be between 70-130% of their known values. If a control spike falls outside the acceptable limits, the entire set of samples should be re-extracted. Any matrix spike outside 70-130% should be evaluated by the analyst to determine if re-extraction is warranted.

4. Any calibration standard found to be a statistical outlier by using the Huge Error Test, may be excluded from the calculation of the calibration curve. However, the total number of calibration standards that could be excluded must not exceed 20% of the total number of standards injected.

5. The correlation coefficient (R) for calibration curves generated must be ≥ 0.9925 ($R^2 \geq 0.985$). If calibration results fall outside these limits, then appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be reanalyzed.

6. Retention times between standards and samples must not drift more than ± 4 % within an analytical run. If retention time drift exceeds this limit within an analytical run then the set must be reanalyzed.

4.7. Performance Criteria

The following two criteria must be performed once as a system suitability test, before the commencement of analysis, when using an instrumentation set-up that has not been used for this method.

First Criterion:
Run a standard solution on LC/MS/MS corresponding to the estimated LOQ (10 ng/mL) in matrix and obtain a signal to noise ratio for the analyte transition of at least 9:1, compared to a reagent blank. If this criterion cannot be met, optimize and change instrument operating parameters (or increase the injection volume, if appropriate).

Second Criterion:
Run a set of standards of five or more concentration levels, from at or below the LOQ, up to the highest concentration level to be included in the analysis. Generate a calibration curve for the analyte and obtain a linear regression with a coefficient of determination (R^2) of at least 0.985 for the analyte. Once this criterion is met, samples may be analyzed with standards interspersed.

4.8. TIME REQUIRED FOR ANALYSIS

One person can take a set of 20 samples through the sample preparation procedure in approximately 4 hours. The LC/MS/MS analysis of the set (containing 20 field samples, 1 matrix blank, 2 laboratory control spikes, 1 matrix spike and 12 standard injections) will take approximately 14 hours.

5. CALCULATIONS

a. Use Equation 1 to calculate the amount of analyte found (in ng/mL, based on peak area) using the standard curve (1/x weighted linear regression parameters) generated by the Masslynx software program.

Equation 1:

$$\text{Analyte found (ng/mL)} = \frac{(\text{peak area} - \text{intercept})}{\text{slope}} \times \text{DF} \times \text{aliquot factor}$$

DF = factor by which the final volume was diluted, if necessary.
Aliquot factor= 10 for liver, 20 for serum and urine

b. For samples fortified with known amounts of analyte prior to extraction, use Equation 2 to calculate the percent recovery.

Equation 2:

$$\text{Recovery (\%)} = \frac{[\text{total analyte found (ng/mL)} - \text{analyte found in control (ng/mL)}]}{\text{analyte added (ng/mL)}} \times 100$$

Note: Subtract analyte found in control (ng/mL) from analyte found (ng/mL), if ng/mL in control is greater than LOQ.

c. Use Equation 3 to calculate the amount of analyte found (in ppb)

Equation 3:

$$\text{Analyte found (ng/g or ng/mL)} = \frac{\text{(analyte found (ng/mL) x FV (mL)}}{\text{sample weight (g) or sample volume (mL)}}$$

FV = final volume

For reporting purposes, samples in which either no peaks are detected or peaks less than the lowest concentration of the calibration standards are detected at the corresponding analyte retention time will be reported as ND (not detected). Samples in which peaks are detected at the corresponding analyte retention time that are less than the LOQ and greater than or equal to the lowest concentration of the calibration standards will be reported as NQ (not quantifiable).

6. SAFETY

The analyst should read the material safety data sheets for all standards and reagents before performing this method. Use universal precautions when handling standards and reagents, including working in fume hoods and wearing laboratory coats, safety glasses, and gloves.

Table 1. Recovery Summary of PFHS in Rat Liver and Serum

Recovery Summary of PFHS in Rat Liver

Sample ID	Analyte Added (ng/g)	Percent Recovery (%)
0201684 Spk A	10	108
0201684 Spk B	10	110
0201684 Spk C	10	126
	Average:	**115**
	Standard Deviation:	**9.9**

Sample ID	Analyte Added (ng/g)	Percent Recovery (%)
0201684 Spk D	50	101
0201684 Spk E	50	98
0201684 Spk F	50	94
	Average:	**98**
	Standard Deviation:	**3.5**

Recovery Summary of PFHS in Rat Serum

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk A	10	112
0201682 Spk B	10	103
0201682 Spk C	10	110
	Average:	**108**
	Standard Deviation:	**4.7**

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk D	50	107
0201682 Spk E	50	104
0201682 Spk F	50	122
	Average:	**111**
	Standard Deviation:	**9.6**

Table 2. Recovery Summary of PFOS in Rat Liver, Serum and Urine

Recovery Summary of PFOS in Rat Liver

Sample ID	Analyte Added (ng/g)	Percent Recovery (%)
0201684 Spk A	10	88
0201684 Spk B	10	95
0201684 Spk C	10	105
	Average:	96
	Standard Deviation:	8.5

Sample ID	Analyte Added (ng/g)	Percent Recovery (%)
0201684 Spk D	50	90
0201684 Spk E	50	88
0201684 Spk F	50	87
	Average:	88
	Standard Deviation:	1.5

Recovery Summary of PFOS in Rat Serum

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk A	10	80
0201682 Spk B	10	85
0201682 Spk C	10	99
	Average:	88
	Standard Deviation:	9.8

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk D	50	118
0201682 Spk E	50	122
0201682 Spk F	50	121
	Average:	120
	Standard Deviation:	2.1

Recovery Summary of PFOS in Rat Urine

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk A	10	98
0201682 Spk B	10	89
0201682 Spk C	10	91
	Average:	93
	Standard Deviation:	4.7

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk D	50	80
0201682 Spk E	50	78
0201682 Spk F	50	78
	Average:	79
	Standard Deviation:	1.2

Table 3. Recovery Summary of PFOA in Rat Liver, Serum and Urine

Recovery Summary of PFOA in Rat Liver

Sample ID	Analyte Added (ng/g)	Percent Recovery (%)
0201684 Spk A	10	95
0201684 Spk B	10	101
0201684 Spk C	10	99
	Average:	98
	Standard Deviation:	3.1

Sample ID	Analyte Added (ng/g)	Percent Recovery (%)
0201684 Spk D	50	97
0201684 Spk E	50	92
0201684 Spk F	50	94
	Average:	94
	Standard Deviation:	2.5

Recovery Summary of PFOA in Rat Serum

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk A	10	118
0201682 Spk B	10	117
0201682 Spk C	10	115
	Average:	117
	Standard Deviation:	1.5

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk D	50	107
0201682 Spk E	50	112
0201682 Spk F	50	115
	Average:	111
	Standard Deviation:	4.0

Recovery Summary of PFOA in Rat Urine

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk A	10	86
0201682 Spk B	10	91
0201682 Spk C	10	89
	Average:	89
	Standard Deviation:	2.5

Sample ID	Analyte Added (ng/mL)	Percent Recovery (%)
0201682 Spk D	50	89
0201682 Spk E	50	88
0201682 Spk F	50	85
	Average:	87
	Standard Deviation:	2.1

Figure 1. Calibration Curve for PFHS

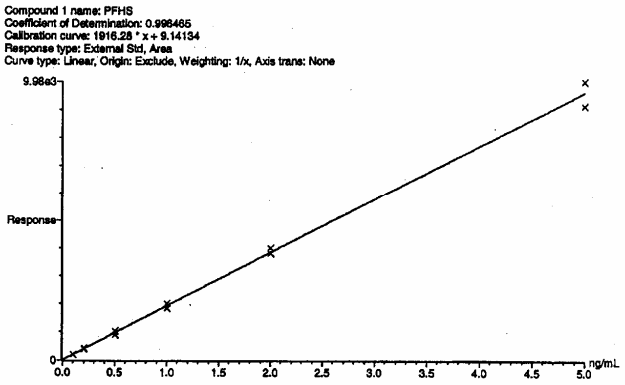


Figure 2. Calibration Curve for PFOS

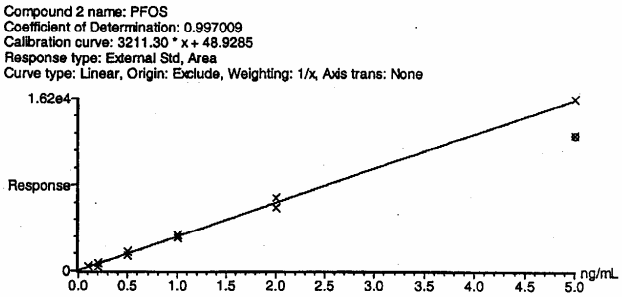


Figure 3. Calibration Curve for PFOA

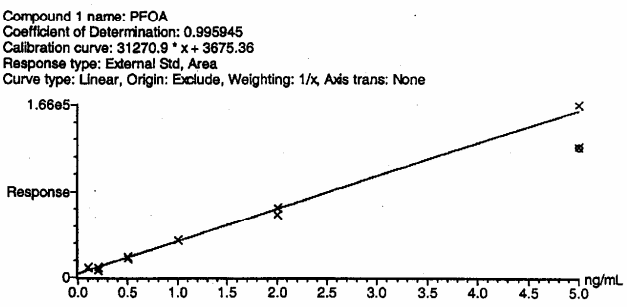


Figure 4. Representative Chromatogram of a 0.1 ng/mL Standard Containing PFHS

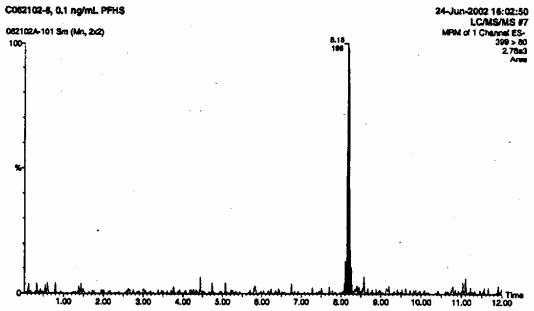


Figure 5. Representative Chromatogram of a 0.1 ng/mL Standard Containing PFOS

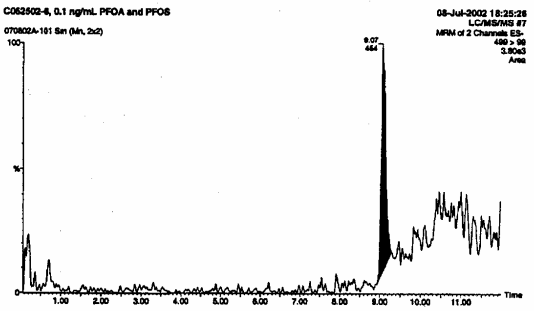


Figure 6. Representative Chromatogram of a 0.1 ng/mL Standard Containing PFOA

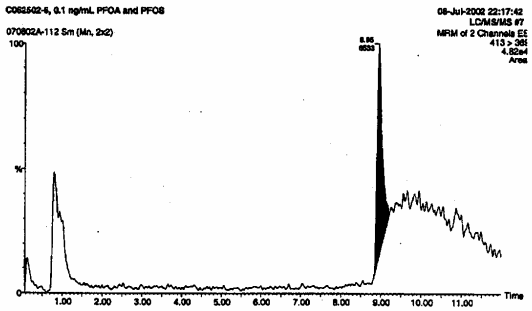


Figure 7. Representative Chromatogram of a 0.5 ng/mL Standard Containing PFHS

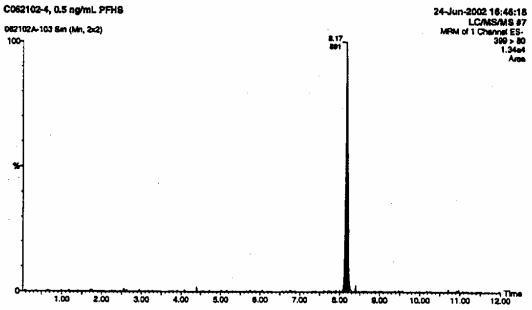


Figure 8. Representative Chromatogram of a 0.5 ng/mL Standard Containing PFOS

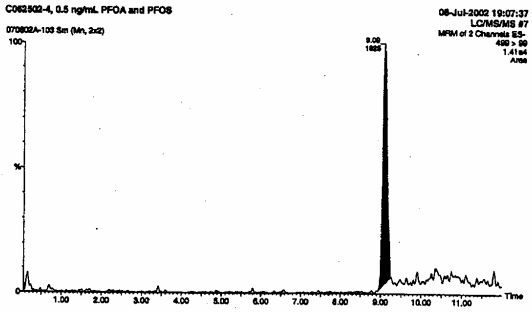


Figure 9. Representative Chromatogram of a 0.5 ng/mL Standard Containing PFOA

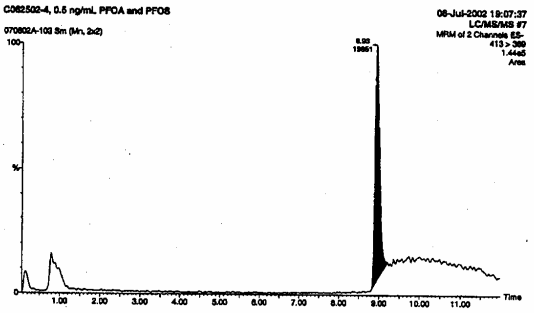


Figure 10. Representative Chromatogram of a 5.0 ng/mL Standard Containing PFHS

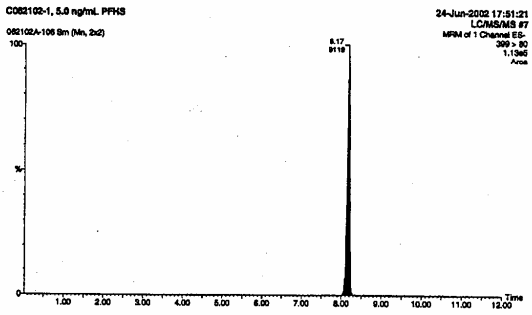


Figure 11. Representative Chromatogram of a 5.0 ng/mL Standard Containing PFOS

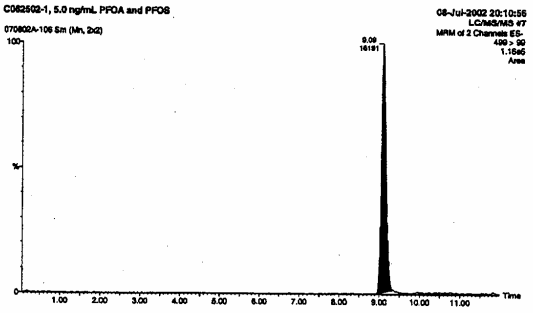


Figure 12. Representative Chromatogram of a 5.0 ng/mL Standard Containing PFOA

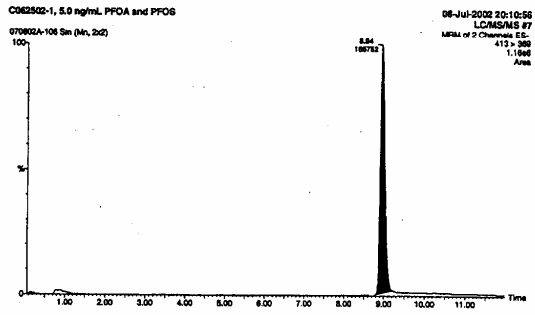


Figure 13. Representative Chromatogram of a Reagent Blank Sample Analyzed for PFHS

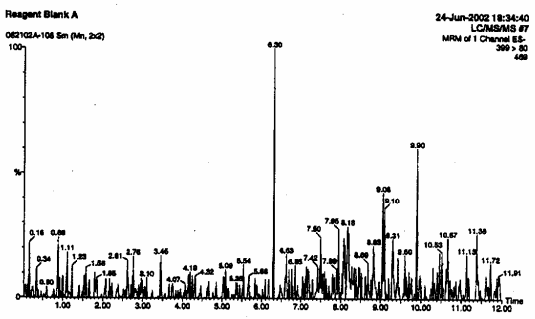
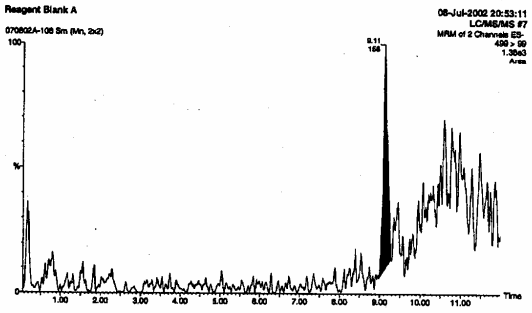


Figure 14. Representative Chromatogram of a Reagent Blank Sample Analyzed for PFOS



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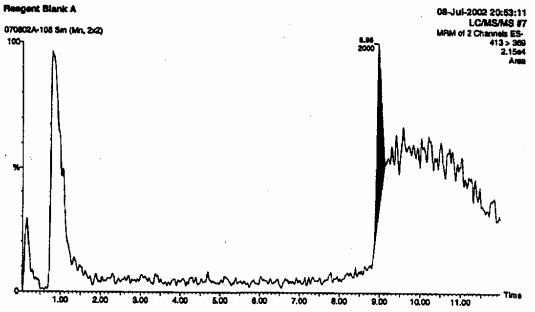

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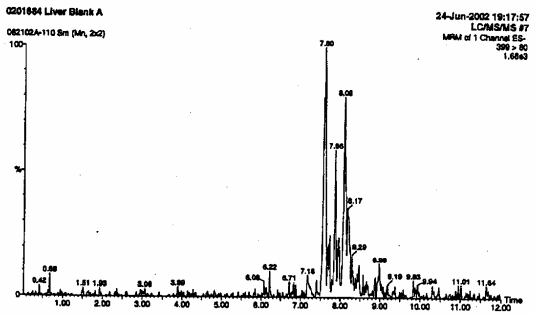


Figure 17. Representative Chromatogram of a Control Liver Sample Analyzed for PFOS

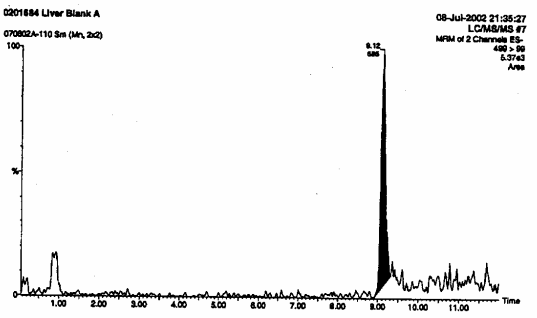
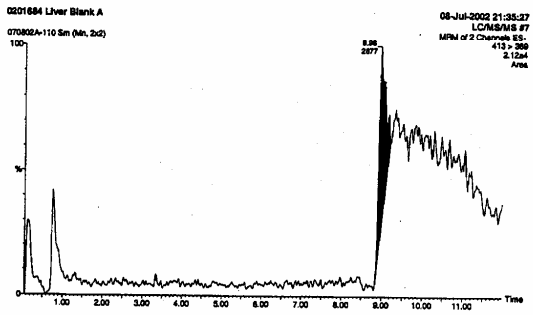


Figure 18. Representative Chromatogram of a Control Liver Sample Analyzed for PFOA



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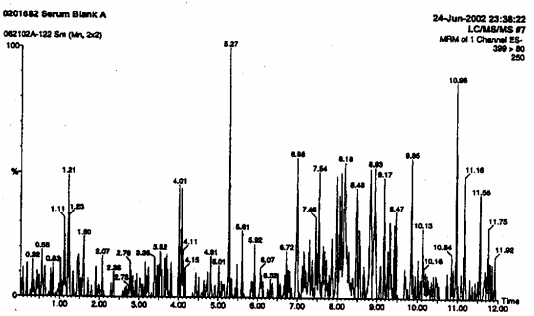


Figure 20. Representative Chromatogram of a Control Serum Sample Analyzed for PFOS

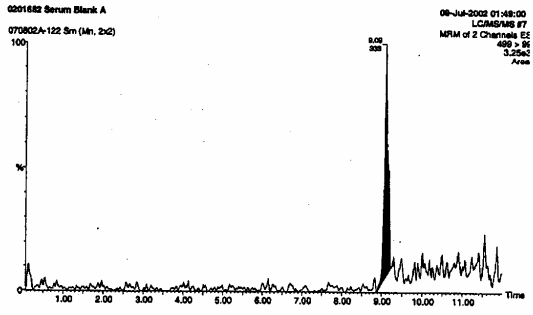
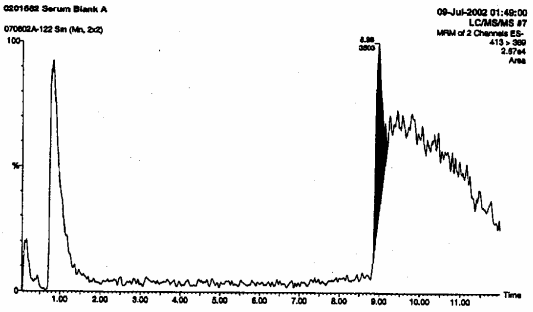


Figure 21. Representative Chromatogram of a Control Serum Sample Analyzed for PFOA



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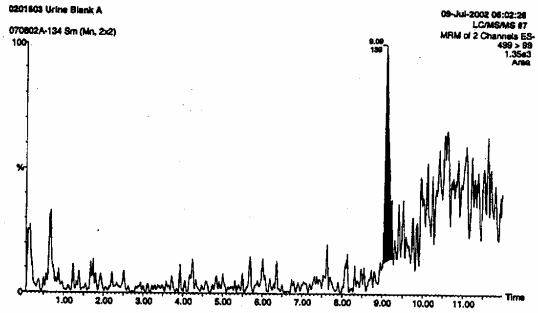


Figure 23. Representative Chromatogram of a Control Urine Sample Analyzed for PFOA

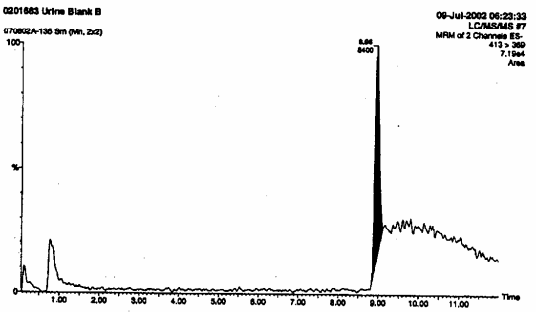


Figure 24. Representative Chromatogram of a Control Liver Sample Fortified at 10 ng/g with PFHS

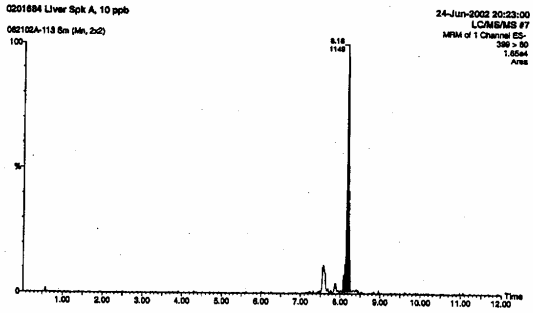


Figure 25. Representative Chromatogram of a Control Liver Sample Fortified at 10 ng/g with PFOS

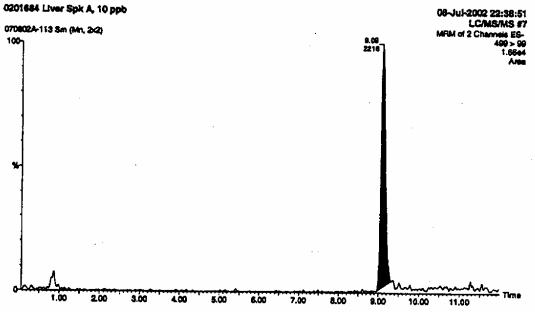


Figure 26. Representative Chromatogram of a Control Liver Sample Fortified at 10 ng/g with PFOA

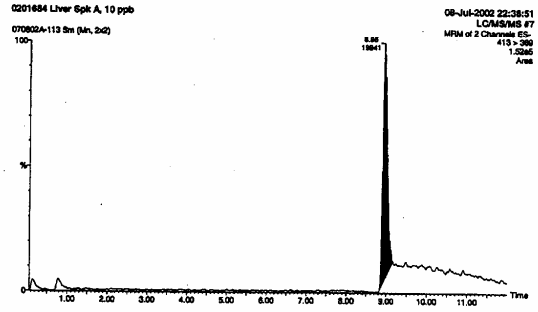


Figure 27. Representative Chromatogram of a Control Liver Sample Fortified at 50 ng/g with PFHS

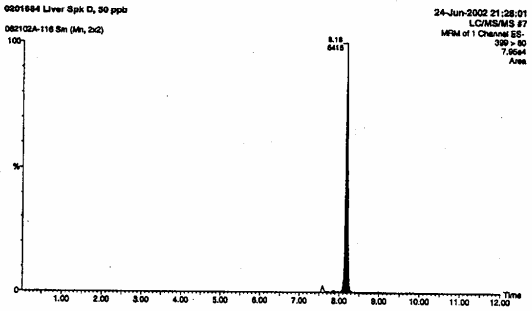


Figure 28. Representative Chromatogram of a Control Liver Sample Fortified at 50 ng/g with PFOS

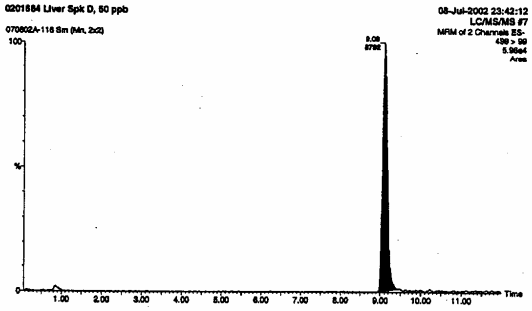


Figure 29. Representative Chromatogram of a Control Liver Sample Fortified at 50 ng/g with PFOA

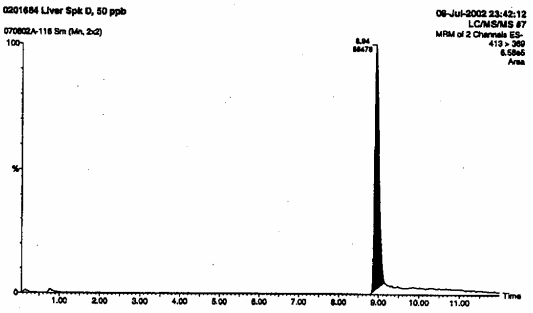


Figure 30. Representative Chromatogram of a Control Serum Sample Fortified at 10 ng/mL with PFHS

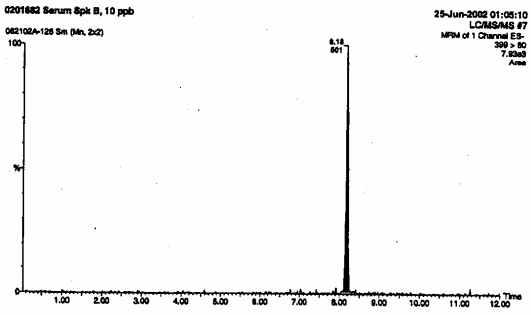
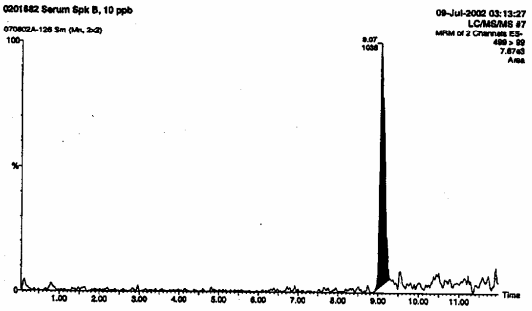


Figure 31. Representative Chromatogram of a Control Serum Sample Fortified at 10 ng/mL with PFOS



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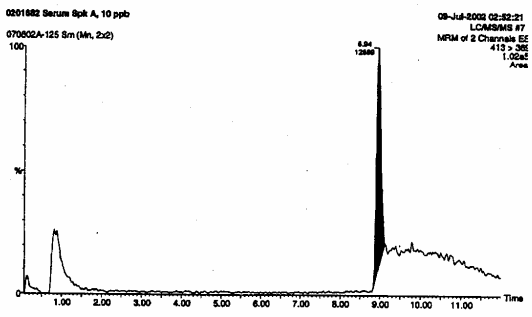


Figure 33. Representative Chromatogram of a Control Serum Sample Fortified at 50 ng/mL with PFHS

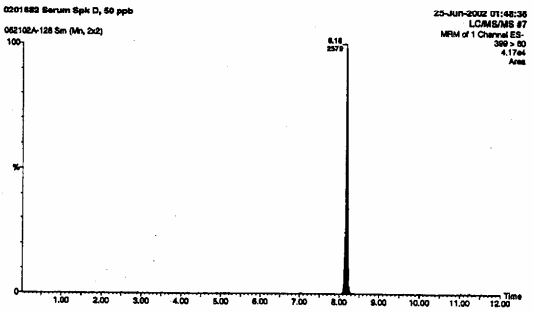
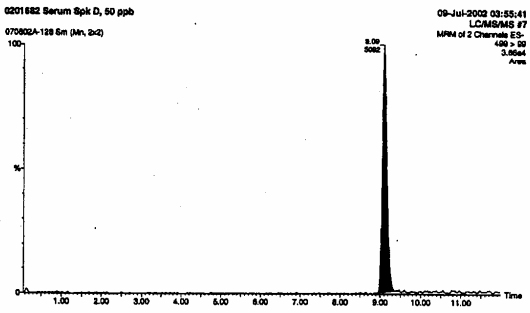


Figure 34. Representative Chromatogram of a Control Serum Sample Fortified at 50 ng/mL with PFOS



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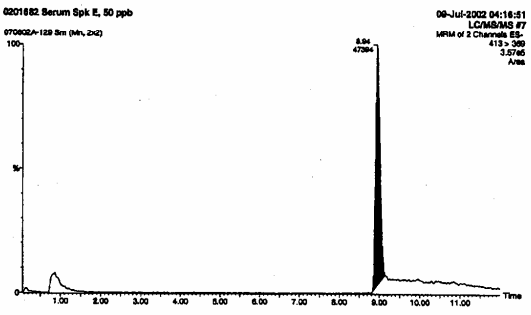


Figure 36. Representative Chromatogram of a Control Urine Sample Fortified at 10 ng/mL with PFOS

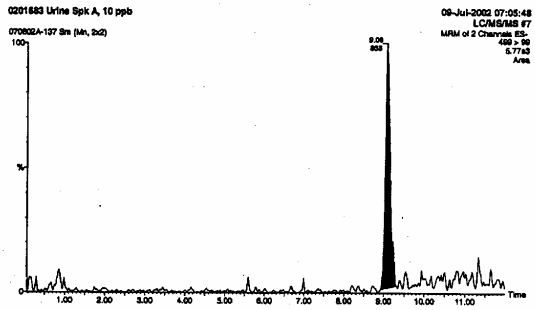
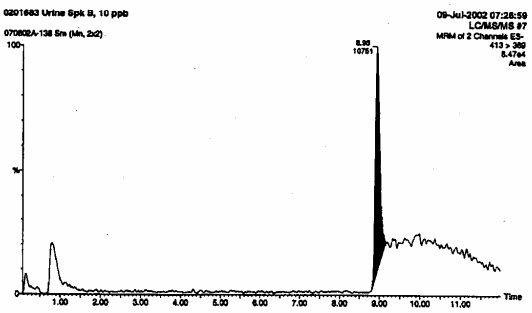


Figure 37. Representative Chromatogram of a Control Urine Sample Fortified at 10 ng/mL with PFOA



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Analytical Method:

Method of Analysis for the Determination of Perfluorooctanesulfonyl Fluoride (POSF) in Rat Urine, Serum, and Liver by GC/MS (Exygen Method No. ExM-023-071A, Revision 2)

TITLE

Method of Analysis for the Determination of Perfluorooctanesulfonyl Fluoride (POSF) in Rat Urine, Serum, and Liver by GC/MS

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1. SUMMARY

This report details a Method of Analysis for the Determination of Perfluorooctanesulfonyl Fluoride (POSF) in Rat Urine, Serum, and Liver by GC/MS.

Analysis of headspace gas is used to detect POSF in rat urine, serum, and liver. Quantification of POSF is accomplished by gas chromatography / mass spectrometry (GC/MS) analysis.

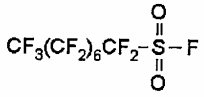
The proposed limit of quantitation (LOQ; the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) for this method is 2.5 µg/mL (for serum and urine) or 2.5 µg/g (for liver) for POSF.

This method was developed using rat urine, rat serum, and rat liver. Typical percent recoveries are shown in **Tables I, II, and III**. A representative calibration curve is shown in **Figure 1**. Representative chromatograms are shown in **Figures 2 to 12**.

2. EXPERIMENTAL COMPOUNDS

The structure for POSF is given below.

POSF
Chemical Name: Perfluorooctanesulfonyl fluoride (POSF)
Chemical Formula: $C_8F_{17}SO_2F$
CAS #: 307-35-7
Molecular Weight: 502



3. CHEMICALS AND SUPPLIES

3.1. CHEMICALS

Chemical	Grade	Source	Catalog No.
Methanol (MeOH)	HPLC	EM Science	MX0475-1

3.2. STANDARDS

Standard	Lot Number	Purity (%)	Source
Perfluorooctanesulfonyl Fluoride (POSF)	15721HQ	98.6	Sigma

3.3. EQUIPMENT AND SUPPLIES

Equipment	Supplier
Balance, analytical (display at least 0.0001 g)	Mettler
125-mL amber glass bottles	Kimble
Disposable glass micropipets (50-100 µL, 100-200 µL)	Drummond
20-mL amber glass vials	Kimble
10mL headspace vials*	Leap Technologies
Class A pipets and volumetric flasks	various suppliers
Standard lab equipment (graduated cylinders, disposable tubes etc.)	various suppliers
GC/MS system	As described in section 4.5.
LEAP Technologies Combi-PAL autosampler	As described in section 4.5.

Note: Equivalent materials may be substituted for those specified in this method if they can be shown to produce satisfactory results. * Actual headspace volume was determined to be 12 mL; these vials were used for the method validation.

3.4. PREPARATION OF STANDARD SOLUTIONS

Analytical standards are used for three purposes:

1. Calibration Standards – These standards are prepared in methanol and are used to calibrate the response of the detector used in the analysis.

2. Laboratory Control Spikes – These fortifications are prepared at concentrations corresponding to the LOQ and 4x LOQ and are used to determine analytical recovery. Laboratory control spikes are prepared in control matrix.
3. Matrix Spikes – These fortifications are prepared by spiking into the field samples at 4x LOQ. Matrix spikes are used to evaluate the effect of the sample matrix on analytical recovery.

The absolute volumes of the standards may be varied by the analyst as long as the correct proportions of solute to solvent are maintained.

3.4.1. Stock solution

Prepare an individual stock solutions of ~2000 µg/mL for POSF by weighing out 200 mg of analytical standard (corrected for purity) and dilute to 100 mL with methanol in a 100-mL volumetric flask. The stock solution (in a 125-mL amber glass bottle) is to be stored in a refrigerator at 2°C to 6°C and is assigned the manufacturer's expiration date.

3.4.2. Fortification / Calibration Standard Solutions

a. Prepare a fortification/calibration standard solution of 1000 µg/mLfor POSF by adding ~25 mL of the ~2000 µg/mL stock solution into a 50-mL volumetric flask and bring up to volume with methanol.
b. Prepare the remaining fortification/calibration standard solutions of 500, 250, 100, 50, and 25 µg/mL from the 1000 µg/mL standard and/or other fortification standards.

Example: Five microliters of the 50 µg/mL solution spiked into 0.1 g of liver (or 100 µL of serum or urine) is equivalent to a 2.5 ppm (2.5 µg/g or 2.5 µg/mL) fortification.

Store all fortification standard solutions in a refrigerator (in 125-mL amber glass bottles) at 2°C to 6°C for a maximum period of one year from the date prepared.

Note: Additional concentrations of Fortification / Calibration Solutions may be prepared if necessary.

3.4.3. Calibration Standards

GC/MS analysis calibration standard levels are determined by spiking a small amount (typically 5 µL) into a sealed vial which contains an appropriate amount of control matrix (100 µL for rat serum and rat urine or 0.1 g rat liver), evaporating the standard, and injecting 1 mL of headspace. Examples of calibration standard levels are shown as follows:

Initial Conc. (µg/mL)	Spiking Volume (µL)	Headspace Volume (mL)	Injection Volume (mL)	Injected Amount (µg)
1000	5	12	1	0.42
500	5	12	1	0.21
250	5	12	1	0.10
100	5	12	1	0.042
50	5	12	1	0.021
25	5	12	1	0.010

4. METHOD

4.1. FLOW DIAGRAM

The flow diagram of the method is given below, followed by a detailed description of each step.

Method Flow Diagram

Weigh ~0.1 g of liver or measure 100 µL of serum (or urine) into headspace vial (seal immediately)
↓
Fortify samples designated as matrix spikes and/or laboratory control spikes
↓
Heat to 80°C for ~4 minutes for manual injection
(heat to 60°C for ~3 minutes if using a LEAP Combi-PAL autosampler)
↓
Inject 1 mL headspace
↓
GC/MS Analysis

4.2. SAMPLE PROCESSING

No sample processing is needed for serum or urine samples. However, frozen serum or urine samples must be allowed to completely thaw to room temperature before use.

For liver samples, remove approximately 0.1 grams (while still frozen) and place into a 12 mL headspace vial. Immediately seal (cap) the vial. Place vials (containing the liver sample) into a freezer if time to analysis exceeds one day.

4.3. BATCH SET UP

a. A batch of samples should not contain more than 20 field samples.

b. Each batch of samples analyzed must include at least one control (method blank using control matrix) and one matrix control fortified at a known concentration (typically between the LOQ and 10x LOQ levels).

c. At least one field sample in each batch must also be separately fortified at a known concentration. Additional samples in the batch may also be fortified based upon the sponsor's requirements.

d. Samples will be analyzed in duplicate.

4.4. Sample Analysis Preparation

4.4.1. Liver Analysis

a. Remove ~0.1 g of liver sample and place into a 12 mL headspace vial. Tightly cap the vial. Fortify sample (if necessary) via syringe.
b. Heat to 80°C for ~4 minutes for manual injection or heat to 60°C for ~3 minutes if using a LEAP Combi-PAL autosampler.
c. Inject 1 mL headspace.
d. Analyze samples using GC/MS.

4.4.2. Serum / Urine Analysis

a. Measure 100 µL of serum (or urine) sample into a 12 mL headspace vial. Tightly cap the vial. Fortify sample (if necessary) via syringe.
b. Heat to 80°C for ~4 minutes for manual injection or heat to 60°C for ~ 3 minutes if using a LEAP Combi-PAL autosampler.
c. Inject 1 mL headspace.
d. Analyze samples using GC/MS.

4.5. QUANTITATION

4.5.1. GC/MS System and Operating Conditions

Instrument:	Hewlett-Packard model 6890 Series Gas Chromatograph/model 5973 mass selective detector
Column:	Restek RTX-502.2, 60 m x 0.25 mm ID, 1.4 µm df
Oven Temperature:	Hold at 40°C for 4 min., ramp 20°C/min. to 100°C, hold for 0 min.
Injector Temperature:	220°C
Transfer Line Temperature:	220°C
Carrier Gas:	Helium
GC Head Pressure	5 psi for 0.25 min., ramp 80 psi/min. to 60 psi, hold 0.5 min., ramp –80 psi/min. to 30 psi, hold at 30 psi for remainder of the analytical run.
GC Flow	Varies with GC head pressure and oven temperature
Injection Mode	Splitless
Injection Liner	4 mm ID straight glass
Injection Purge Delay	2 min.
Purge Flow to Split Vent	10 mL/min.
Injection Volume	1 mL
Electron Multiplier Voltage	From ATUNE + 200V
Scan Mode	SIM
Monitored Ions	m/z's 69 (quantitation ion); 131, 219 (qualifier ions)
Dwell Time	75 ms
Integrator	Hewlett-Packard ChemStation Software
Retention Times	~3.4 minutes
Total run time	~7 minutes

4.5.2. Autosampler Operating Conditions

Study Plan No. ExP-023-075 Exygen Study No. 023-075

Instrument:	LEAP Technologies Combi-PAL autosampler
Injection / Volume:	Headspace / 1mL
Incubator / Agitator Temp:	60°C
Incubator / Agitator Time:	3 min.
Incubator / Agitator Speed:	400 RPM – 2 sec. on, 18 sec. off
Syringe Temp:	70°C
Syringe Fill Speed:	50 µL/sec.
Syringe Inject Speed:	750 µL/sec.
Syringe Pullup Delay:	1 sec.
Pre-Injection Delay:	100 msec.
Post-Injection Delay:	200 msec.

4.5.3. Calibration Curve Procedures

a. Prepare calibration standards by spiking an appropriate volume of standard solution into a sealed (capped) headspace vial which contains an appropriate amount of control matrix (100 µL for rat serum and rat urine or 0.1 g rat liver). After the vial is conditioned (heated) for (approximately) four minutes (at 80°C), manually inject the same amount of headspace (1 mL) of each calibration standard (ranging from the lowest level standard to the highest level prepared), into the GC/MS. If using a LEAP Combi-PAL autosampler for injection, the vial is conditioned (heated) for (approximately) three minutes at 60°C (the syringe is heated to 70°C).

b. Use weighted linear standard curves for quantitation. Linear standard curves are generated for each analyte by linear regression using 1/x weighting of peak area versus calibration standard concentration using HP Chemstation software. Any calibration standard found to be a statistical outlier may be excluded from the calculation of the calibration curve. However, the total number of calibration standards that may be excluded must not exceed 20% of the total number of standards injected.

c. The correlation coefficient (R) for calibration curves generated must be ≥0.9925 (R^2 ≥0.985). If calibration results fall outside these limits, then appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be re-analyzed.

A typical calibration curve for POSF can be found in Figure 1.

4.5.4. Sample Analysis

a. Prepare samples by weighing (for liver) or measuring (for serum and urine) the sample into a headspace vial. If necessary, spike an appropriate volume of standard solution into the sealed (capped) headspace sample vial. After the vial is conditioned (heated) for approximately four minutes (at 80°C), manually inject the same aliquot (1 mL) of each standard, sample, recovery, control, etc. into the GC/MS system. If using a LEAP Combi-PAL autosampler for injection, the vial is conditioned (heated) for (approximately) three minutes at 60°C (the syringe is heated to 70°C).

b. Standards corresponding to at least five or more concentration levels (starting with the LOQ level or below) must be included in an analytical set.

c. An entire set of calibration standards should be injected at the beginning of a set followed by a batch of samples. However, a batch may not consist of

more than twenty samples (excluding spikes and duplicates) until a new calibration curve must be generated.

d. The concentration of each sample/fortification/control is determined from the standard curve, based on the peak area of each analyte. The standard responses should bracket responses of the residue found in each sample set. Results may be quantitated up to 10% outside the curve by extrapolation. If necessary, a smaller sample aliquot may be used if compound response exceeds the calibration curve.

e. Fortification recoveries falling within 60 to 140% are considered acceptable. Recoveries outside these limits may be accepted with sponsor approval.

f. Rat serum and urine samples must be stored frozen at -10°C, or refrigerated between 2°C to 6°C one day prior to analysis, and rat liver must be stored frozen at -10°C until analysis.

g. Field samples in which no peaks are detected at the corresponding analyte retention time will be reported as ND (not detected). Samples in which peaks are detected at the corresponding analyte retention time that are less than the LOQ will be reported as NQ (not quantifiable).

4.6. Acceptance Criteria

The following criteria must be met to ensure the presence of POSF:

1. The instrument must have adequate sensitivity (s/n $\geq$ 10) at the LOQ level.

2. Method blanks must not contain POSF at levels greater than the LOQ. If a blank contains the analyte at levels greater than the LOQ, analysis will cease until the source of the contamination is found.

3. Recoveries of control spikes and matrix spikes should be between 60-140% of their known values. If a control spike falls outside the acceptable limits, another spike should be prepared and analyzed. Any matrix spike outside 60-140% should be evaluated by the analyst to determine if re-analysis is warranted.

4. Any calibration standard found to be a statistical outlier may be excluded from the calculation of the calibration curve. However, the total number of calibration standards that could be excluded must not exceed 20% of the total number of standards injected.

5. The correlation coefficient (R) for calibration curves generated must be $\geq$0.9925 (R^2 $\geq$0.985). If calibration results fall outside these limits, then

appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be reanalyzed.

4.7. Performance Criteria

The following two criteria must be performed as a system suitability test, before the commencement of analysis when using an instrumentation set-up that has not been used for this method.

First Criterion:

Prepare and inject a GC/MS standard corresponding to the estimated LOQ to determine if the instrument has adequate sensitivity. If this criteria cannot be met, optimize by changing instrument operating parameters.

Second Criterion:

Run a set of standards of five or more concentration levels, from at or below the LOQ, up to the highest concentration level to be included in the analysis. Generate a calibration curve for the analyte and obtain a linear regression with a coefficient of determination (R^2) of at least 0.985 for the analyte. Once this criterion is met, samples may be analyzed.

4.8. Time Required for Analysis

One person can take a set of 20 samples through the sample preparation procedure in approximately 2 hours. The GC/MS analysis of the set (containing 6 standard injections, 20 field samples, 1 matrix blank, 1 laboratory control spike, and 1 matrix spike) will take approximately 5 hours.

5. CALCULATIONS

a. Use Equation 1 to calculate the amount of POSF found (in µg/mL, based on peak area) using the standard curve (1/x weighted linear regression parameters) generated by the HP Chemstation software program.

Equation 1:

$$\text{Analyte found (µg/mL)} = \frac{\text{(Peak Area - Intercept)}}{\text{Slope}} \times \text{DF}$$

DF = factor by which the final volume was diluted, if necessary.

b. Equation 2 was used to calculate the amount of POSF found (in µg/mL or µg/g).

Equation 2:

Total Analyte Found (µg/mL or µg/g) =

$$\frac{\text{Analyte Found (µg/mL) x Standard Volume Added (mL)}}{\text{SW (g) or SV (mL)}}$$

SW = sample weight, SV = sample volume

c. Equation 3 was used to calculate the percent recovery for samples fortified with known amounts of POSF prior to extraction.

Equation 3:

Recovery (%) =

$$\frac{\text{Total Analyte Found (µg/mL*) – Analyte Found in Sample (µg/mL*)}}{\text{Analyte Added (µg/mL*)}} \times 100$$

* = (or µg/g)

For reporting purposes, field samples in which no peaks are detected at the corresponding analyte retention time will be reported as ND (not detected). Samples in which peaks are detected at the corresponding analyte retention time that are less than the LOQ will be reported as NQ (not quantifiable).

6. SAFETY

The analyst should read the material safety data sheets for all standards and reagents before performing this method. Use universal precautions when handling standards and reagents, including working in fume hoods and wearing laboratory coats, safety glasses, and gloves.

Table I. Recovery Summary of POSF in Rat Serum

Summary of Rat Serum Recoveries

Sample Trial	Sample Injected Amount (µg)	Sample Area Counts	Std Injected Amount (µg)	Std Area Counts	% Recovery
#1	0.1	603475	0.1	536407	113
#2	0.1	506452	0.1	536407	94.4
#1	0.01	46925	0.01	51030	92.0

Table II. Recovery Summary of POSF in Rat Urine

Summary of Rat Urine Recoveries

Sample Trial	Sample Injected Amount (µg)	Sample Area Counts	Std Injected Amount (µg)	Std Area Counts	% Recovery
#1	0.1	468310	0.1	662599	70.7
#2	0.1	513880	0.1	662599	77.6
#1	0.01	48036	0.01	51030	94.1

Table III. Recovery Summary of POSF in Rat Liver

Summary of Rat Liver Recoveries

Sample Trial	Sample Injected Amount (µg)	Sample Area Counts	Std Injected Amount (µg)	Std Area Counts	% Recovery
#1	0.1	702597	0.1	629578	112
#2	0.1	552876	0.1	629578	87.8
#1	0.01	45864	0.01	51030	89.9

Figure 1. Calibration curve for POSF

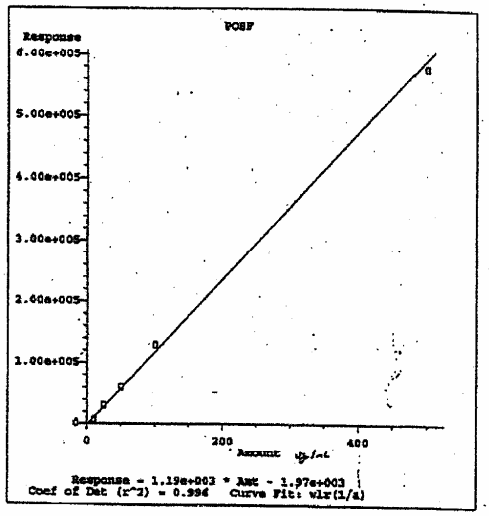


Figure 2. Representative Chromatogram of a 0.10 µg Standard Injection of POSF

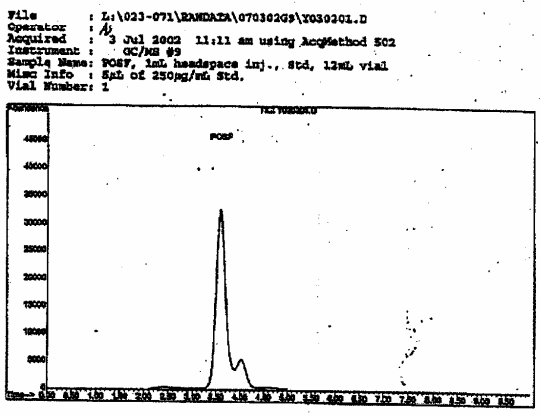


Figure 3. Representative Chromatogram of a 0.01 µg Standard Injection of POSF

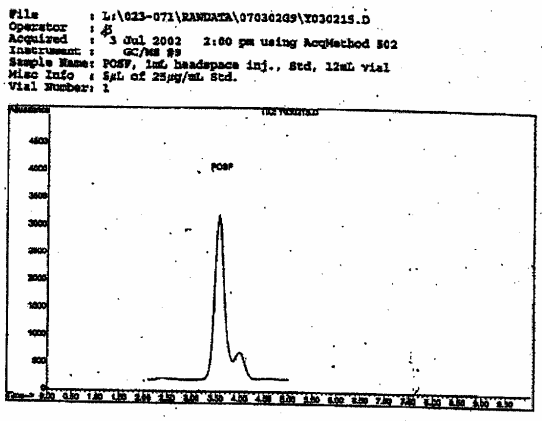


Figure 4. Representative Chromatogram of a Control Sample (100 µL Rat Serum)

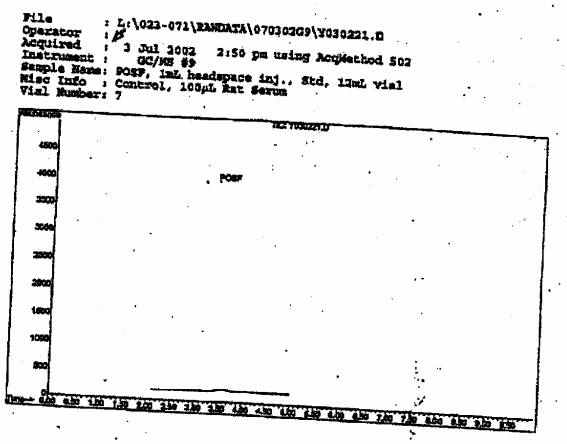


Figure 5. Representative Chromatogram of a Control Sample (100 µL Rat Urine)

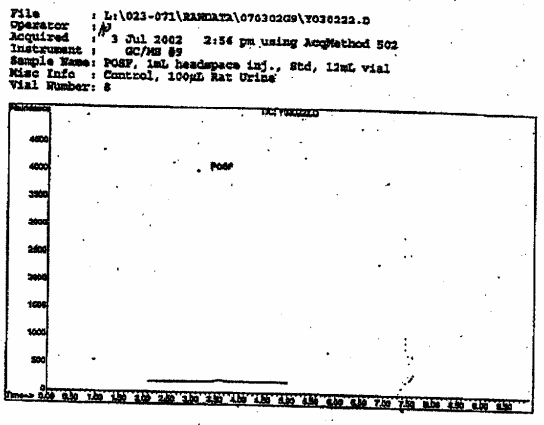


Figure 6. Representative Chromatogram of a Control Sample (0.1 g Rat Liver)

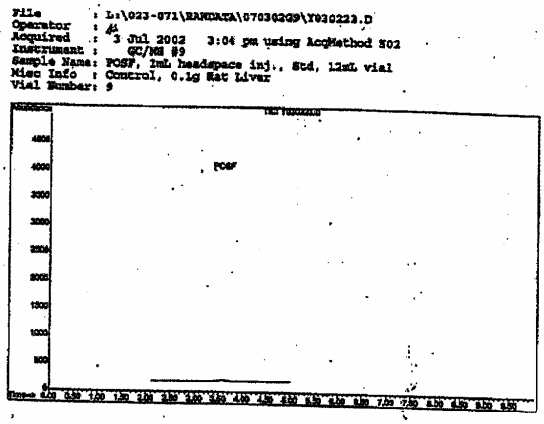


Figure 7. Representative Chromatogram of a Fortified Rat Serum Control Sample (0.1 µg Injection of POSF)

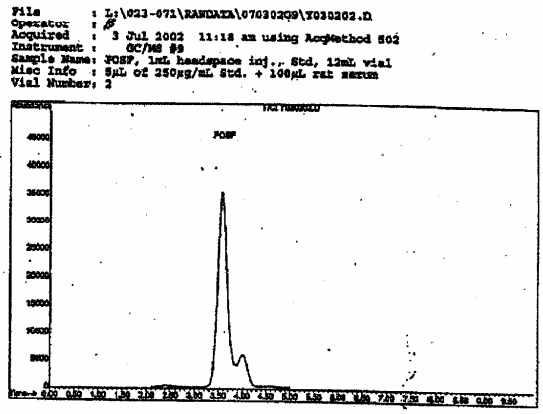


Figure 8. Representative Chromatogram of a Fortified Rat Urine Control Sample (0.1 µg Injection of POSF)

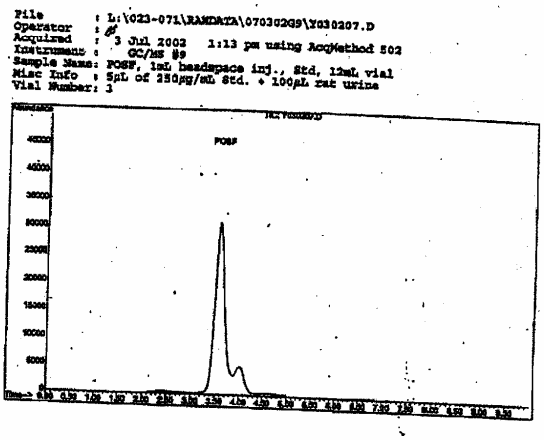


Figure 9. Representative Chromatogram of a Fortified Rat Liver Control Sample (0.1 µg Injection of POSF)

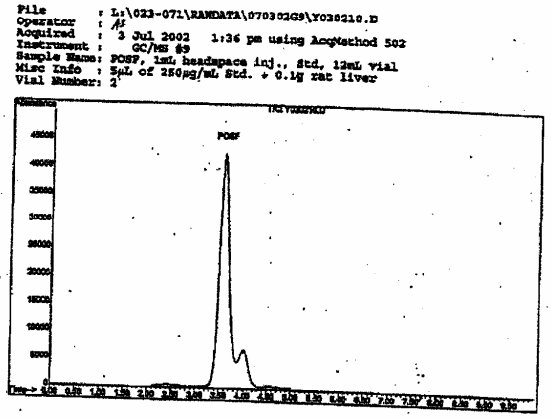


Figure 10. Representative Chromatogram of a Fortified Rat Serum Control Sample (0.01 µg Injection of POSF)

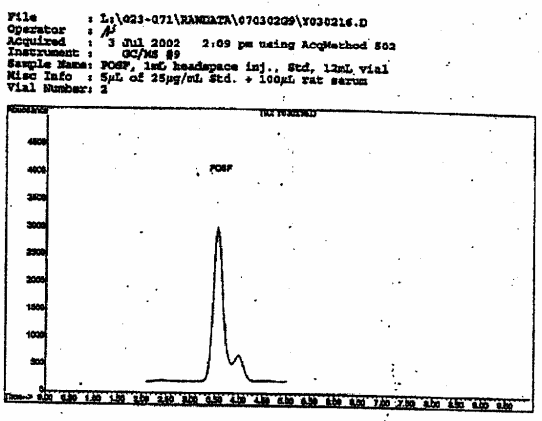


Figure 11. Representative Chromatogram of a Fortified Rat Urine Control Sample (0.01 µg Injection of POSF)

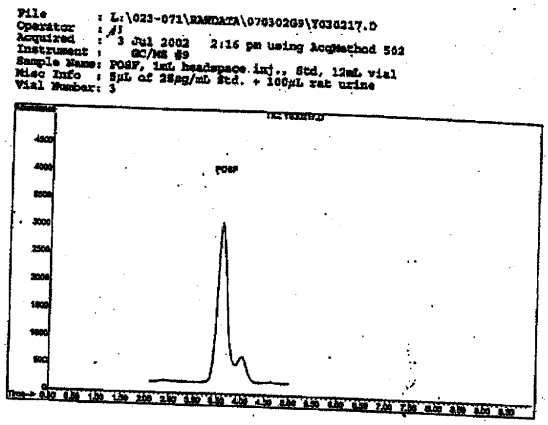
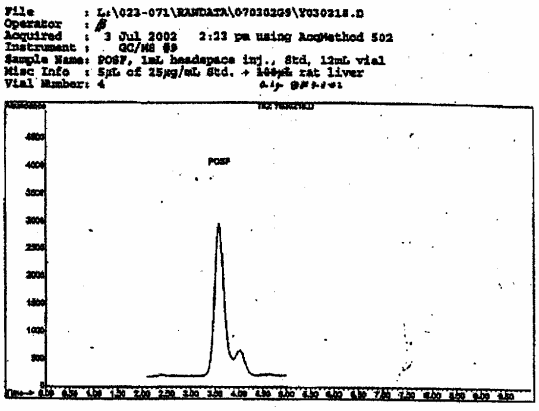


Figure 12. Representative Chromatogram of a Fortified Rat Liver Control Sample (0.01 µg Injection of POSF)



SCANNING ELECTRON MICROSCOPE EXAMINATION AND X-RAY MICROANALYSIS

Plymouth Electron Microscope Unit

Scanning Electron Microscope Examination

and

X-Ray Microanalysis.

Study Number: MIN312

For:

Huntingdon Life Sciences
Huntingdon
Cambs
PE28 4HS

Plymouth Electron Microscope Unit
University of Plymouth
Drake Circus
Plymouth
PL4 8AA

01752 233092

Materials and Methods

1. SEM examination of urinary bladder tissue samples:

Subsamples (approximately 0.5 x 0.5cm) were cut from fixed tissue samples (supplied in 70% ethanol) and critical point dried (protocol attached). Dry samples were mounted on specimen stubs with colloidal silver adhesive, sputter coated with gold and examined in a JEOL 5300 SEM at 15kV.

Each sample was imaged at 50x magnification to provide an overview of the bladder epithelium, and four images, each at 500x magnification, were collected to illustrate typical epithelial morphology.

2. SEM/X-Ray microanalysis of urine samples:

Samples as supplied were carbon coated and examined in a JEOL 6100 SEM at 15kV. X-ray data were collected and analysed with an Oxford Instruments ISIS X-Ray Analysis system.

Specimen Preparation for Scanning Electron Microscopy

Critical Point Drying (CPD)

1. Fix freshly excised sample appropriately (eg. 2.5% Glutaraldehyde in 0.1M phosphate buffer)

2. Wash in buffer - 3x 15 minutes

3. Dehydrate in ethanol series
   - 30% - 15 minutes
   - 50% - 15 minutes
   - 70% - 15 minutes
   - 90% - 15 minutes
   - 100% - 15 minutes
   - 100% - 15 minutes

4. Fill CPD chamber with 100%ethanol and cool to 10°C or less

5. Place specimens in CPD chamber

6. Close and flood with liquid carbon dioxide

7. Exhaust alcohol from chamber.

8. Leave sample 30 minutes in chamber to infiltrate with carbon dioxide.

9. Exhaust remaining ethanol

10. Heat chamber to critical point

11. Vent carbon dioxide gas from chamber

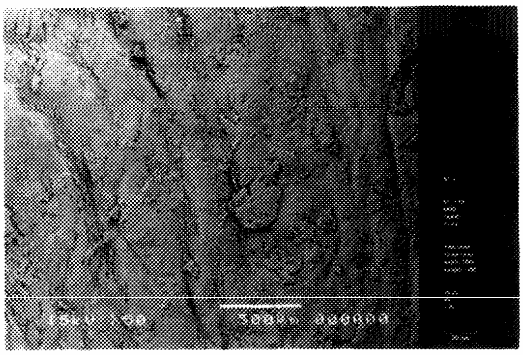
12. Remove sample from chamber and mount for SEM examination.

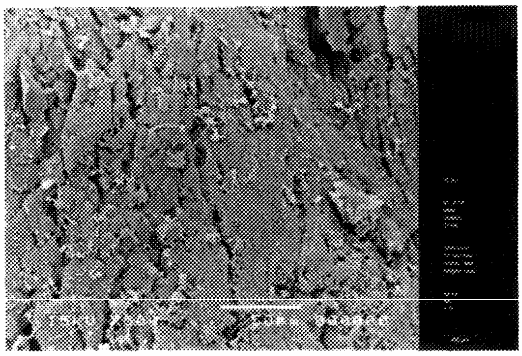
Results

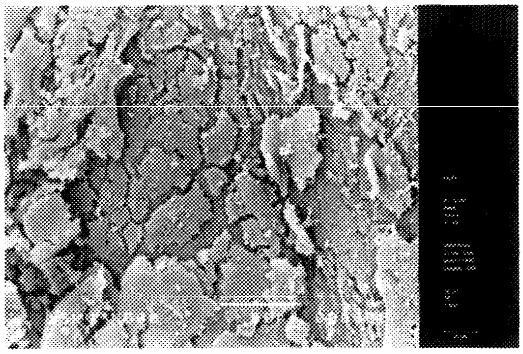
1. SEM Examination of Urinary Bladder Epithelium.

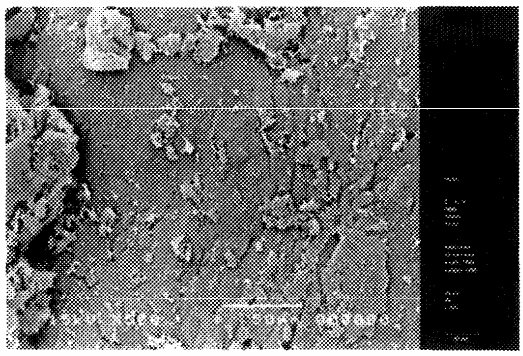
Sample 5 - no low power image was collected due to poor image signal from the sample.

Sample 1

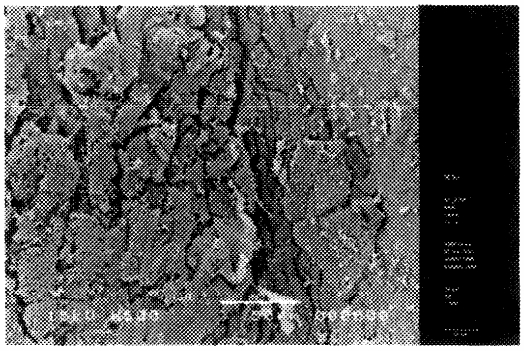




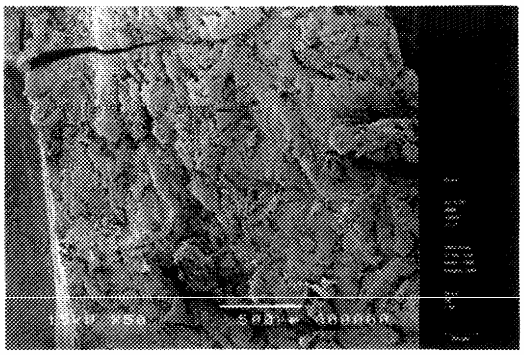


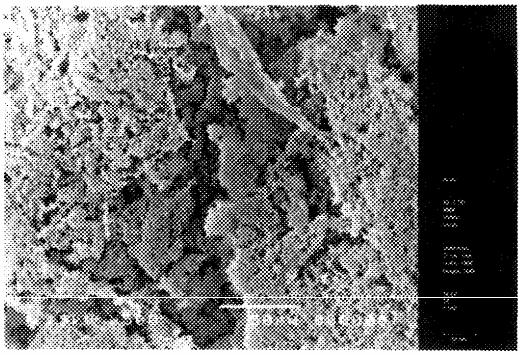


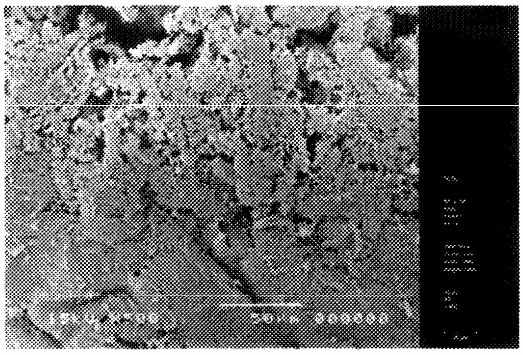
Sample 1

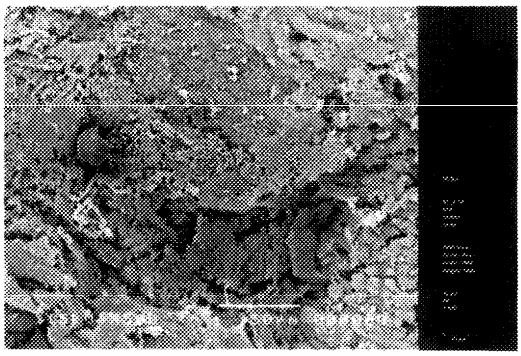


Sample 2

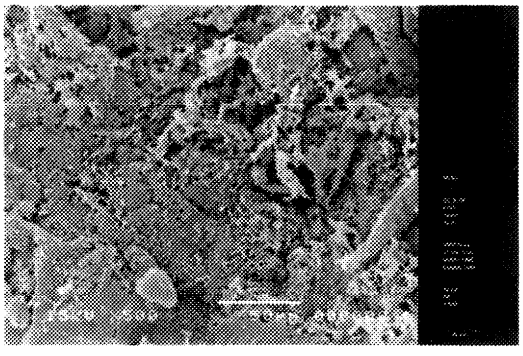




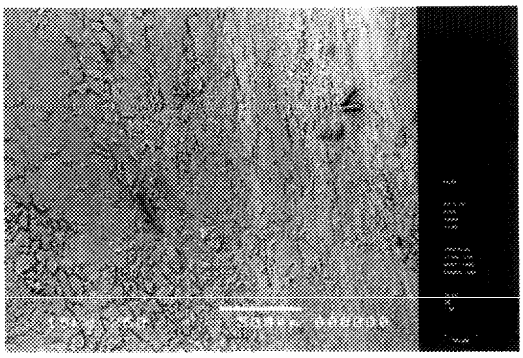


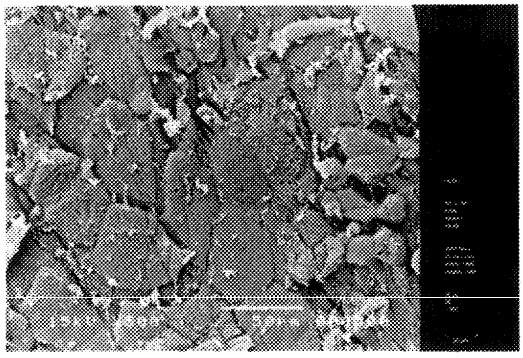


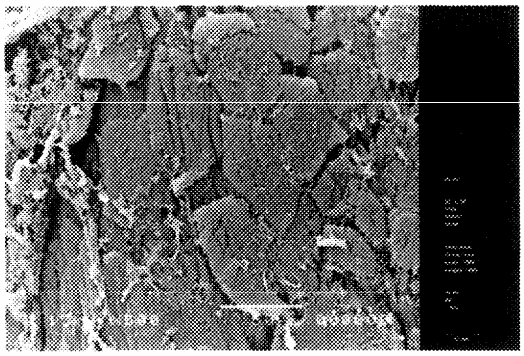
Sample 2

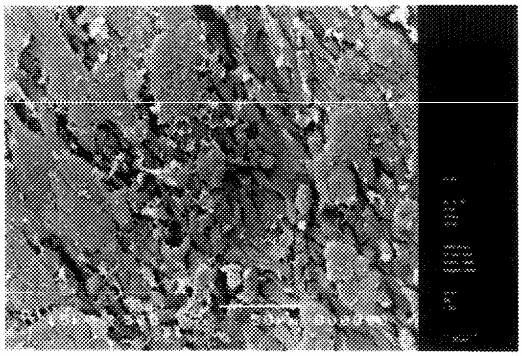


Sample 3

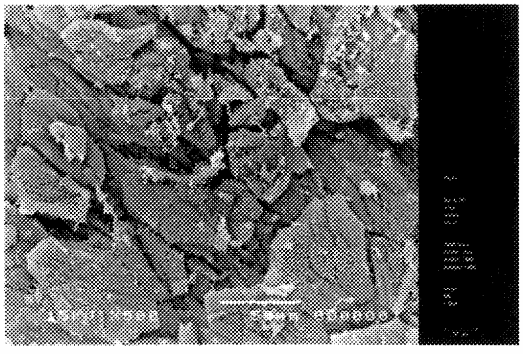




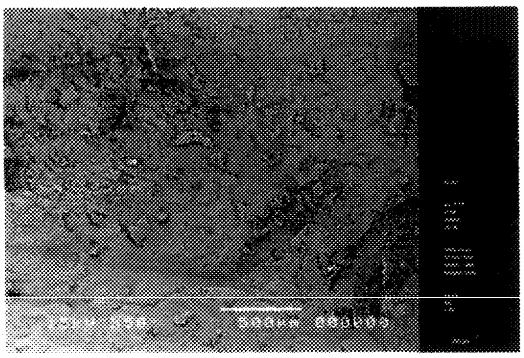


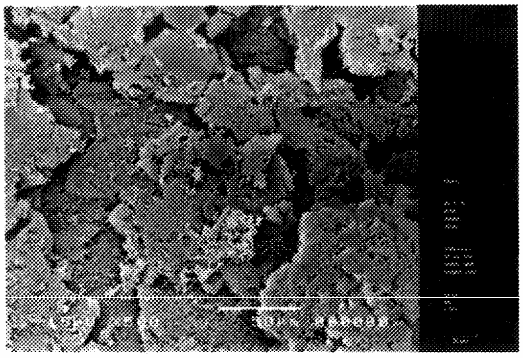


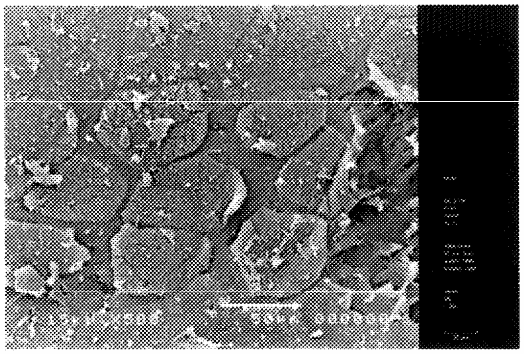
Sample 3

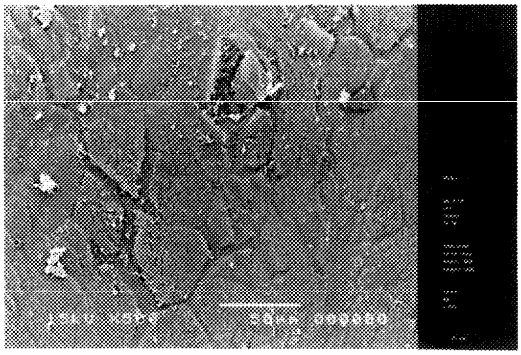


Sample 4

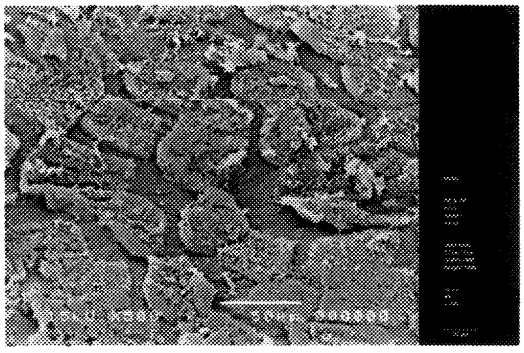


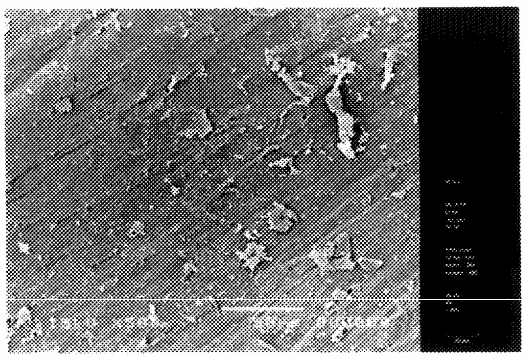


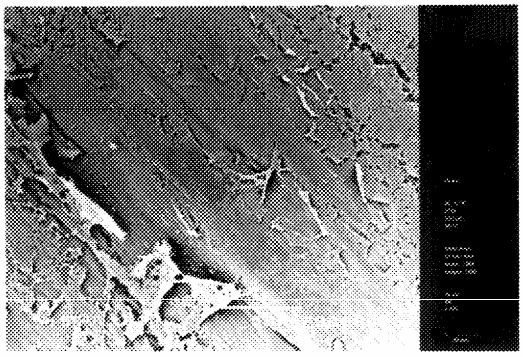


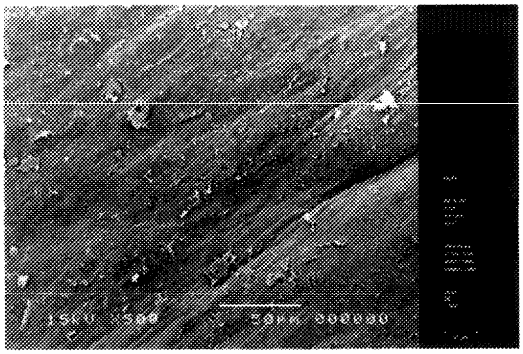


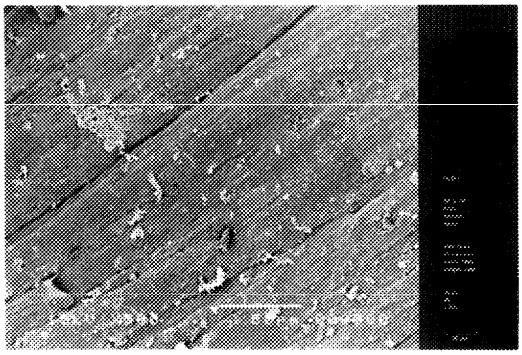
Sample 4











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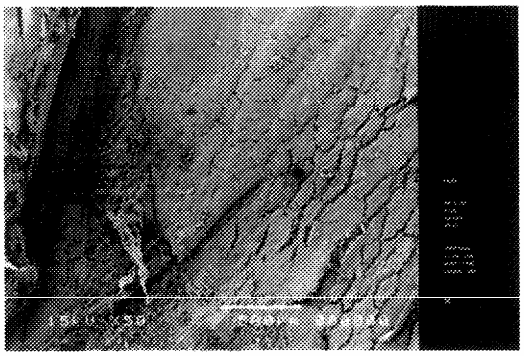

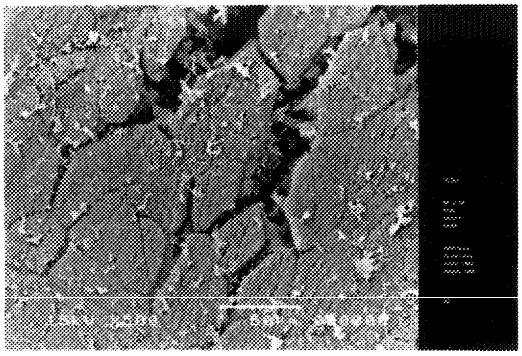

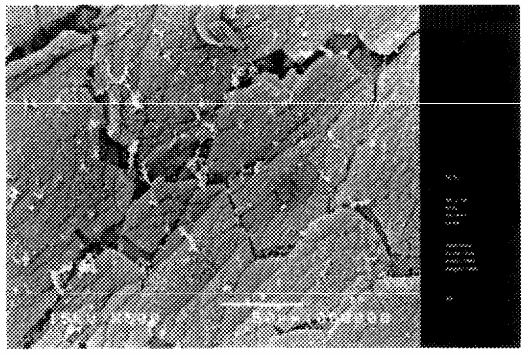

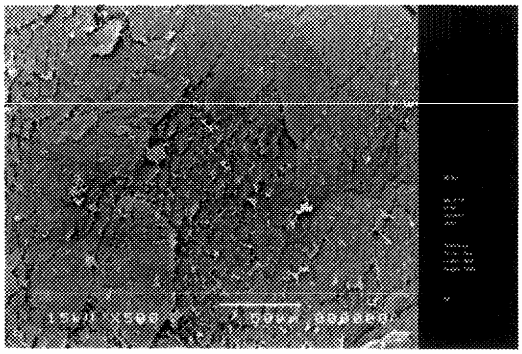

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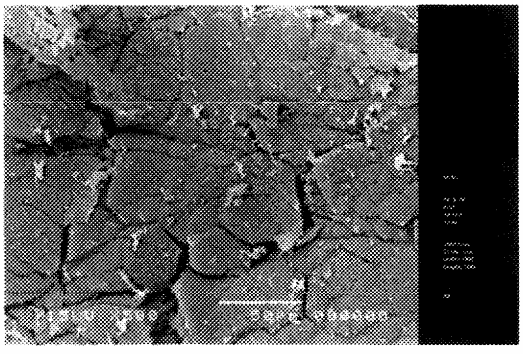

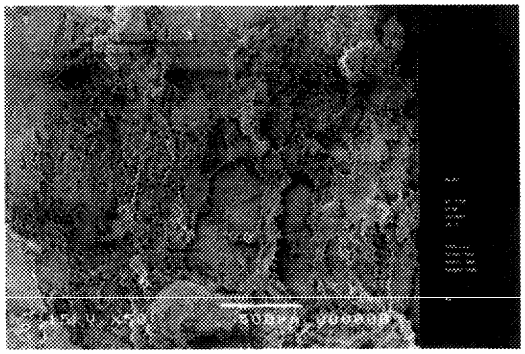

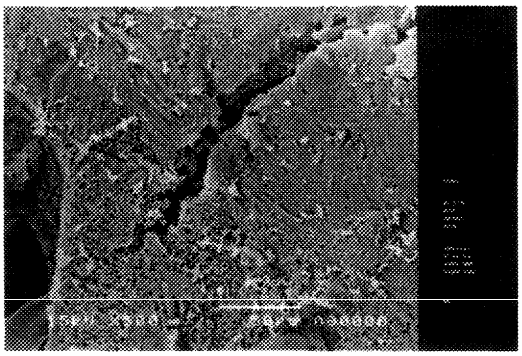

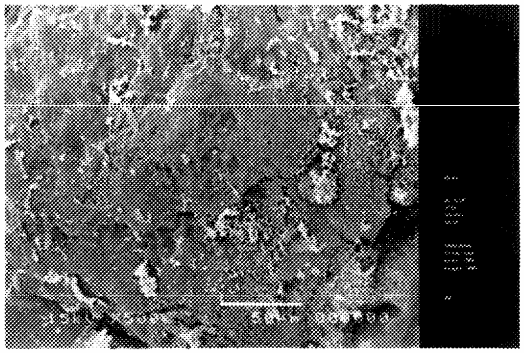

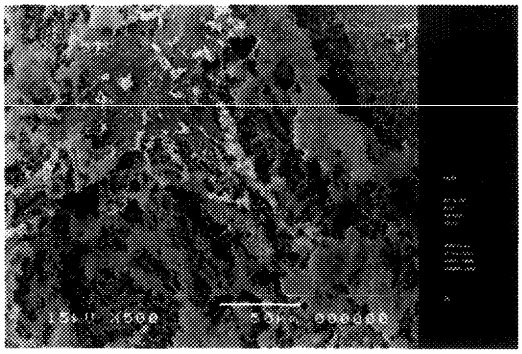

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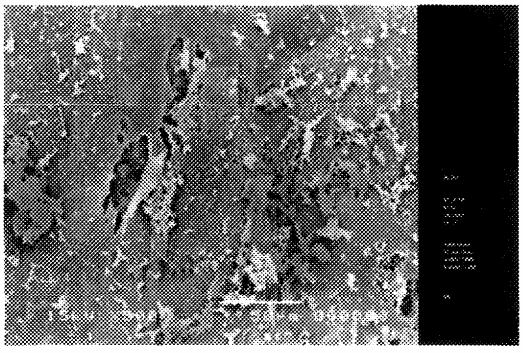

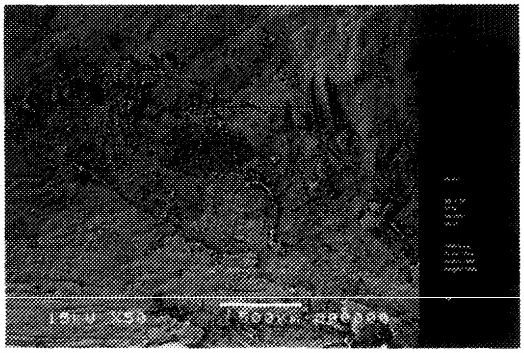

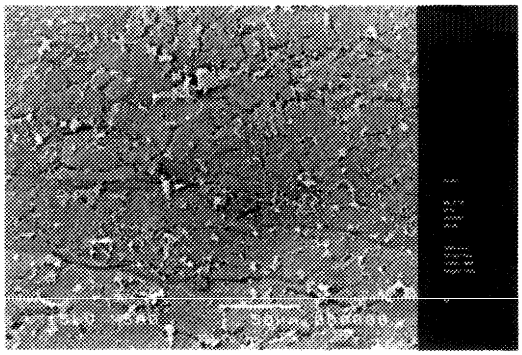

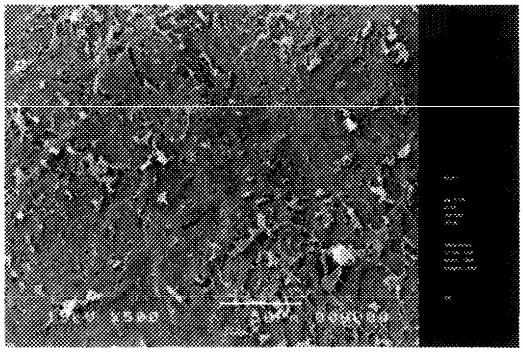

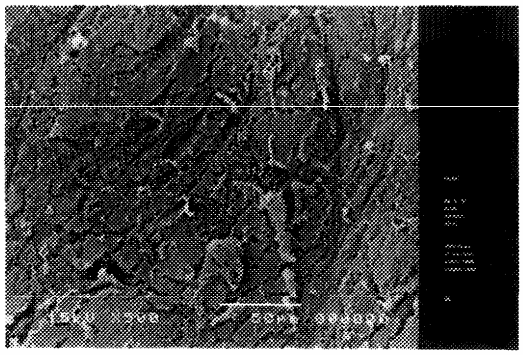

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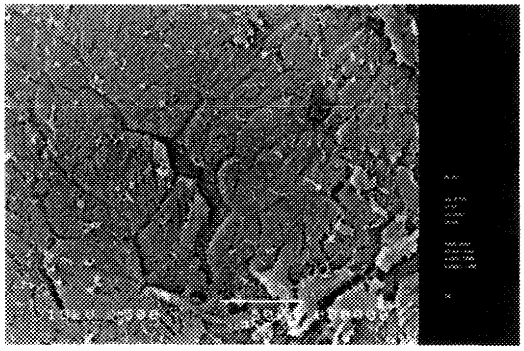
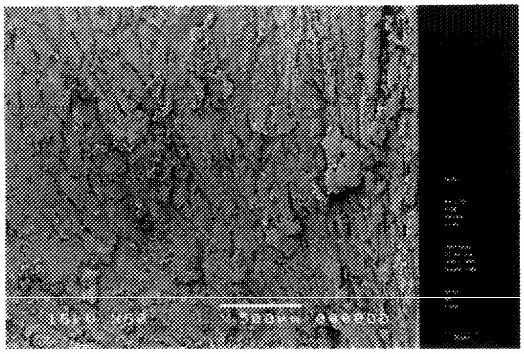
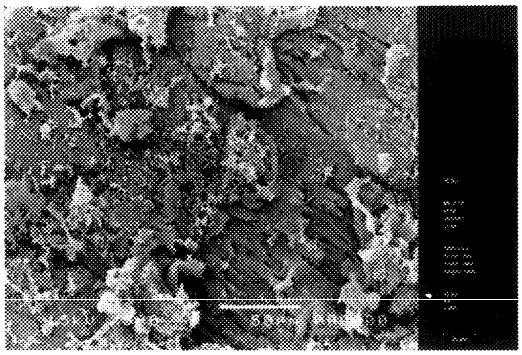
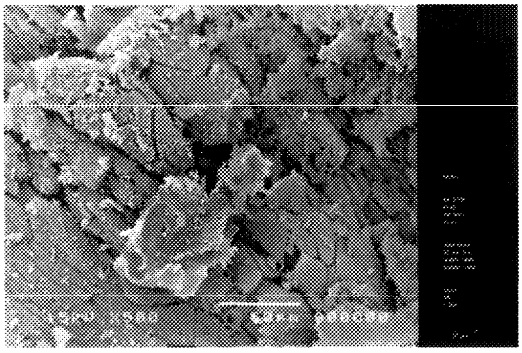
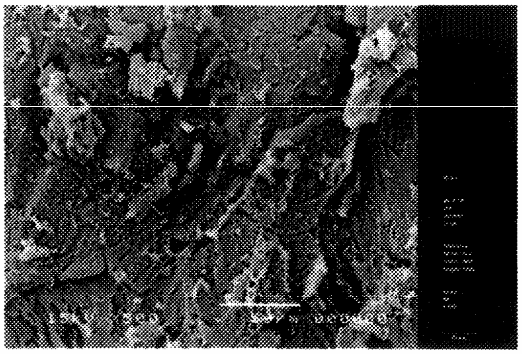


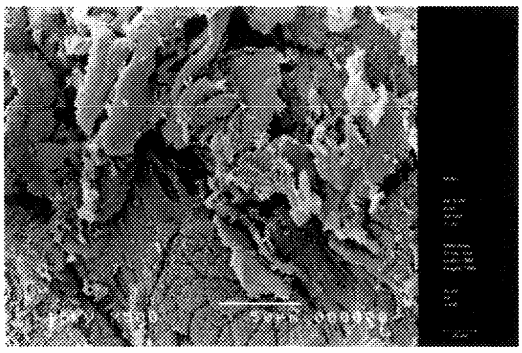


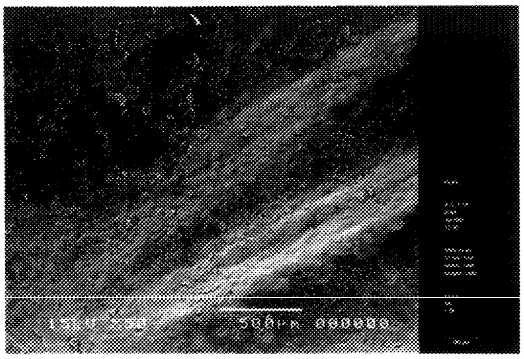


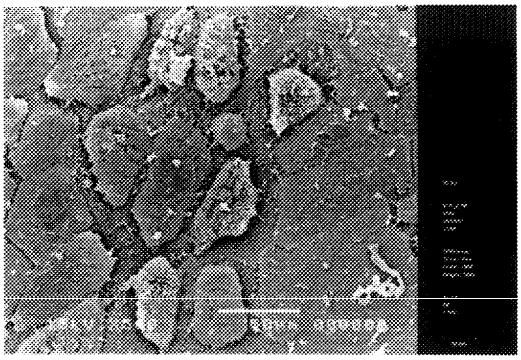


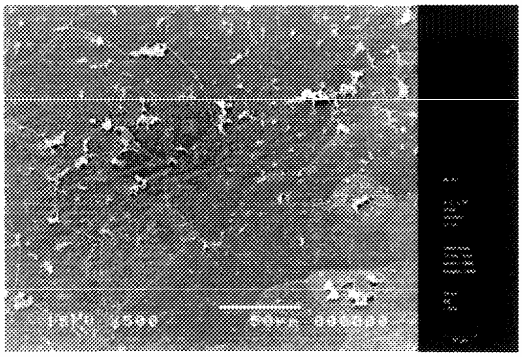


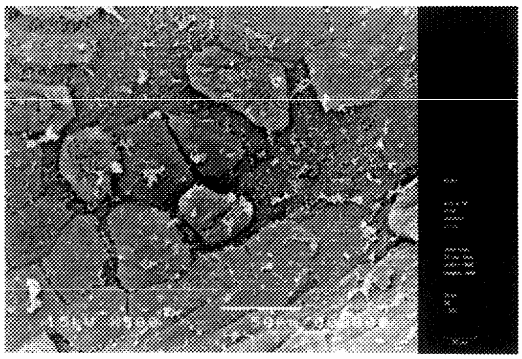
Sample 9



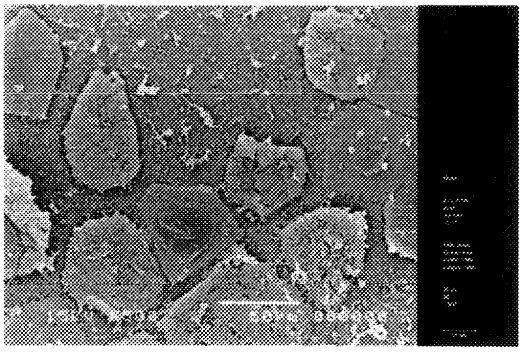


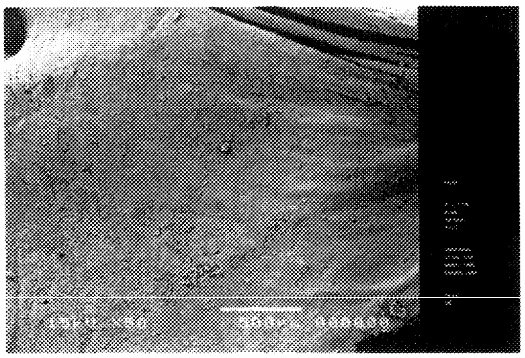


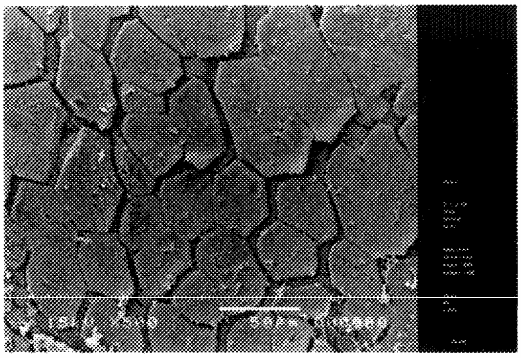


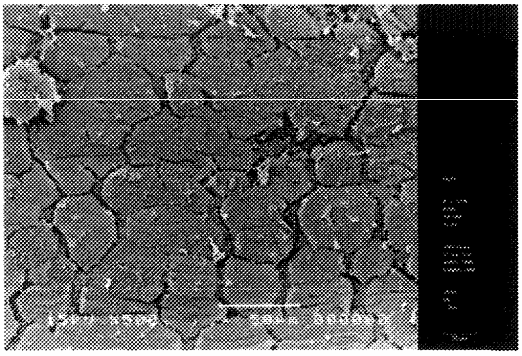


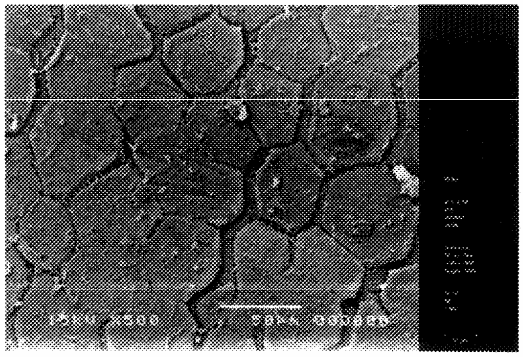
Sample 10



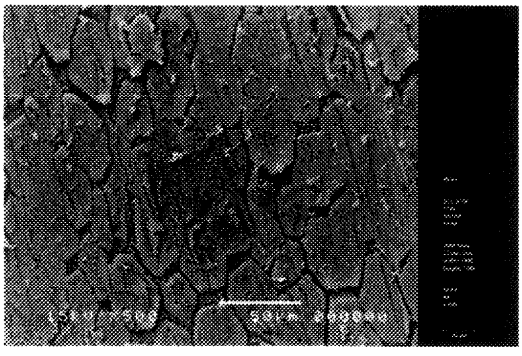


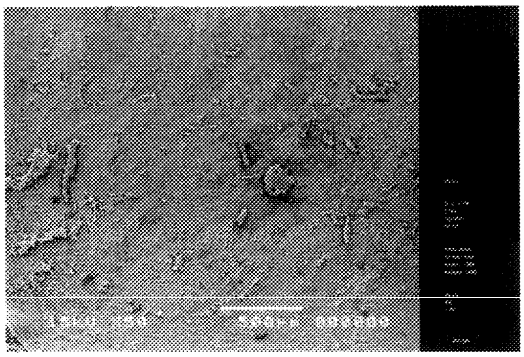


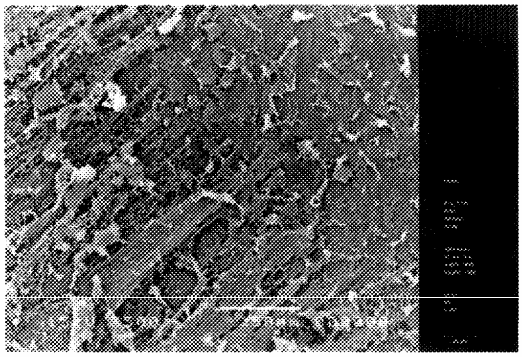


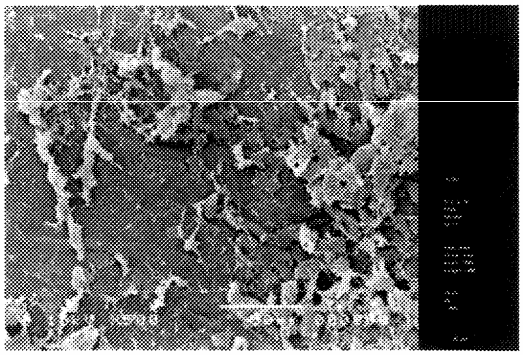


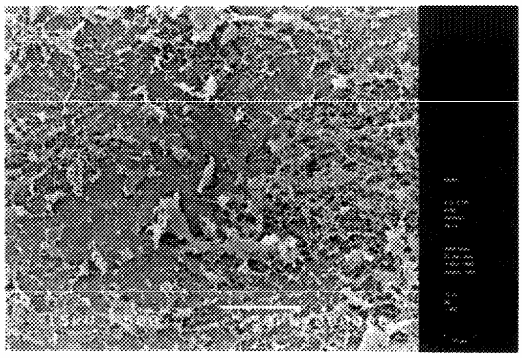
Sample 11



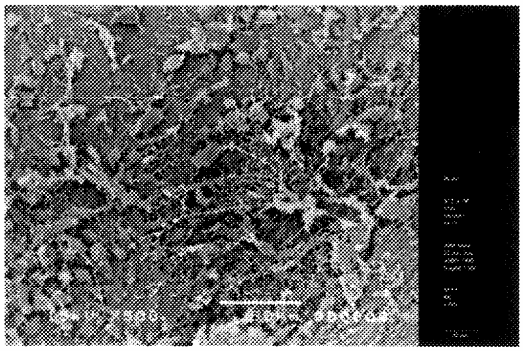


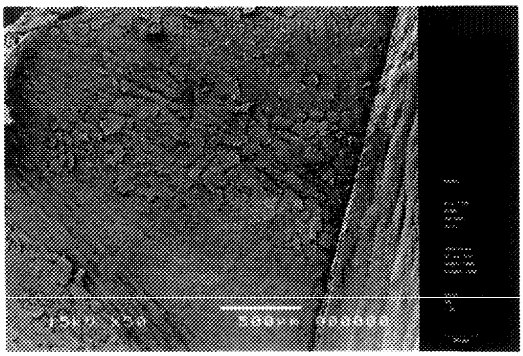


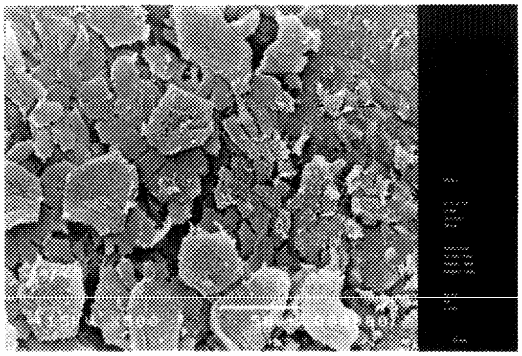


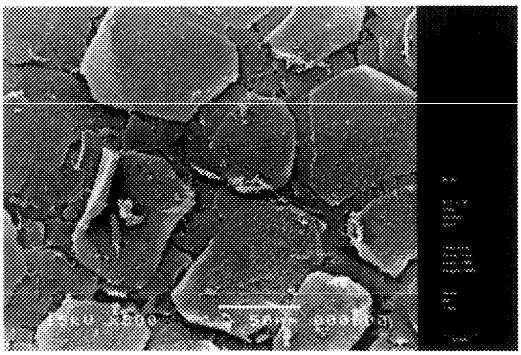


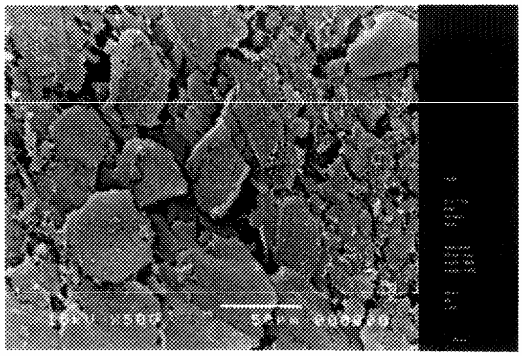
Sample 12



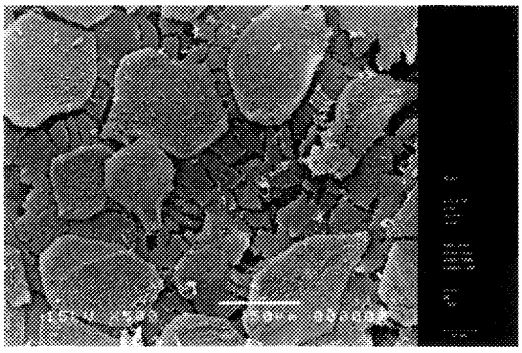


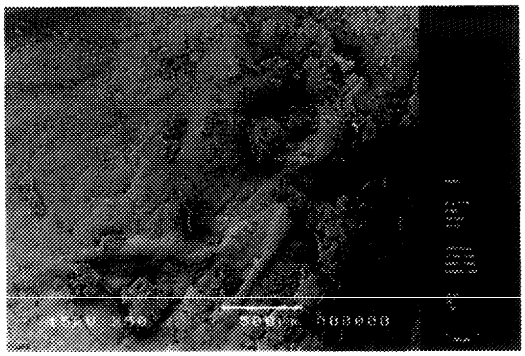


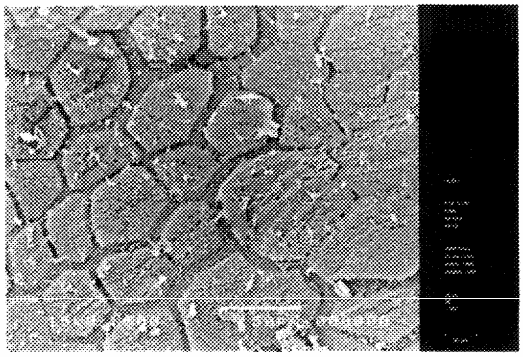


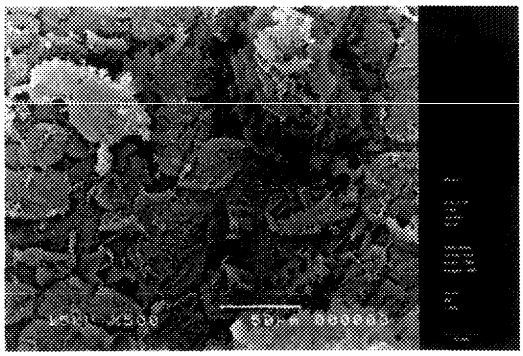


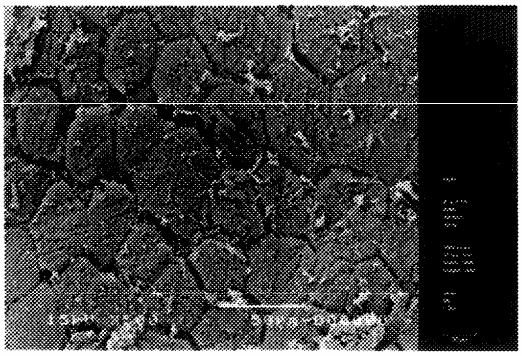
Sample 13



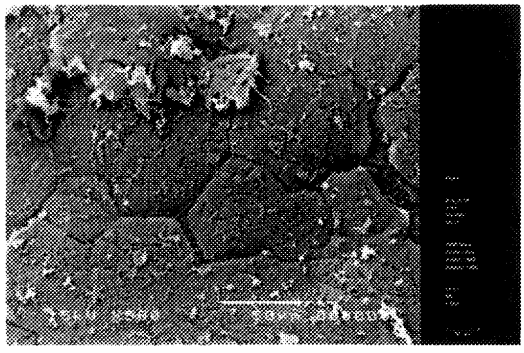


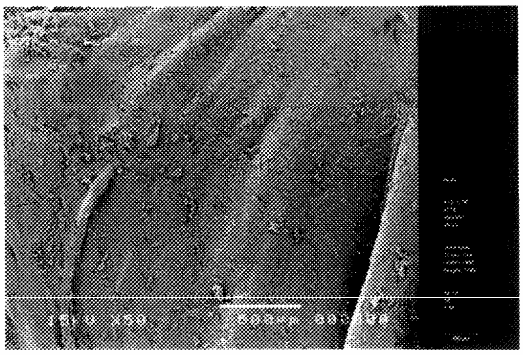


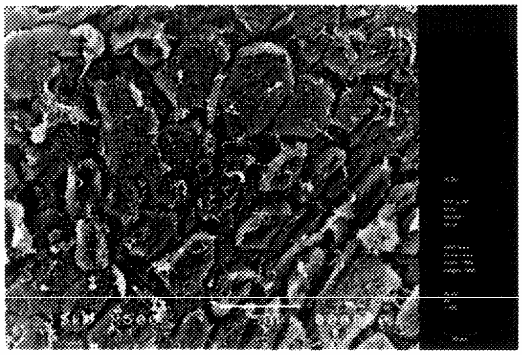


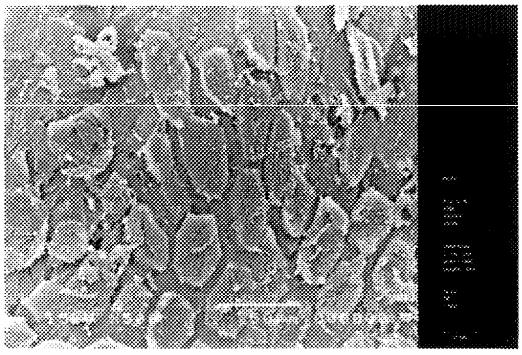


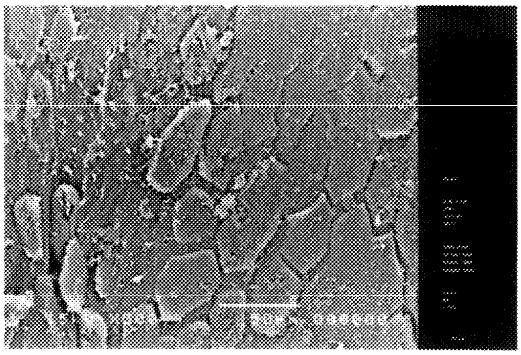
Sample 14



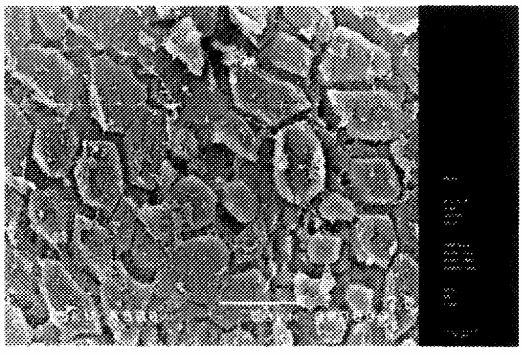


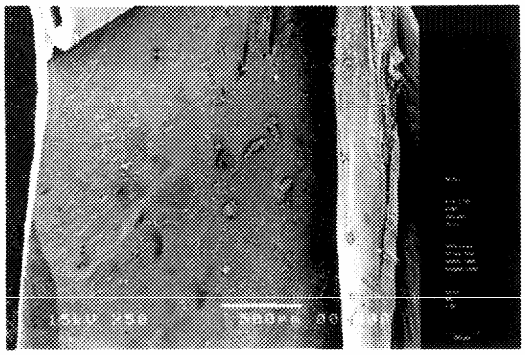


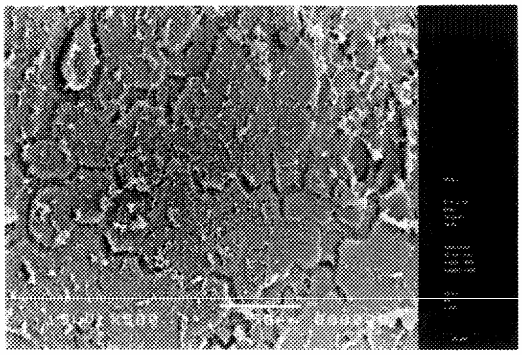


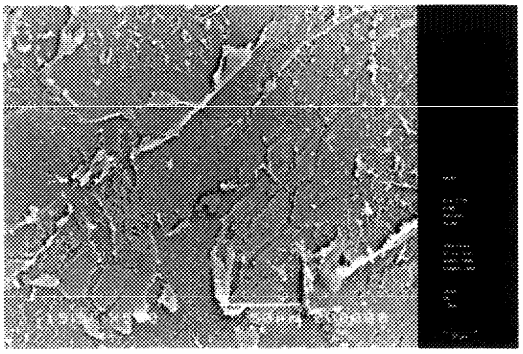


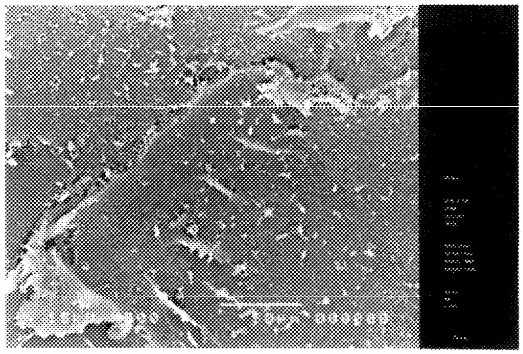
Sample 15



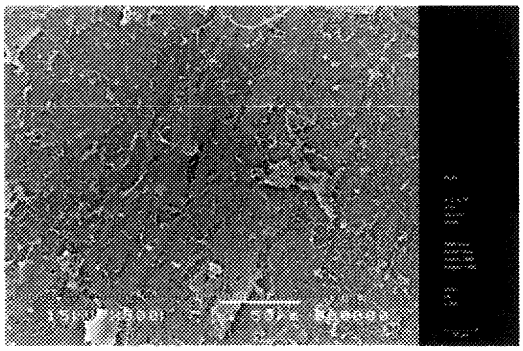


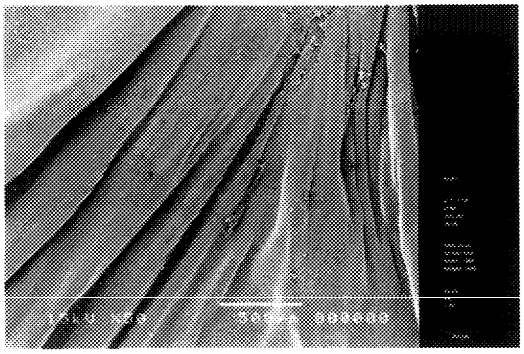


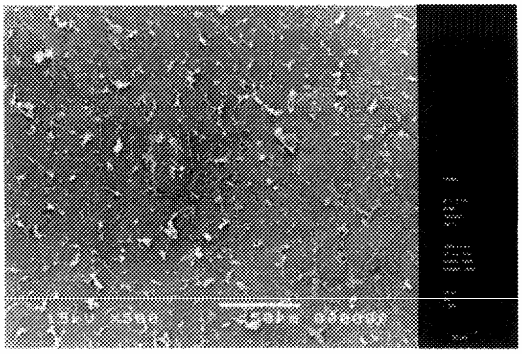


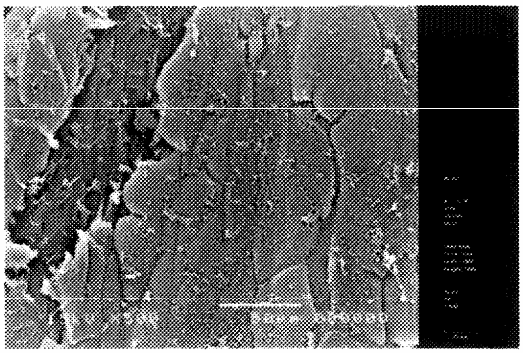


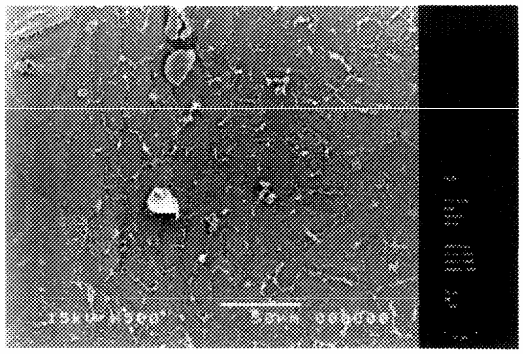
Sample 16



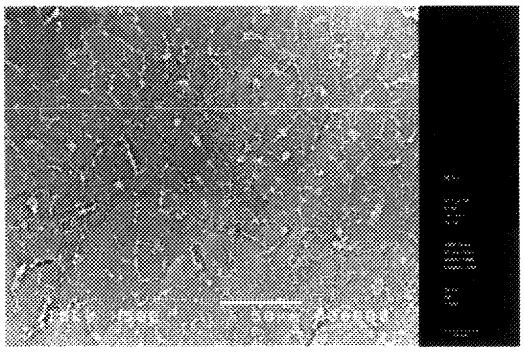


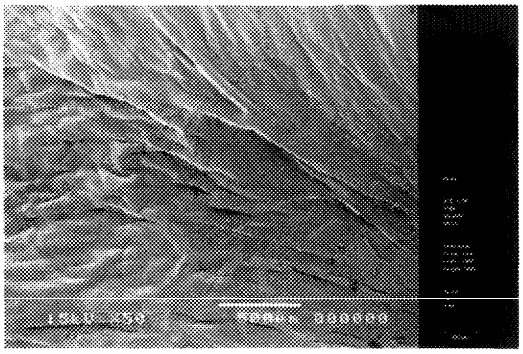


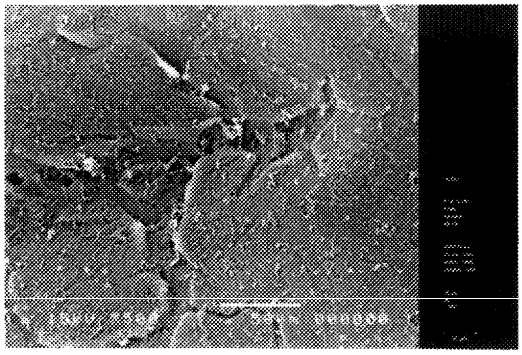


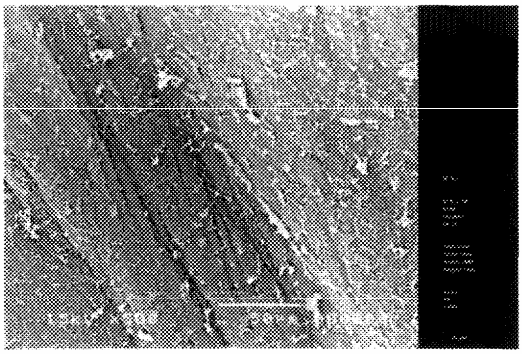


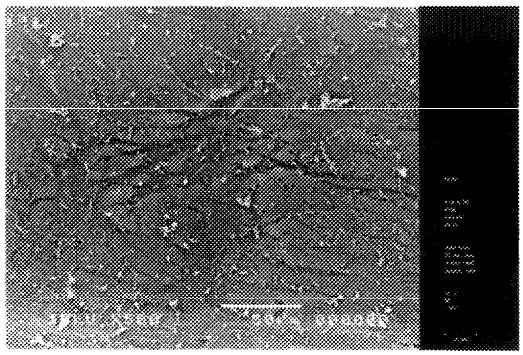
Sample 17



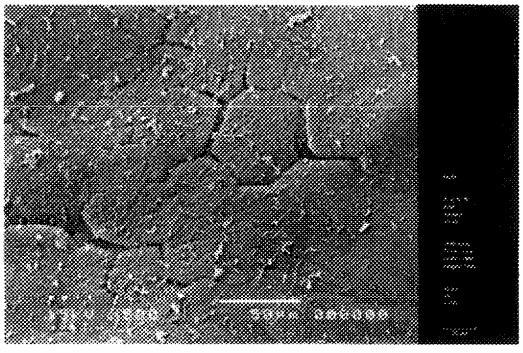


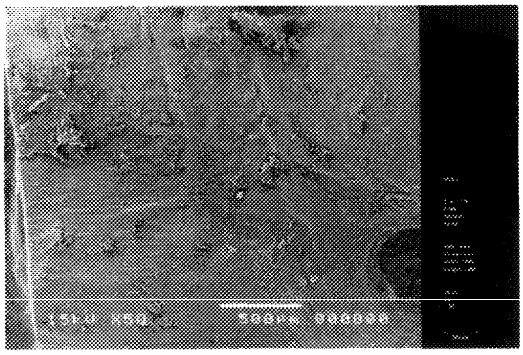


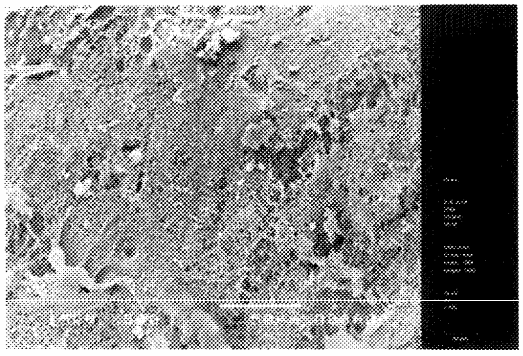


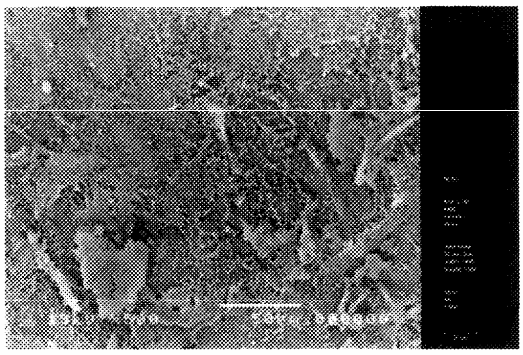


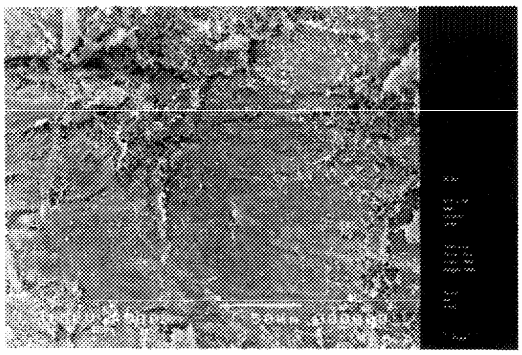
Sample 18



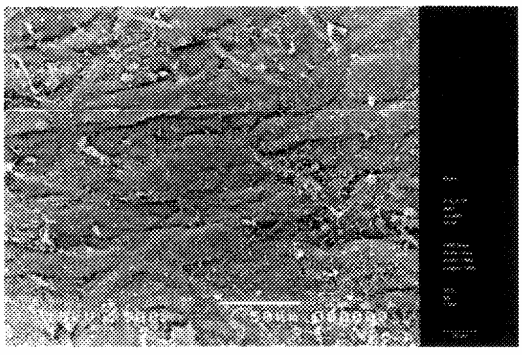


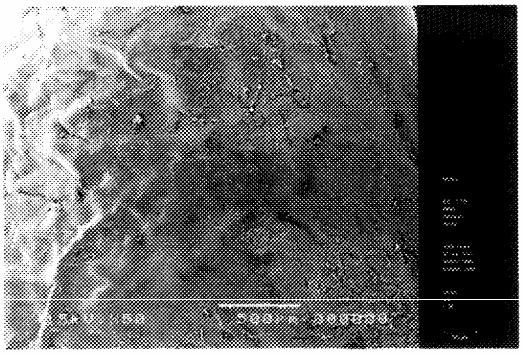


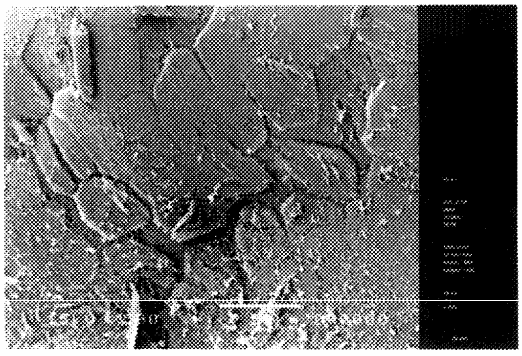


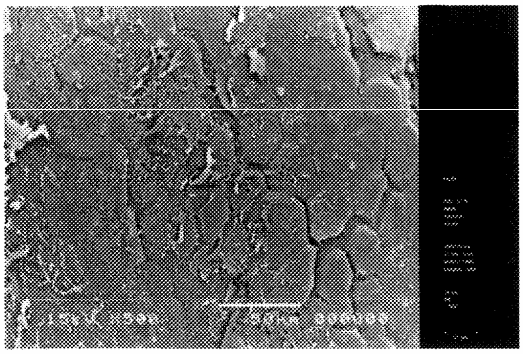


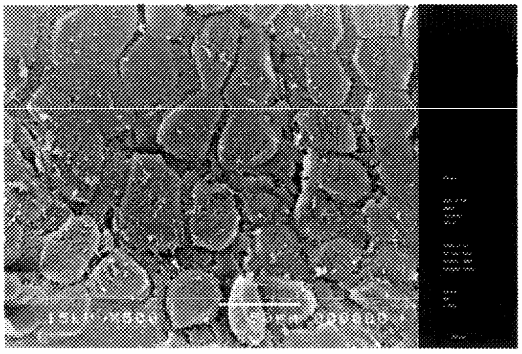
Sample 19



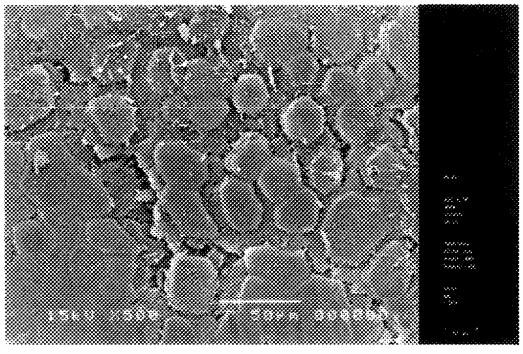








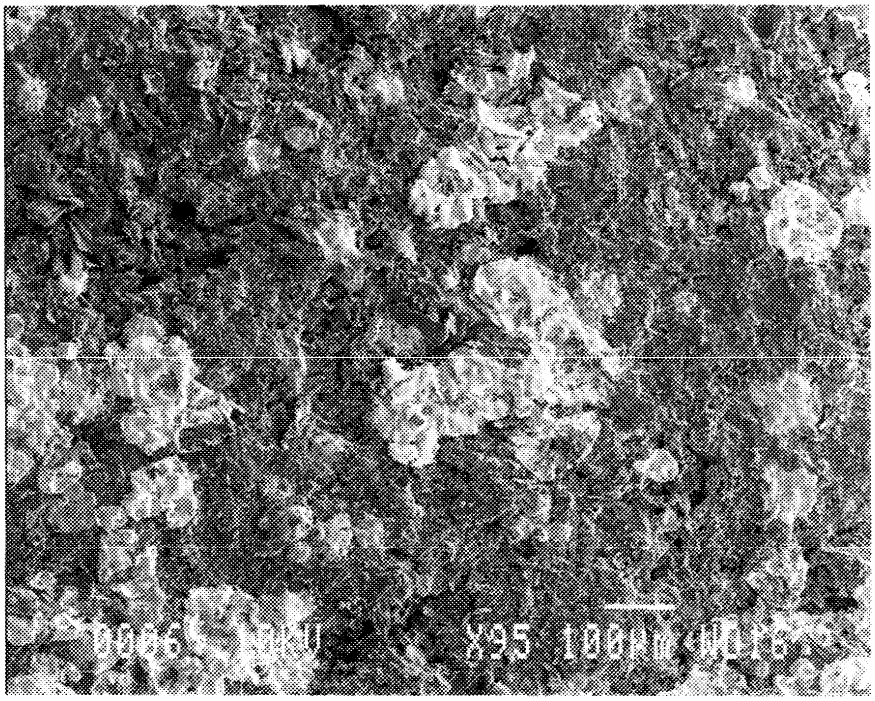
Sample 20



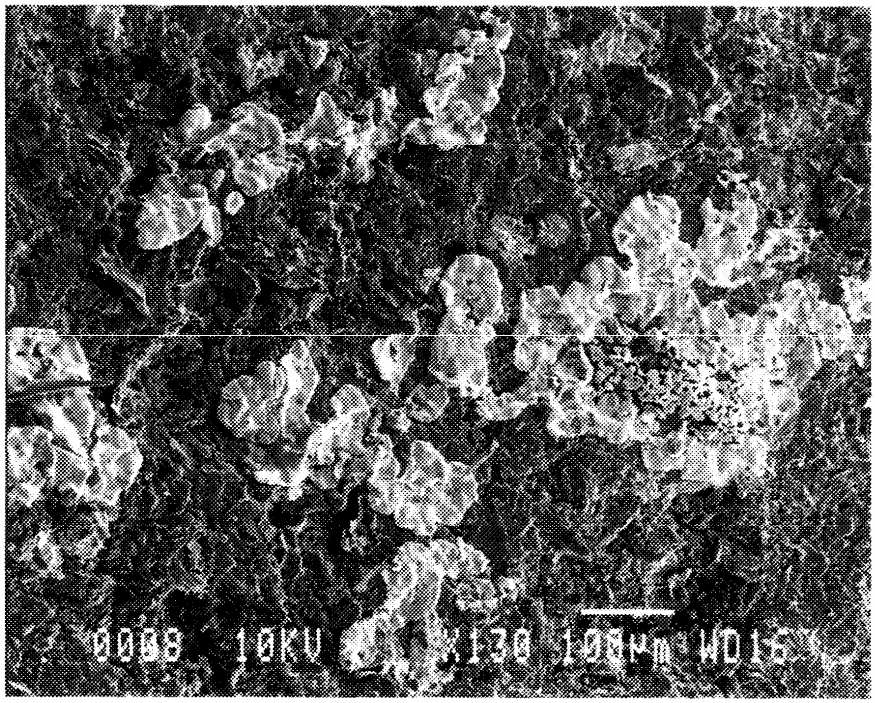
2. SEM/X-Ray analysis of Urine samples

Representative images are provided to illustrate typical crystal structure and deposit morphology.

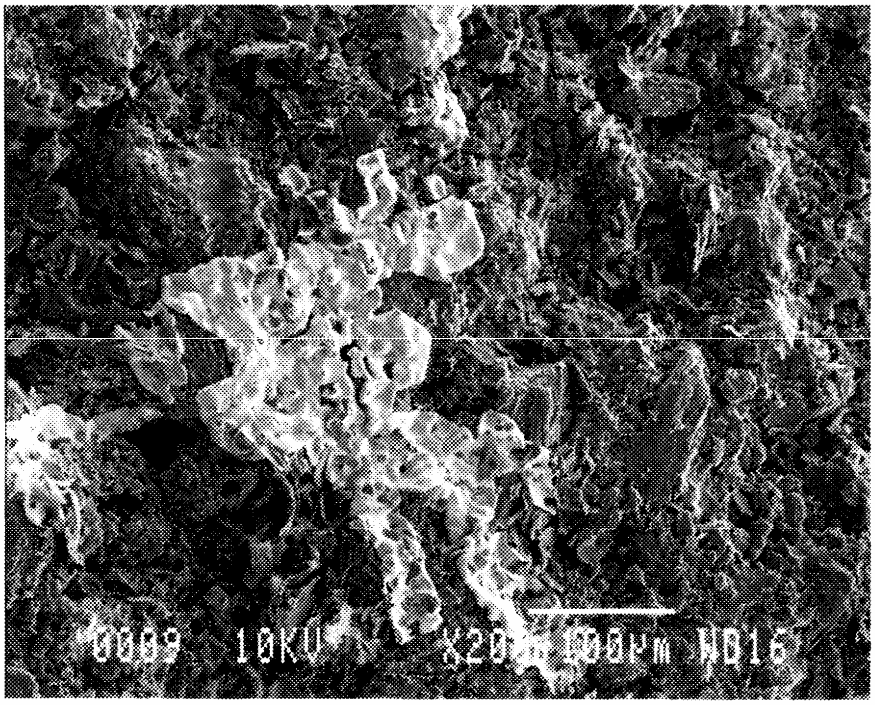
X-Ray analysis spectra from each sample, to show general elemental composition, and composition of specific crystals and deposits (as labelled) are provided.



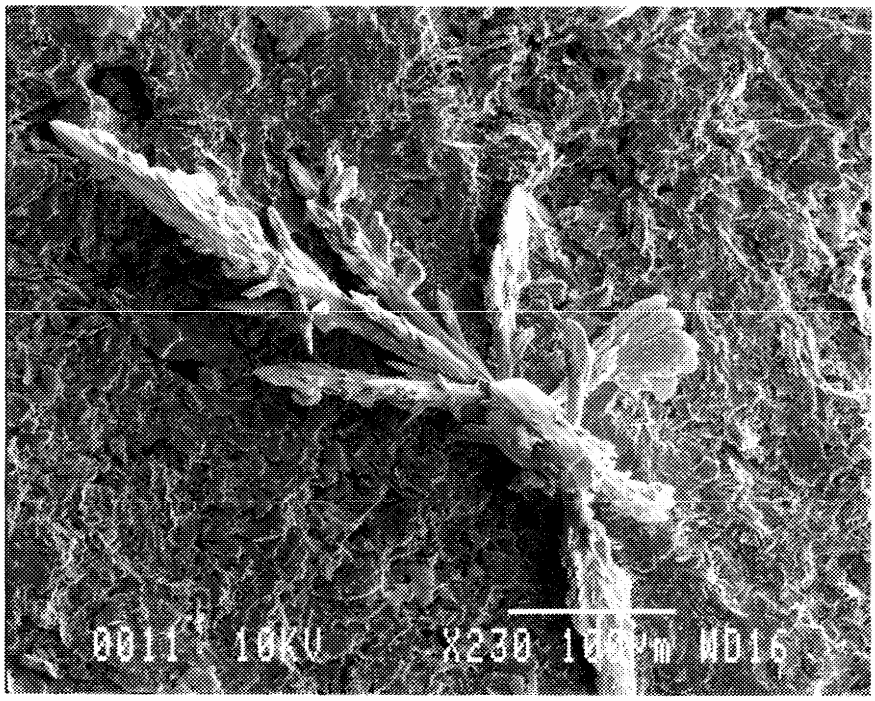
G1 11F



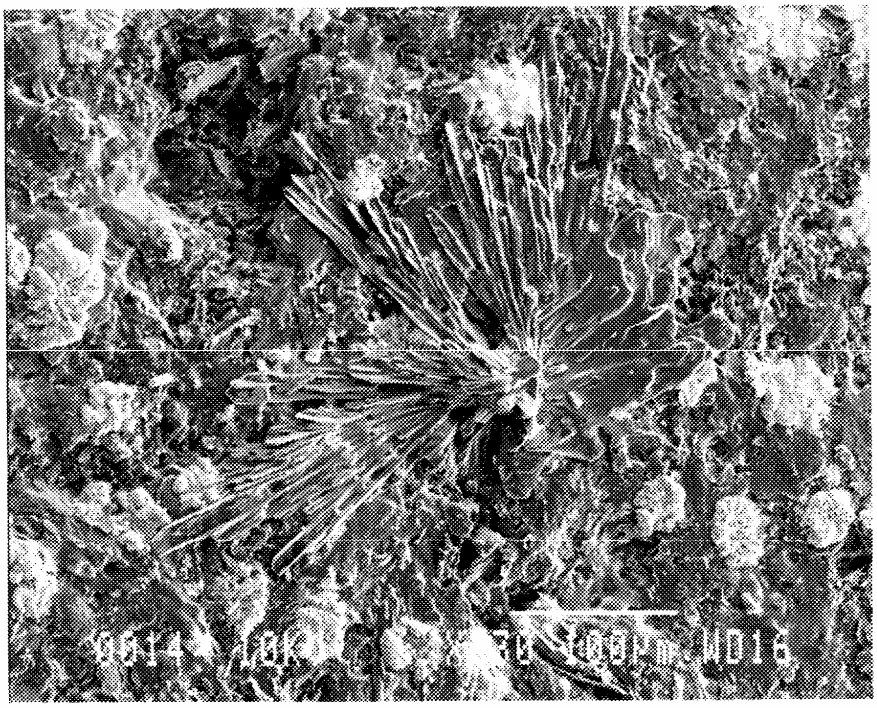
G1 13F light deposit



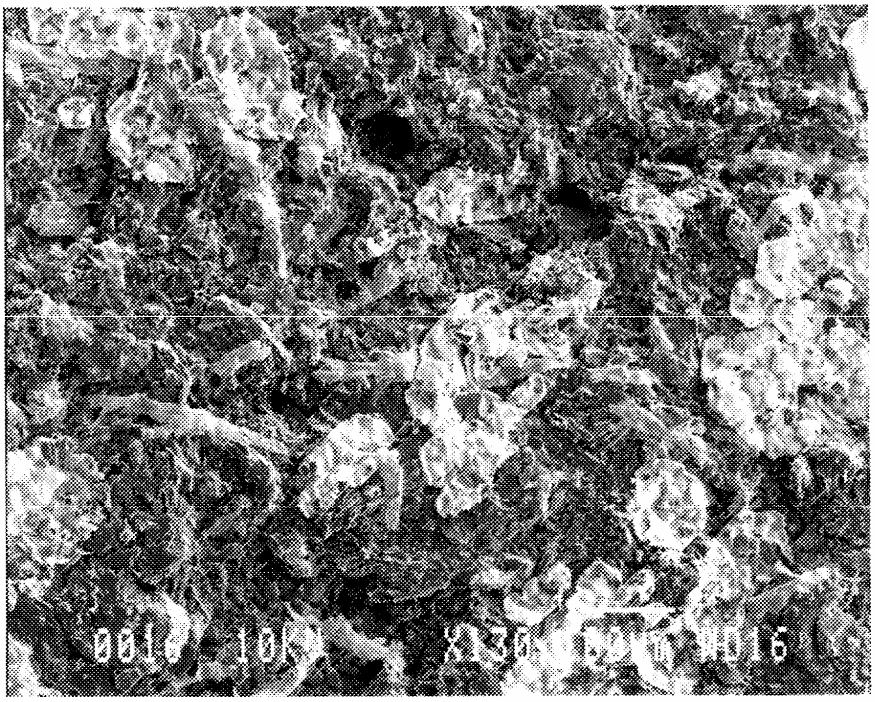
G1 14F



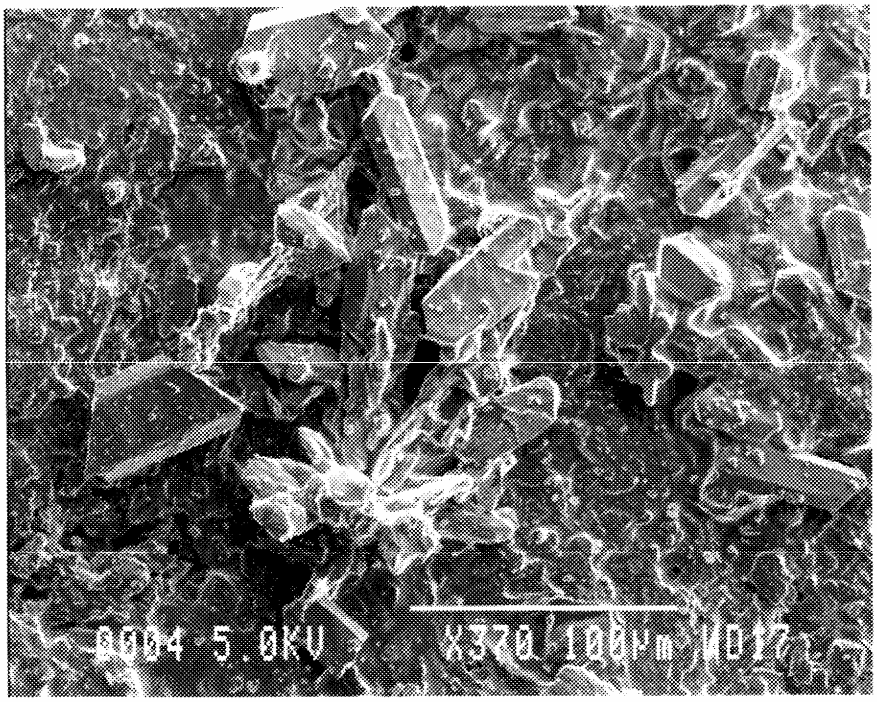
G1 15F



G2 16F

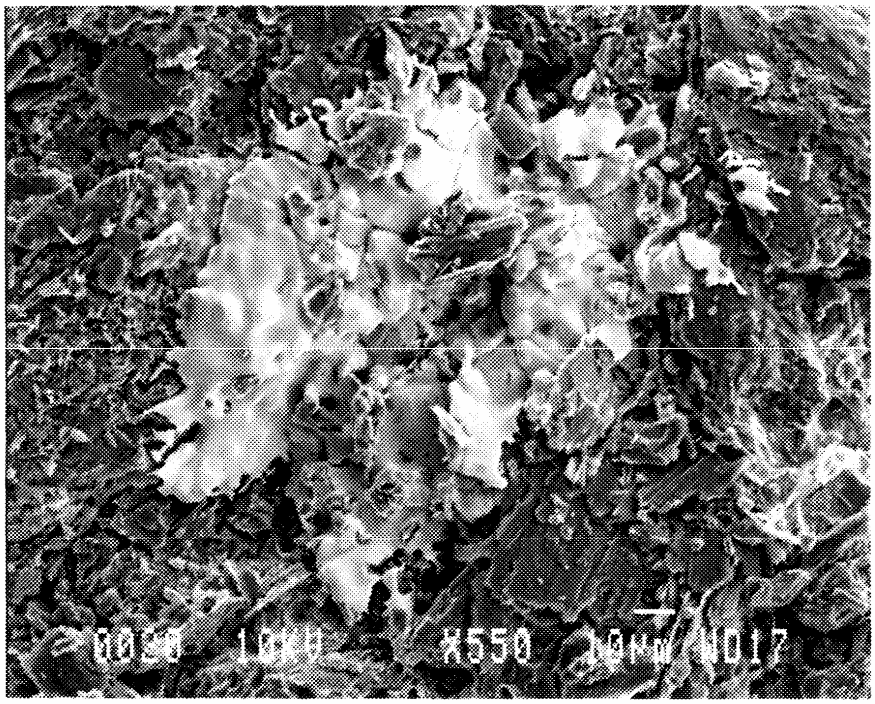


G2 17F

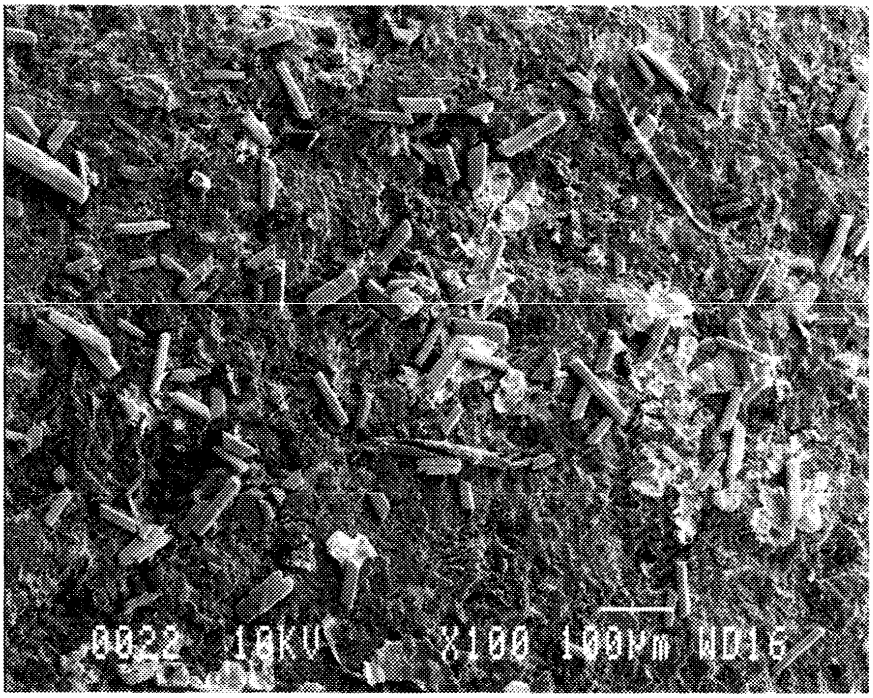


G2 7F

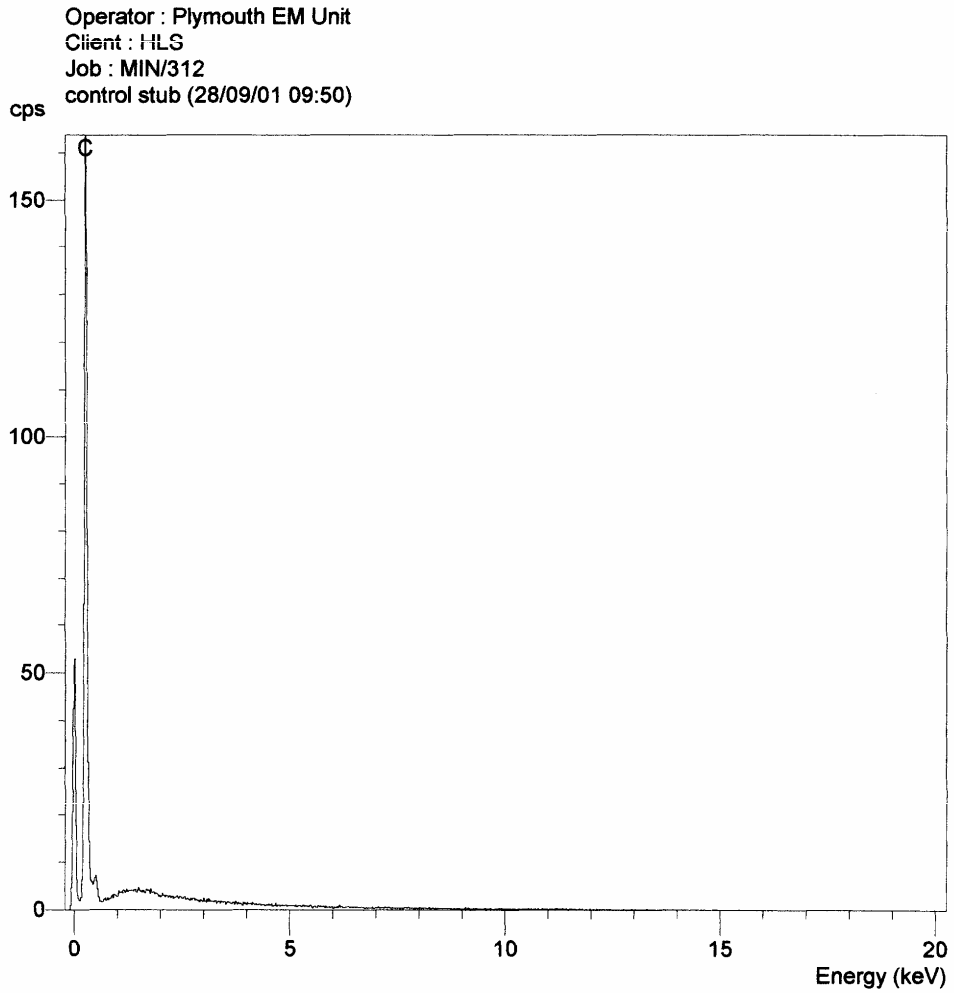
G2 18F



G2 19F



G2 20F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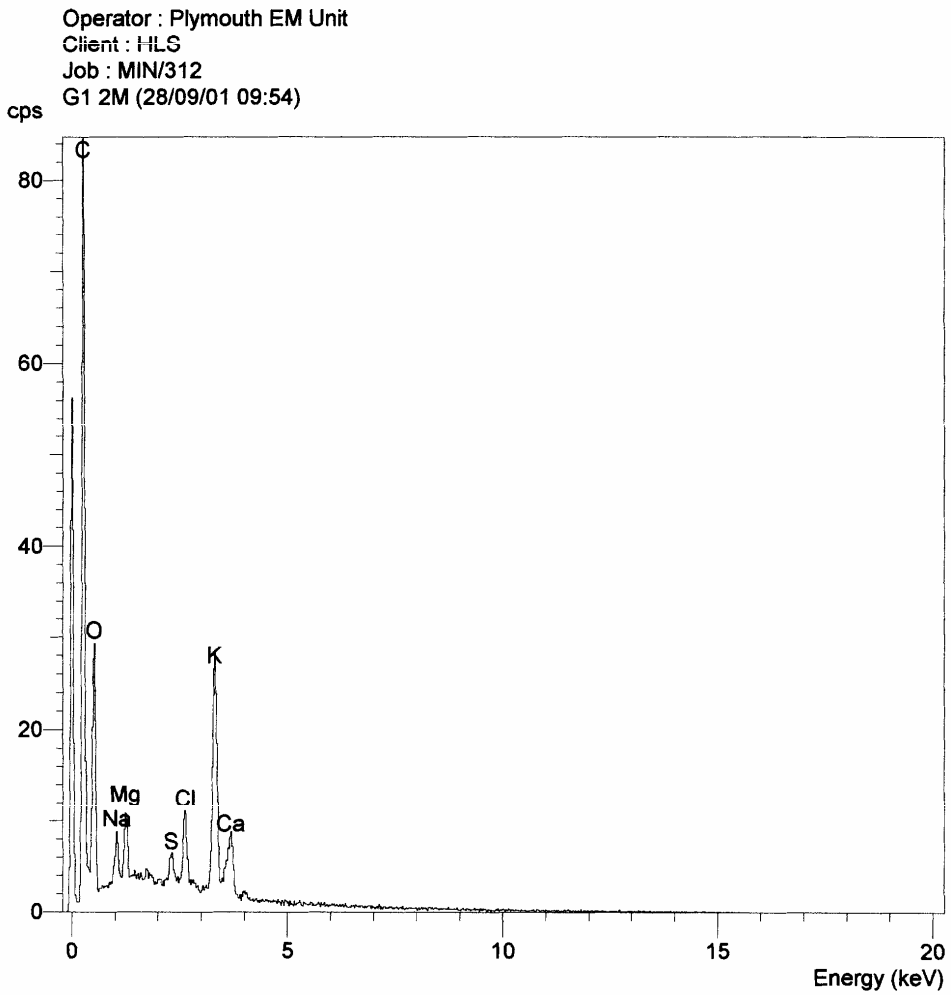
cps
C
150
100
50
0
0
5
10
15
20
Energy (keV)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Client : HLS
Job : MIN/312
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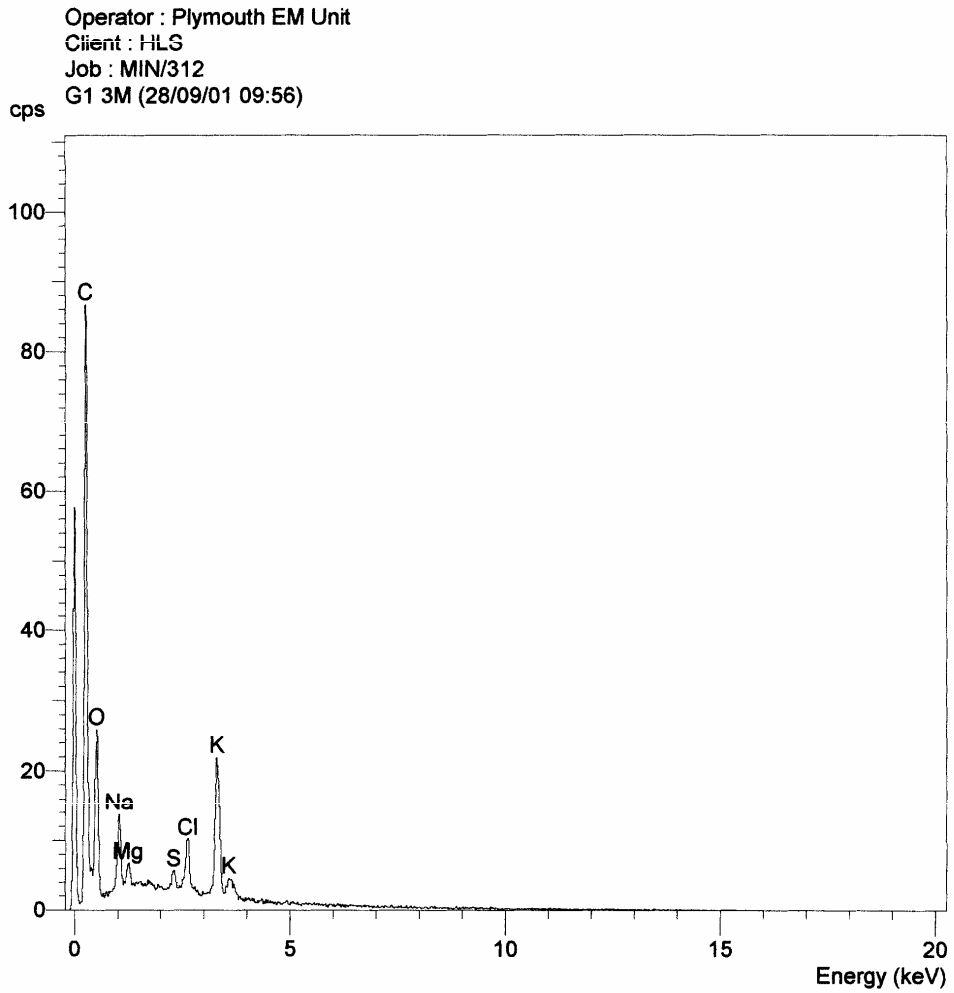
cps
80
60
40
20
0
C
O
Mg
Na
S
Cl
K
Ca
0
5
10
15
20
Energy (keV)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cps
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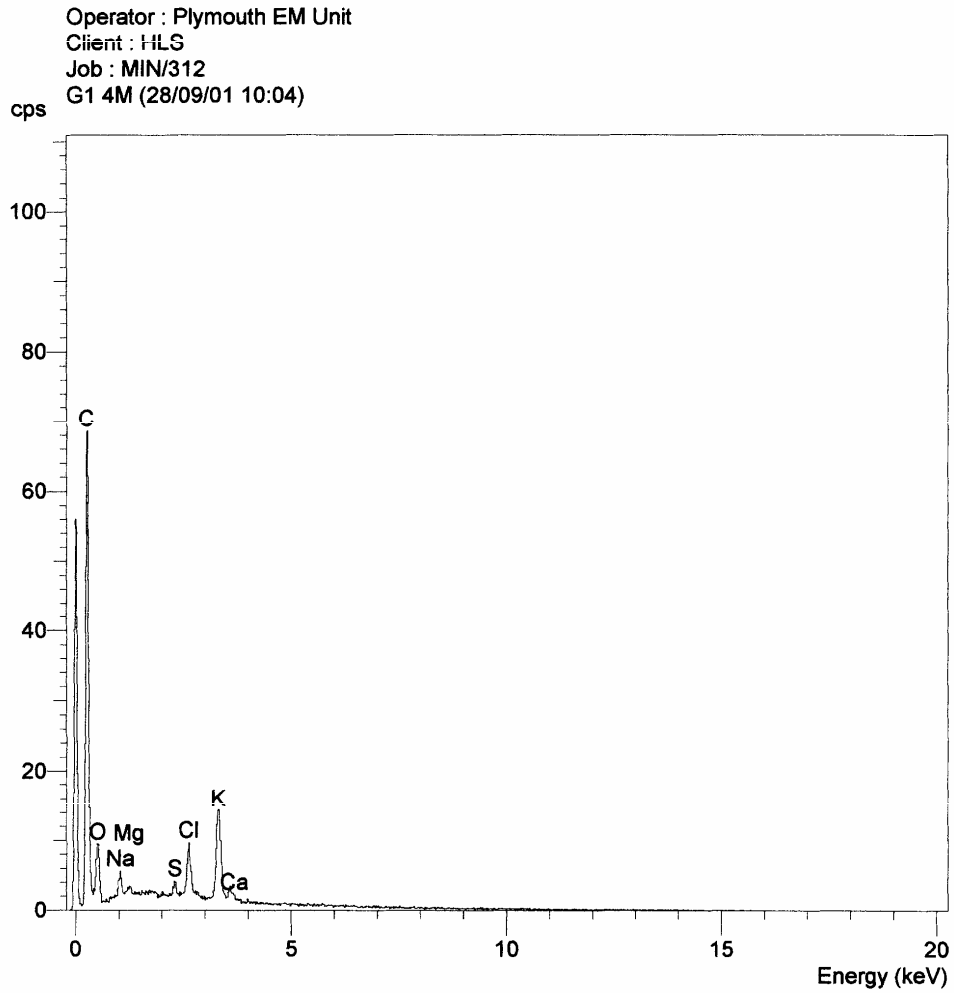
20
0
C
O
Na
Mg
S
Cl
K
K
0
5
10
15
20
Energy (keV)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Job : MIN/312
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cps
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40
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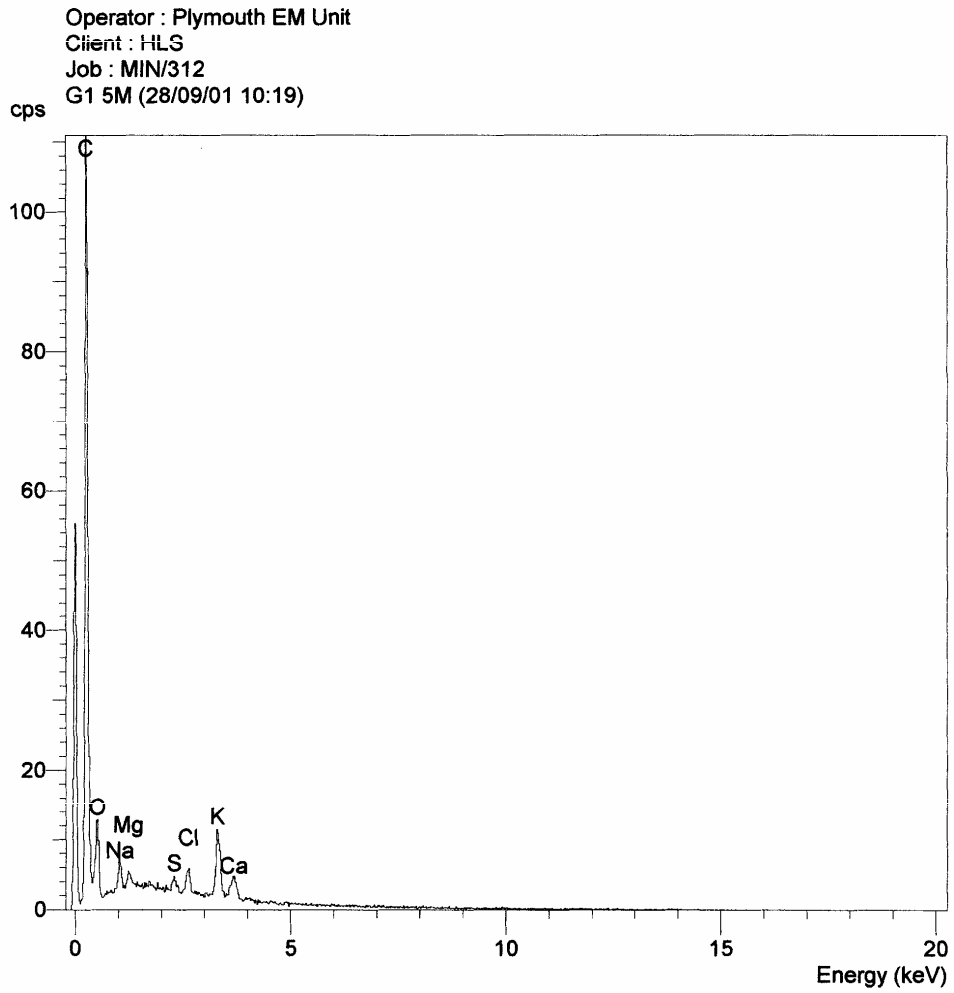
0
C
O
Mg
Na
S
Cl
K
Ca
0
5
10
15
20
Energy (keV)


Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G1 5M (28/09/01 10:19)
cps
C
O
Mg
Na
S
Cl
K
Ca
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80
60
40
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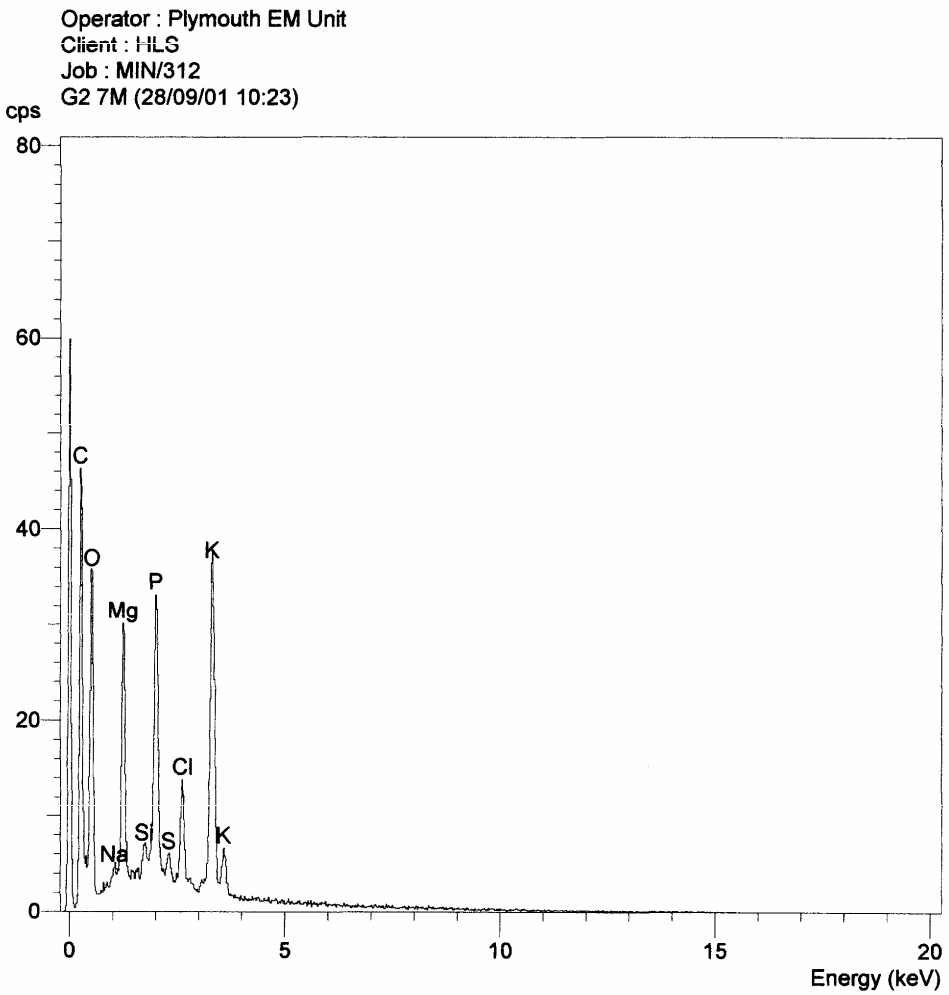
0
0
5
10
15
20
Energy (keV)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Job : MIN/312
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cps
80
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Mg
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Si
P
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K
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0
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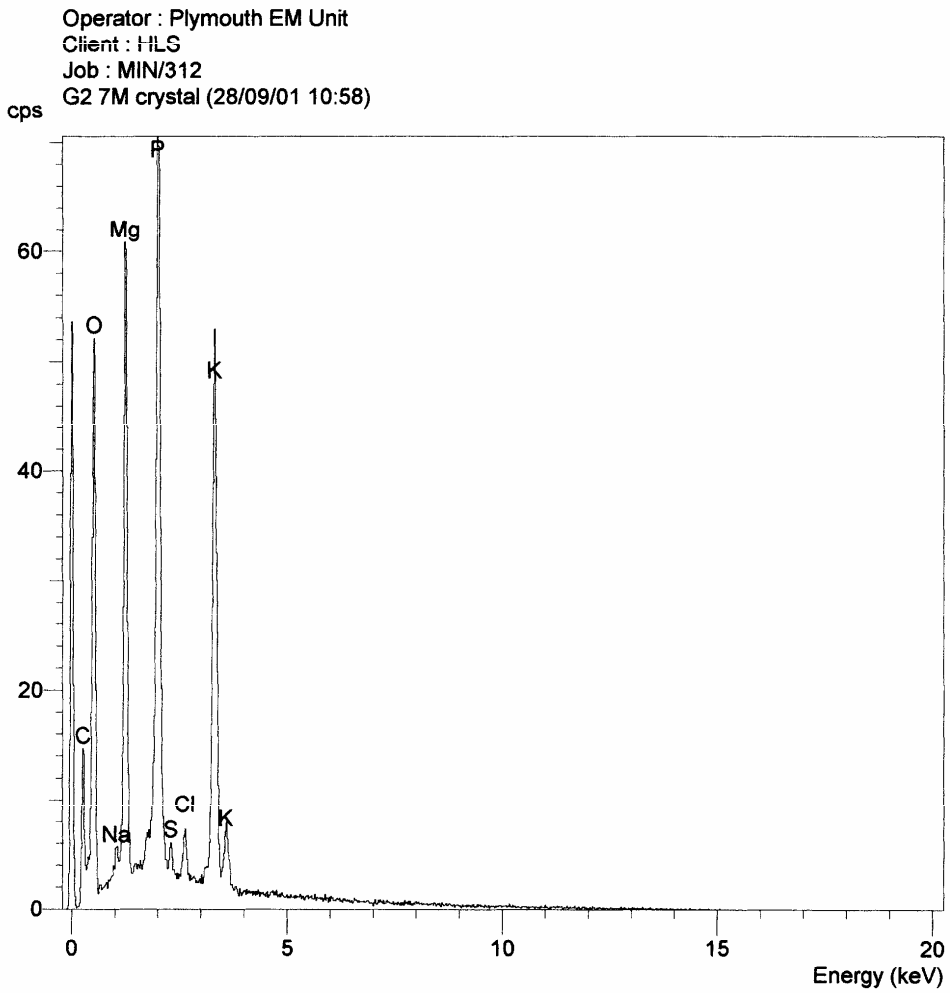
20
Energy (keV)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Job : MIN/312
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cps
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P
Mg
O
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Na
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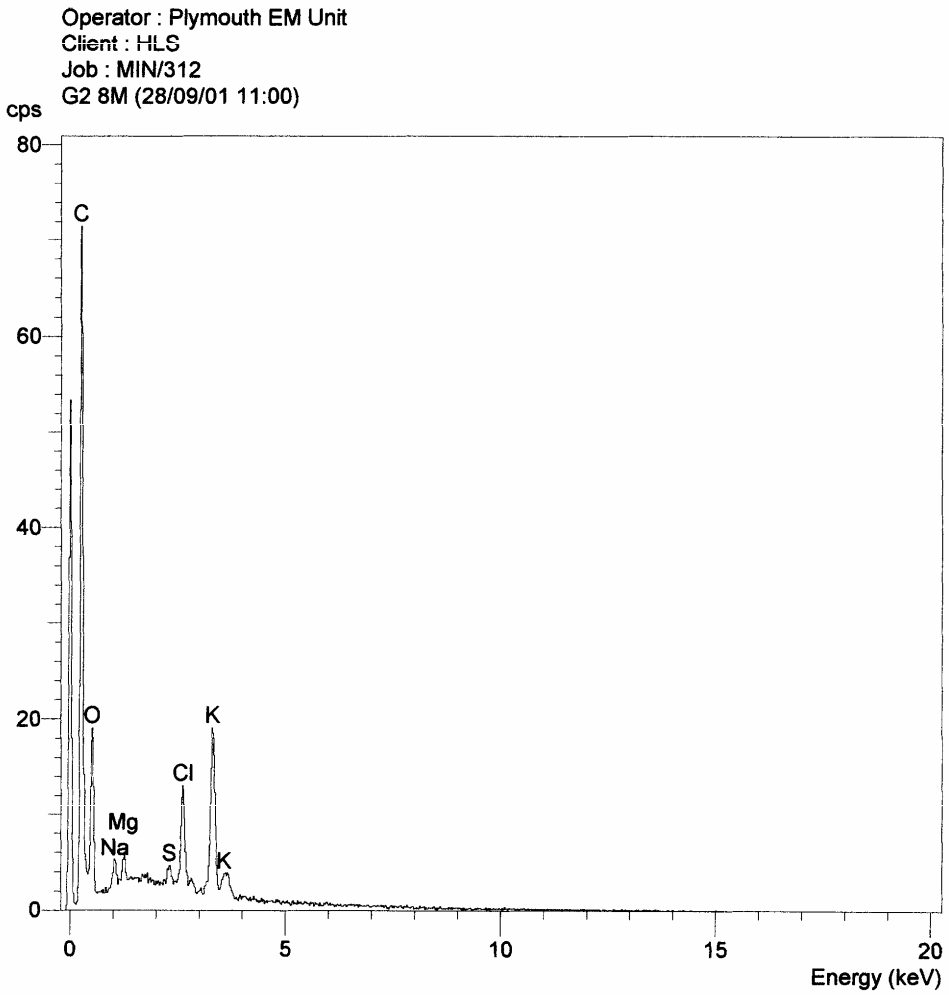
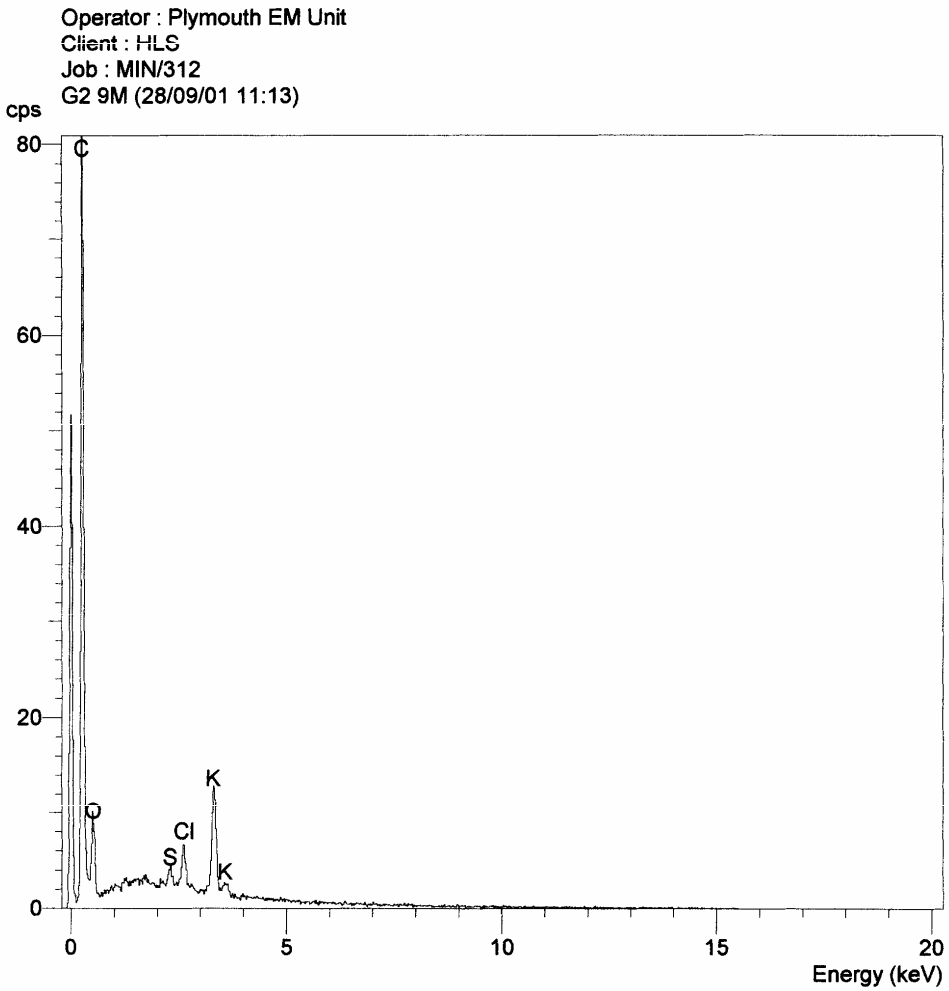
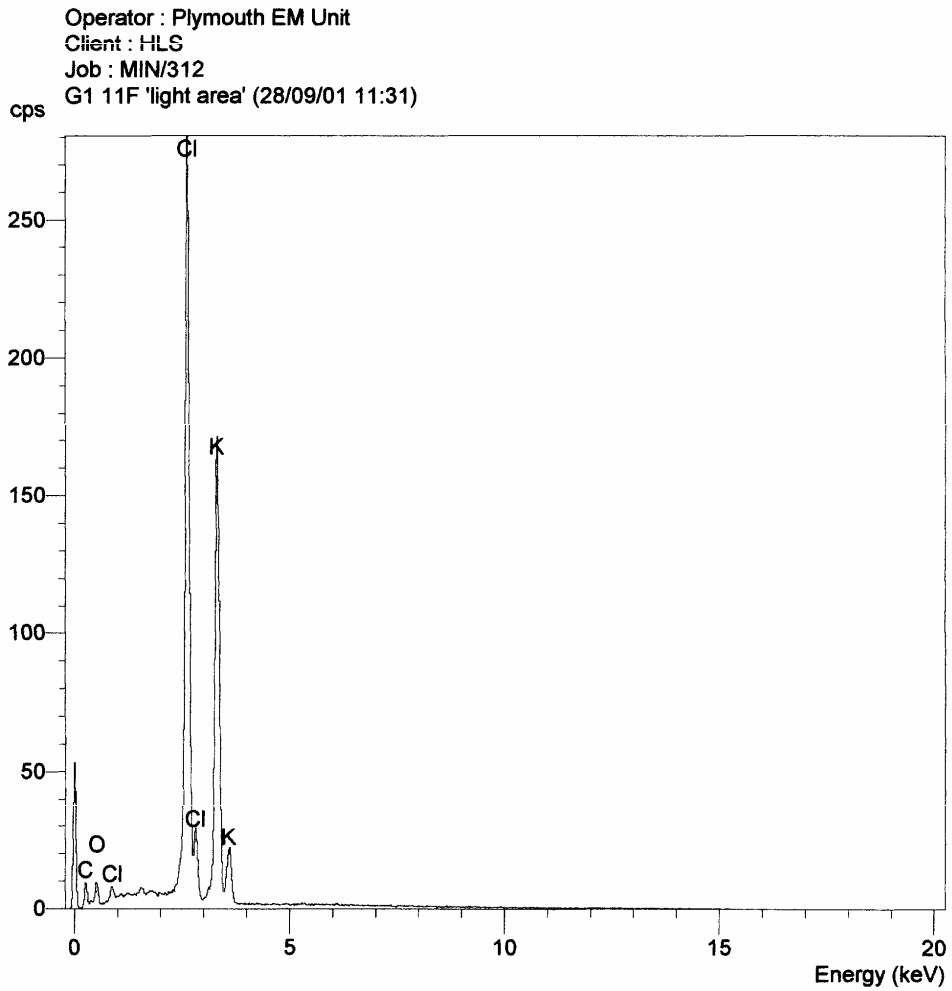
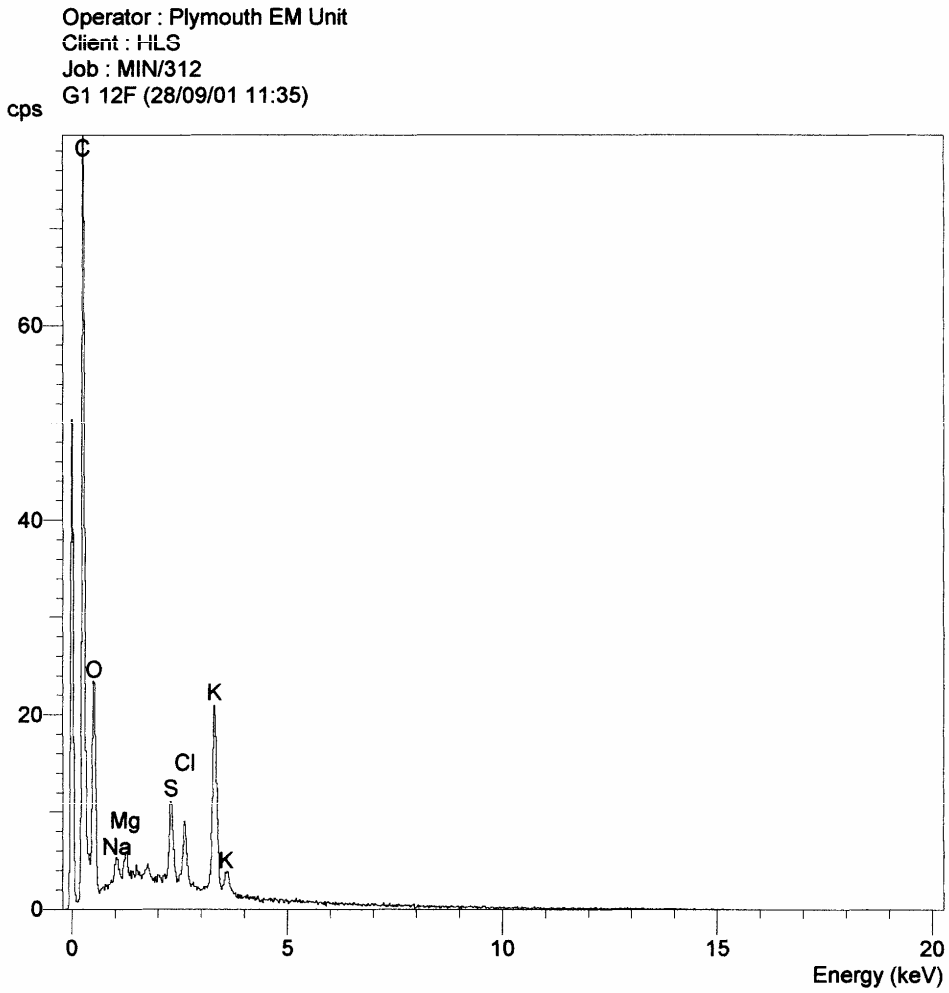
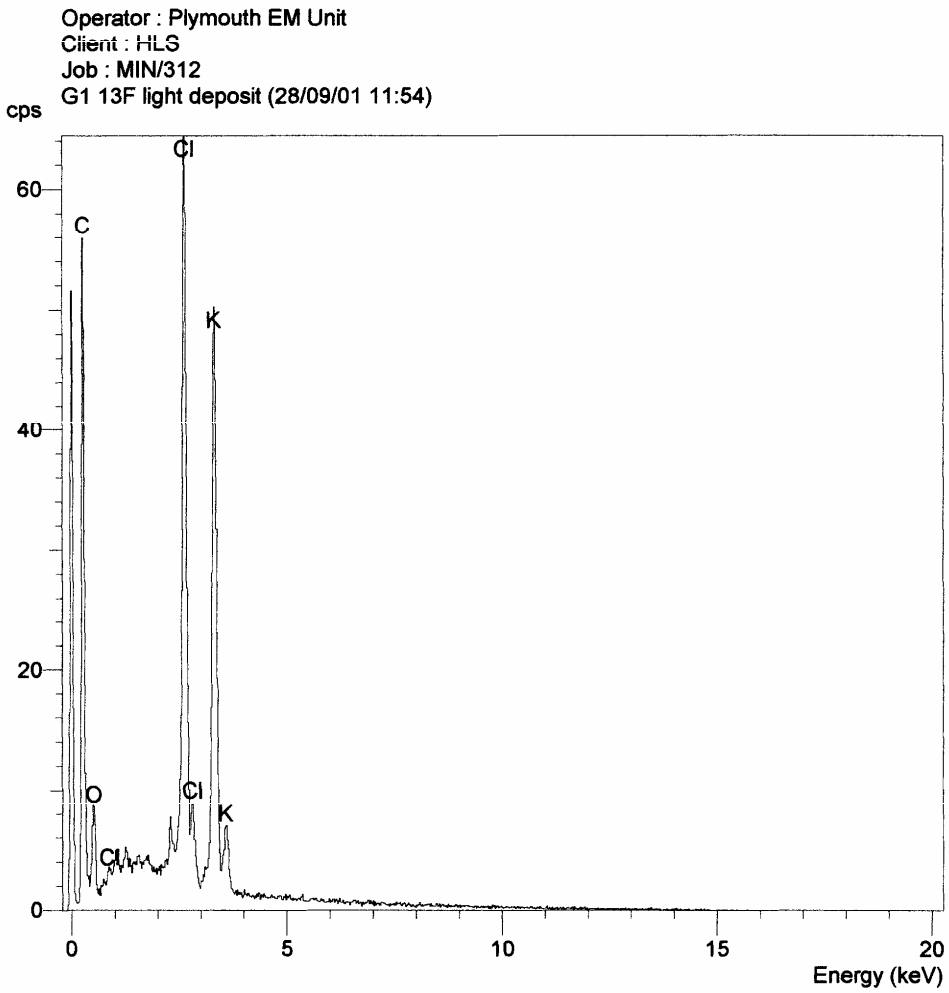
Cl
K
0
5
10
15
20
Energy (keV)

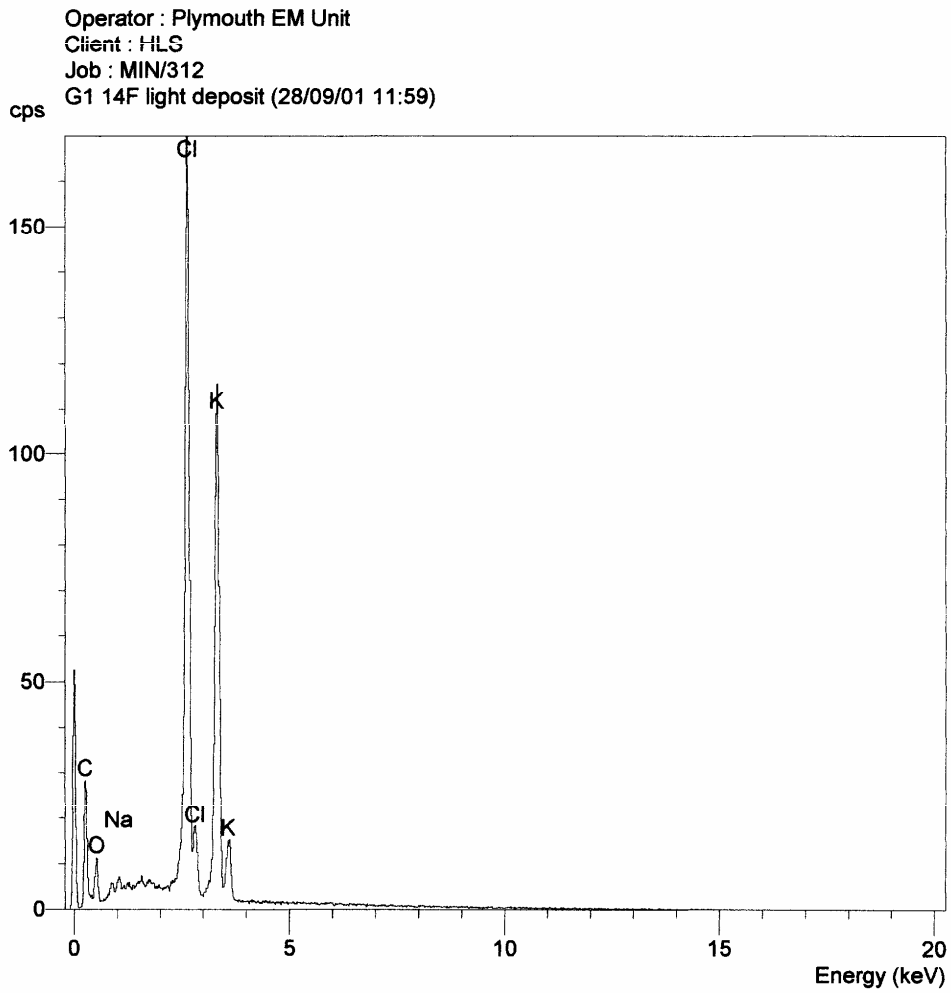

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 8M (28/09/01 11:00)
cps
80
60
40
20
0
C
O
Mg
Na
S
Cl
K
K
0
5
10
15
20
Energy (keV)

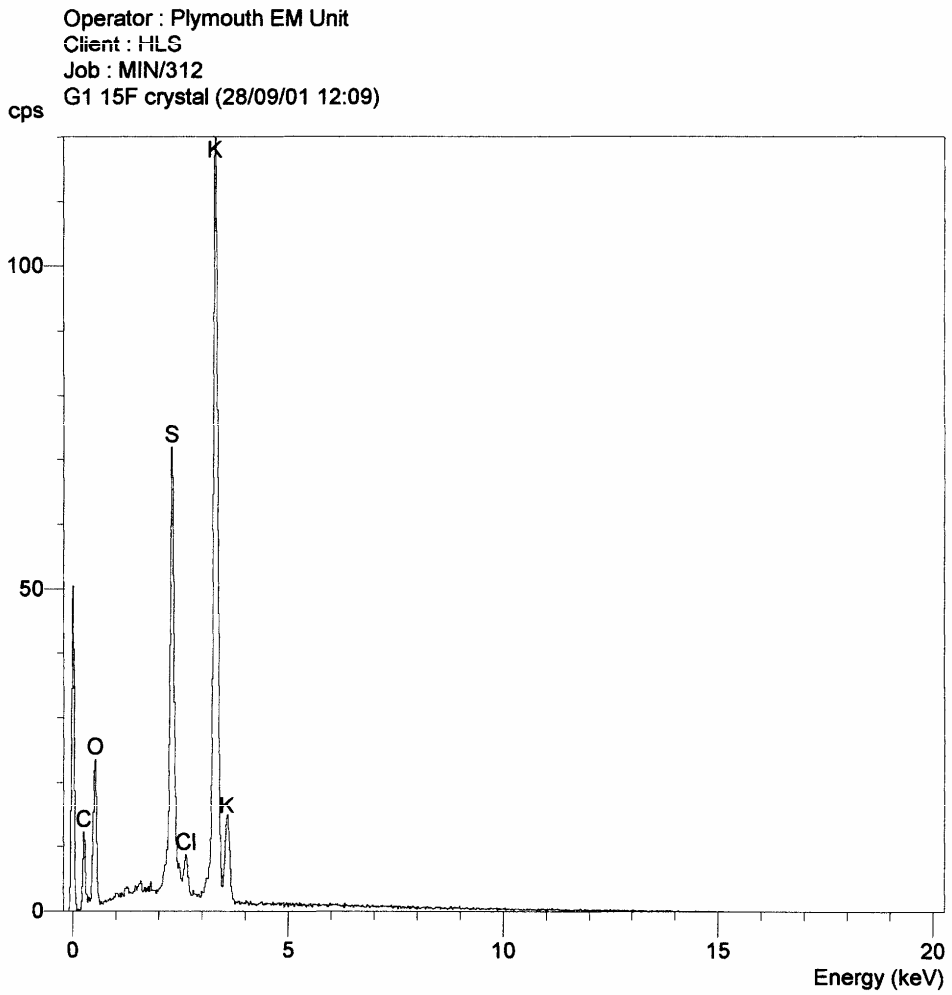

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 9M (28/09/01 11:13)
cps
80
60
40
20
0
C
O
S
Cl
K
K
0
5
10
15
20
Energy (keV)

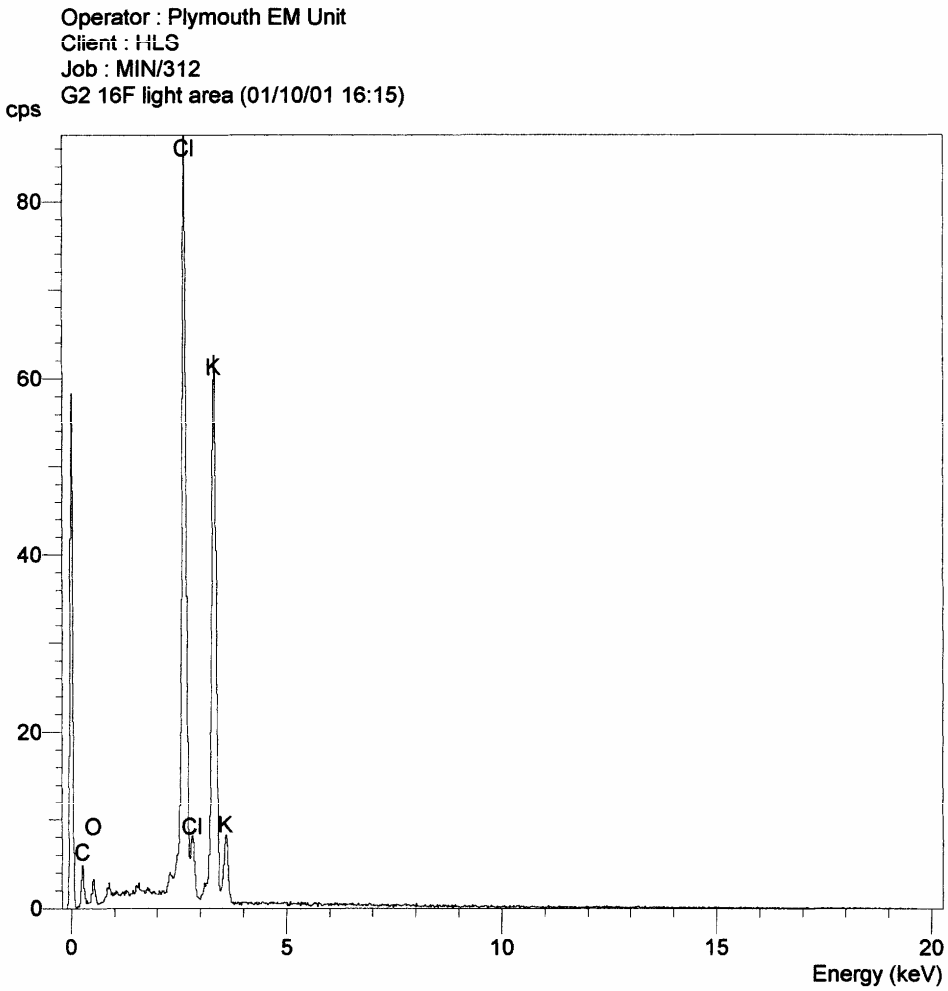

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G1 11F 'light area' (28/09/01 11:31)
cps
250
200
150
100
50
0
Cl
K
O
C
Cl
Cl
K
0
5
10
15
20
Energy (keV)

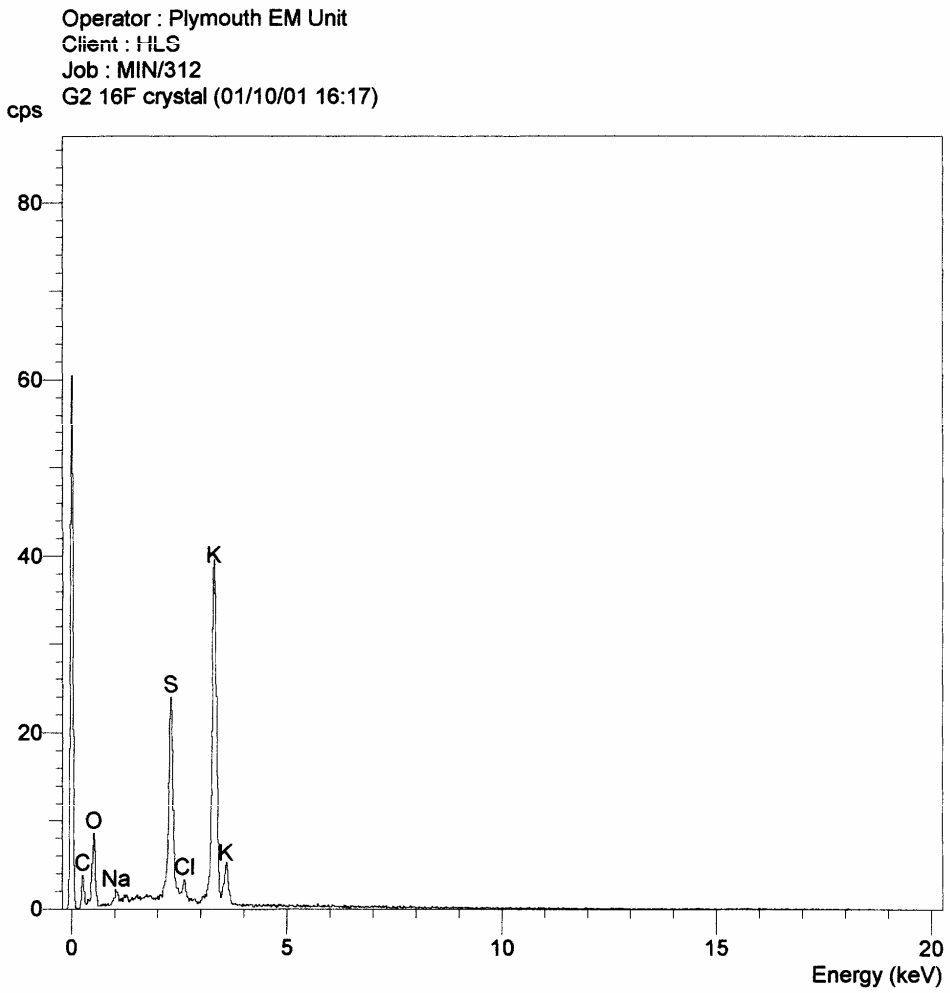

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G1 12F (28/09/01 11:35)
cps
C
O
Mg
Na
S
Cl
K
K
60
40
20
0
0
5
10
15
20
Energy (keV)

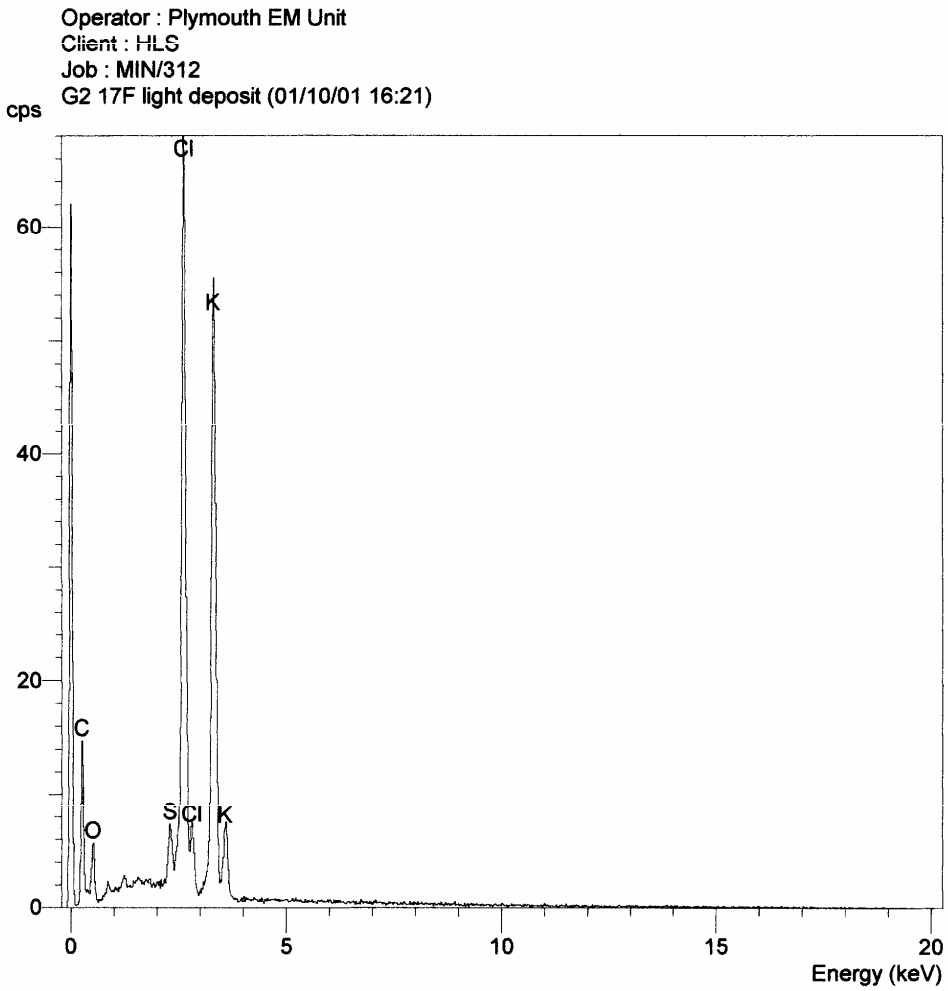

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G1 13F light deposit (28/09/01 11:54)
cps
60
40
20
0
C
O
Cl
Cl
K
Cl
K
0
5
10
15
20
Energy (keV)

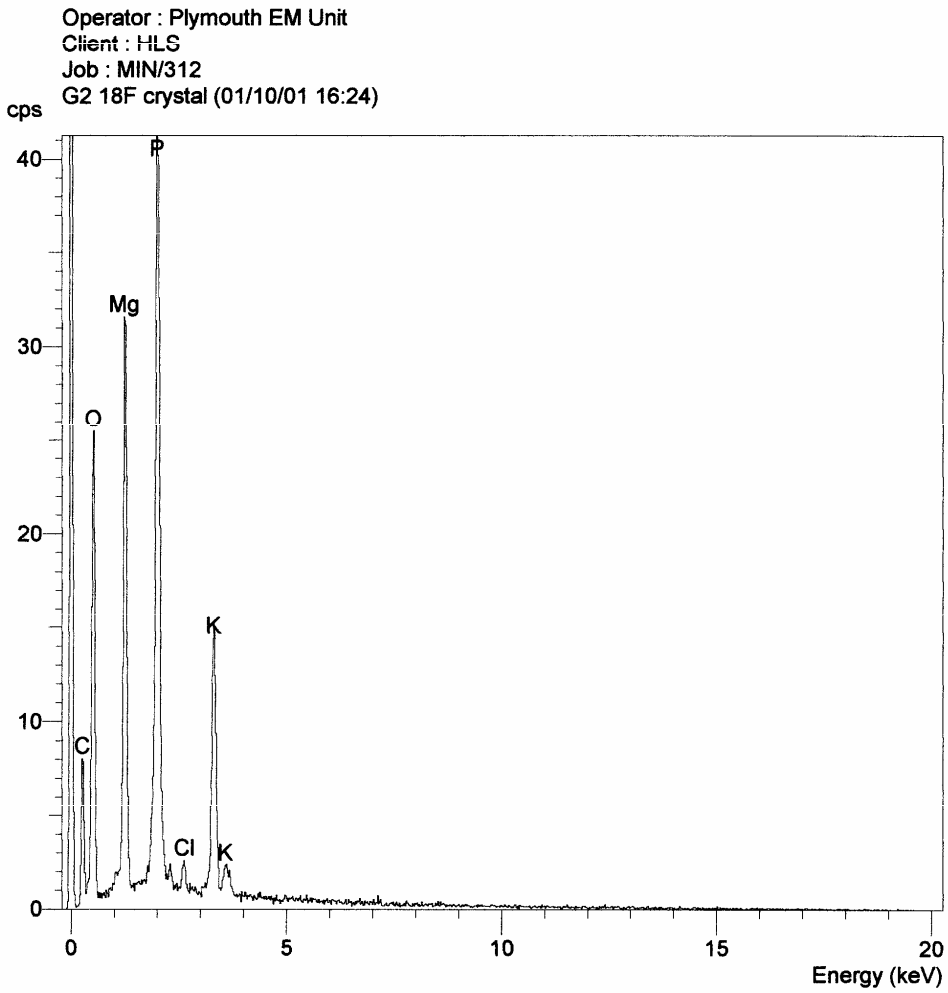

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G1 14F light deposit (28/09/01 11:59)
cps
150
100
50
0
Cl
K
C
Na
O
Cl
K
0
5
10
15
20
Energy (keV)

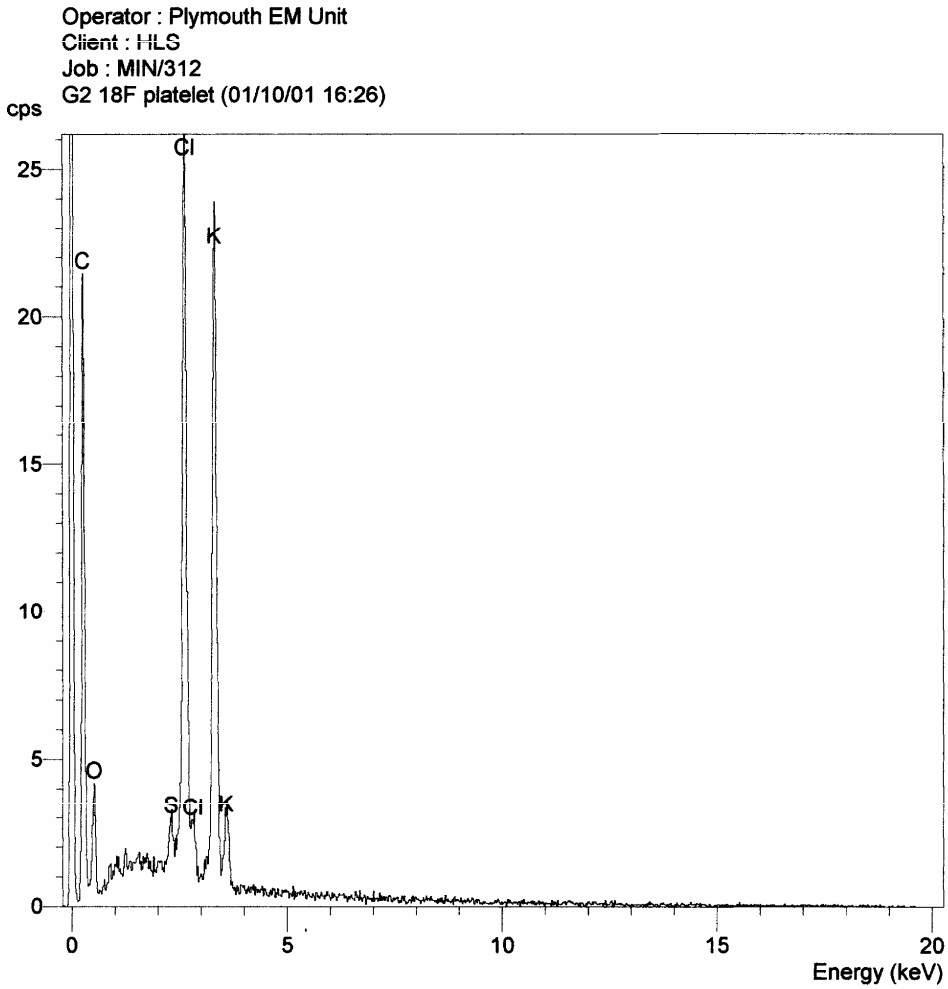

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G1 15F crystal (28/09/01 12:09)
cps
100
50
0
K
S
O
C
Cl
K
0
5
10
15
20
Energy (keV)

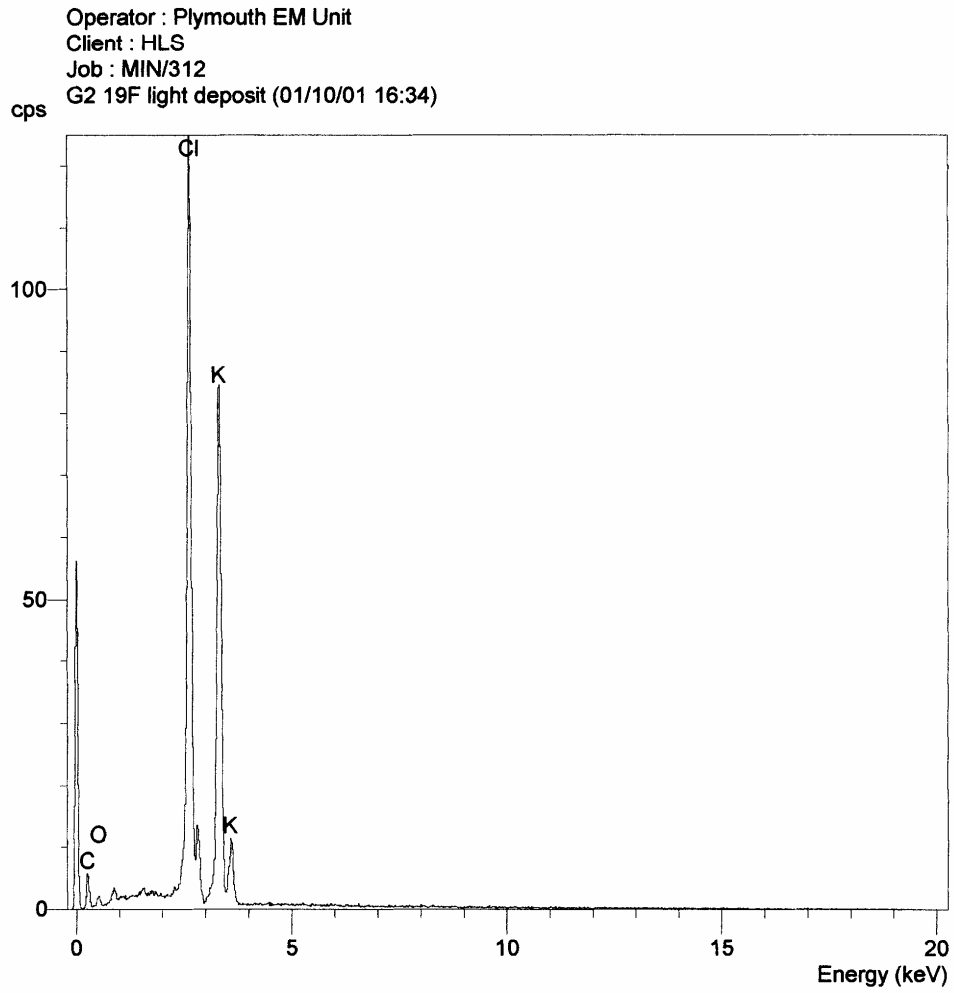

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 16F light area (01/10/01 16:15)
cps
80
60
40
20
0
Cl
K
O
C
Cl
K
0
5
10
15
20
Energy (keV)

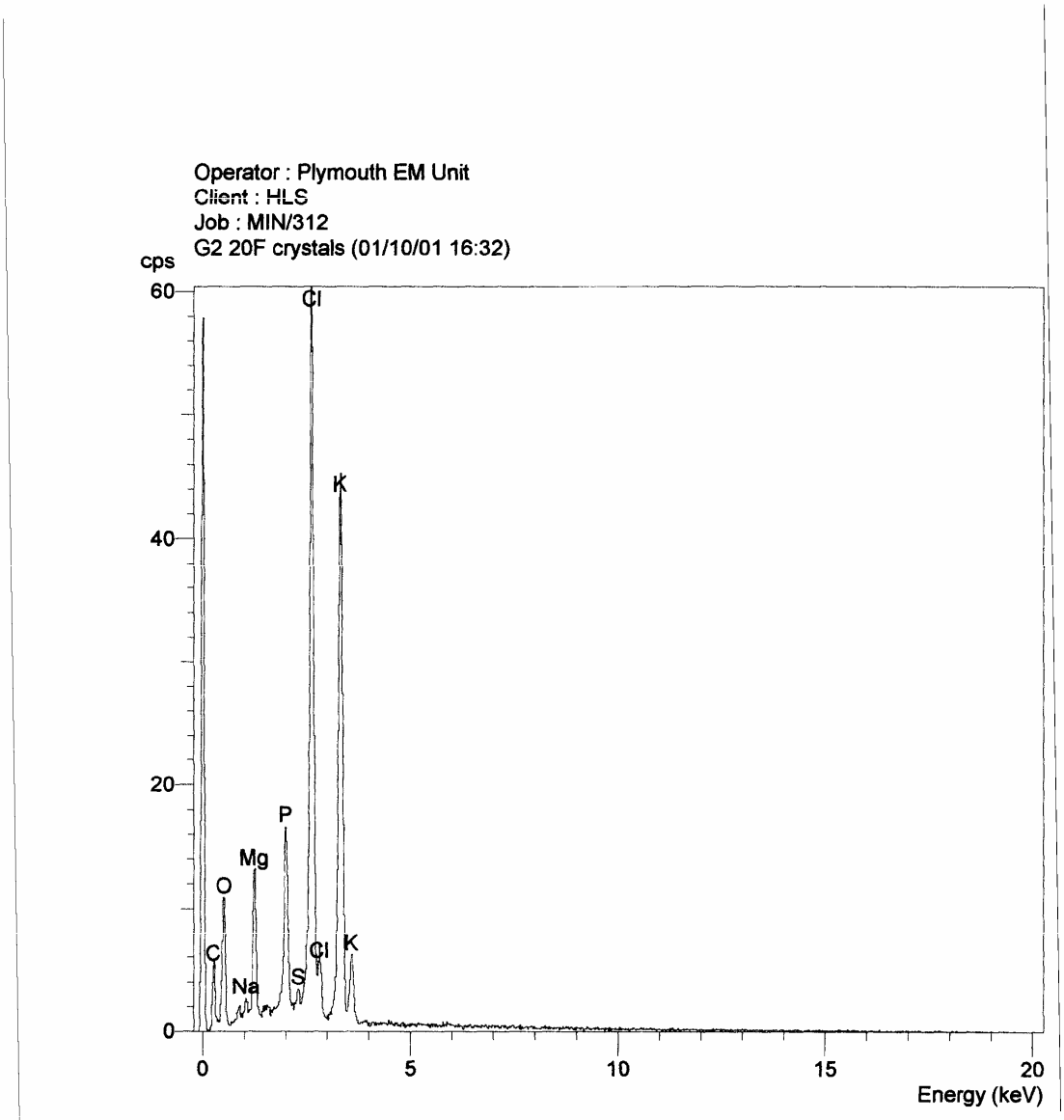

Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 16F crystal (01/10/01 16:17)
cps
80
60
40
20
0
0
5
10
15
20
Energy (keV)
C
O
Na
S
Cl
K
K


Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 17F light deposit (01/10/01 16:21)
cps
60
40
20
0
Cl
K
C
O
S
Cl
K
0
5
10
15
20
Energy (keV)


Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 18F crystal (01/10/01 16:24)
cps
40
30
20
10
0
P
Mg
O
C
Cl
K
K
0
5
10
15
20
Energy (keV)


Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 18F platelet (01/10/01 16:26)
cps
25
20
15
10
5
0
C
O
Cl
K
S
Cl
K
0
5
10
15
20
Energy (keV)


Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 19F light deposit (01/10/01 16:34)
cps
Cl
K
O
C
K
100
50
0
0
5
10
15
20
Energy (keV)


Operator : Plymouth EM Unit
Client : HLS
Job : MIN/312
G2 20F crystals (01/10/01 16:32)
cps
60
40
20
0
Cl
K
P
Mg
O
C
Na
S
Cl
K
0
5
10
15
20
Energy (keV)

HUNTINGDON RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2001

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Huntingdon Research Centre** **Wooley Road** **Alconbury** **Huntingdon** **Cambs.** **PE28 4HS**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION
15th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger J. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

HUNTINGDON RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2003

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Huntingdon Research Centre** **Woolley Road** **Alconbury** **Huntingdon** **Cambs.** **PE28 4HS**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Toxicology** **Phys/Chem Tests**

DATE OF INSPECTION

07th April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander 1/8/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

HUNTINGDON RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2005

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY

Huntingdon Life Sciences
~~**Huntingdon Research Centre**~~
Woolley Road
Alconbury
Cambridgeshire
PE28 4HS

TEST TYPE

Analytical Chemistry
~~**Clinical Chemistry**~~
Ecosystems
Environmental Fate
Environmental Toxicity
Toxicology
Phys/Chem Testing

DATE OF INSPECTION

7th March 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Bryan J Wright 9/6/05

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority