Chapter 1: Literature Study

1.1 Metabolism of trace elements

1.1.1 Absorption

A major site for the absorption of the trace elements is the small intestine, with a net absorption of Cu, Fe, Mn and Zn from the large intestine also being reported (Grace & Clark, 1991).

The amount absorbed is influenced by the physiological status of the animal, mineral intake and dietary factors. During growth, pregnancy, and lactation, the demands for trace elements increases (Grace & Clark, 1991).

For many of the trace elements, such as Fe, Mn and Zn, the efficiency of absorption is inversely related to the mineral status of the animal, as homeostatic mechanisms operate to prevent the excessive absorption and tissue accumulation of the trace element. An exception is the liver, which acts as a store for Cu and vitamin B_{12} On the other hand there appears to be little control over the amount of Se absorbed, as blood and tissue Se levels reflect the intake of Se (Grace & Clark, 1991).

Dietary factors can greatly influence the absorption and utilization of trace elements. Interactions arise when elements of similar physical and chemical properties act as antagonists in biological systems. For example, when interactions occur between elements and lead to the formation of insoluble complexes (Grace & Clark, 1991).

1.1.2 Distribution of trace elements between tissues and organs

Trace elements are transported in the blood and stored in tissues in association with proteins and amino acids.

Table 1.1.1 Distribution of Cu, Fe, Mn, Mo, Se, and Zn between various organs and tissues of a 40 kg sheep with a 3 kg fleece [a]

Body part	Cu	Fe	Mn	Mo	Se	Zn
Brain	0.15	0.08	0.11	0.10	0.46	0.07
Lung	0.75	2.53	0.32	2.71	2.63	0.46
Heart	0.45	0.42	0.20	0.15	0.99	0.16
Spleen	0.04	0.54	0.07	0.15	0.48	0.09
Kidney	0.31	1.04	0.58	2.94	6.55	0.16
Pancreas	0.04	0.06	0.38	0.26	0.46	0.05
Liver	65.12	3.99	11.97	62.20	4.40	1.55
Blood	1.68	37.52	0.54	-	8.57	1.64
Digestive tract	2.13	7.32	20.02	5.25	10.08	2.48
Muscle	11.56	24.59	7.88	9.16	33.51	32.15
Bone	0.88	9.45	5.24	10.86	7.30	11.88
Skin	2.87	7.72	13.24	2.04	7.16	2.87
Wool	14.02	4.77	39.45	4.18	17.41	46.44

[a] Values given as % of total (Grace & Clark, 1991).

1.1.3 Excretion

The major route of excretion for most trace elements is the faeces, with smaller amounts being lost via the urine. Many of the trace elements such as Cu, Fe, Mn, and Zn are returned to the digestive tract via the saliva, bile, pancreatic juice and other intestinal secretions. The quantities of trace elements secreted in the digestive tract together with the amounts associated with the spent mucosal cells lining the gut, contribute to the faecal trace element endogenous loss (Grace & Clark, 1991).

1.1.4 Animal reserves

Animals have the ability to accumulate reserves of minerals during times of adequacy and to utilize these reserves during times of deficiency.

Reserves of Cu and vitamin B_{12} in liver, Se in muscle and liver and Ca and P in bone reduce the need to meet the daily requirements of these nutrients. For other minerals, the animal has limited tissue reserves (e.g. Mn and Zn) or limited readily available reserves (e.g. Mg) and hence is at risk of transient deficiencies in these minerals (Judson & McFarlane, 1998).

1.2 The three important principals of biochemical diagnosis: relationship with intake, time and function

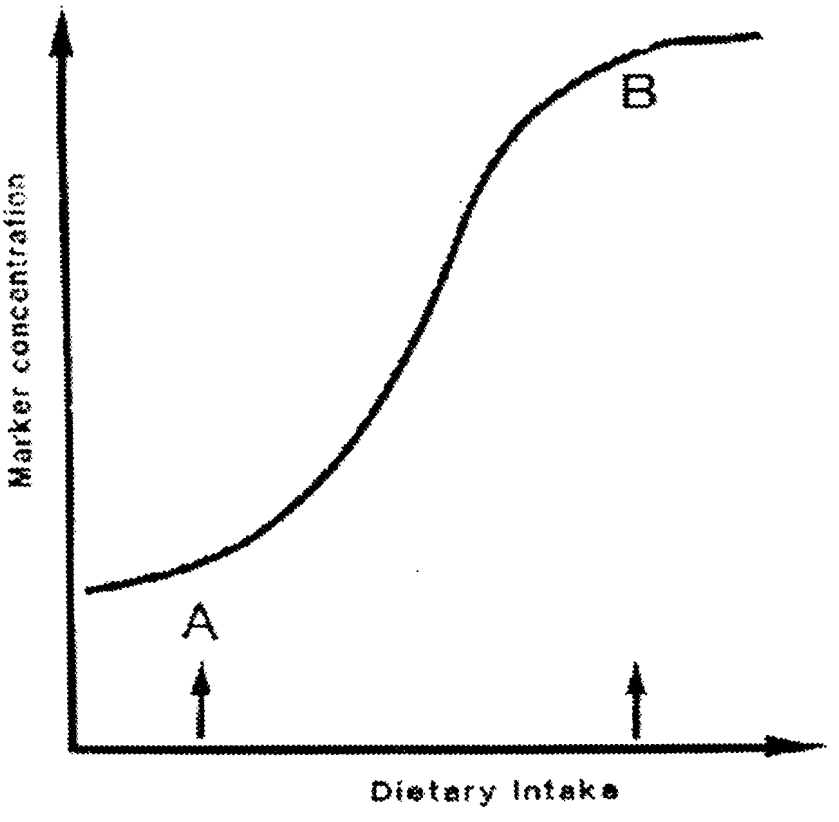

Figure 1.2.1 Schematic representation of the relationship between a direct biochemical marker of trace element status in blood or tissues and the intake of the element at a fixed time. A and B denote alternative hypothetical points at which the animal's requirement for the element is just met (Suttle, 1986)

1.2.1 Relationship with trace element intake

The relationship between the tissue concentration of a 'direct' (i.e. trace-element based) marker and the intake of the element will generally be sigmoidal in shape (Fig. 1.2.1) and the important point on the curve is the intake at which the animal's requirement for the trace element is passed.

For several markers of trace element status, the position on the X-axis at which the requirement is passed coincides with the end of the lower plateau of the response in marker concentration (A. Fig. 1.2.1). Under these conditions the marker is an excellent index of surfeit and the extent of body reserves but an insensitive index of a deficiency of the nutrient. If requirement is passed at B at the beginning of the upper plateau, however, the marker is a poor index of surfeit but a good index of deficiency. This first principal allows direct markers to be divided into 'storage' and 'non-storage' types, corresponding to A and B respectively (Suttle, 1986).

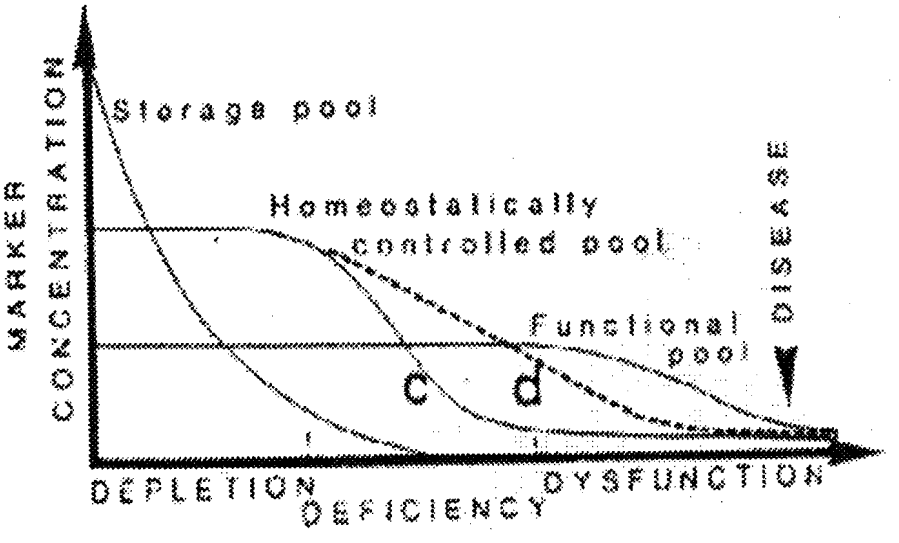

Figure 1.2.2 Schematic representation of the relationship between direct and biochemical markers of trace element status in blood or tissues and the duration of a dietary deficiency (Suttle, 1983). Two types of response in blood components are indicated, rapid exponential and slow linear (Suttle, 1986)

1.2.2 Relationship to time

A second principal allows 'non-storage' criteria to be divided into indicators of acute and chronic deficiency. Two types of relationships can be distinguished, a rapid early decline in marker concentration followed by a plateau (c) and a slow linear rate of decline (d) (Fig. 1.2.2). Markers showing slow linear responses (d) will be good indices of chronic deficiency but bad indices of acute deficiency because they can't respond quickly enough. Conversely, the marker showing a rapid early decline (c) will be a good index of acute deficiency but a poor indicator for chronic deficiency if the low plateau is reached before functions are impaired. Those biochemical criteria which are based on metallo-enzyme or metallo-protein concentrations in erythrocytes are of type d because the marker is incorporated into the erythrocyte before its release into the bloodstream and thereafter its half-life is determined by that of the erythrocyte (150 days or more). Metallo-enzymes or metallo-proteins in the plasma, which have short half-lives, provide markers of type c (Suttle, 1986).

1.2.3 Relationship to function

The development of a deficiency can be divided into 4 phases, depletion, deficiency (marginal), dysfunction and disease (Fig. 1.2.2). During the depletion phase there is a loss of trace element from any storage sites such as the liver and the plasma concentrations of the trace element may remain constant. Eventually, however, there are departures from normality and concentrations of the nutrient in the circulation may decline during the second phase of marginal deficiency. It may be some time before the concentrations or activities of trace-element-containing enzymes in the tissues begin to decline and it is not until this happens that the phase of dysfunction is entered. There may be a further lag before the changes in cellular function are manifested as clinical signs of disease. Biochemical criteria can be divided, according to the phase during which they change, into indicators of marginal deficiency and dysfunction. Few 'direct' markers are reliable indicators of dysfunction and for this purpose indirect markers, that reflect metabolic disturbance, are often needed (Suttle, 1986).

1.3. Interpretation of biochemical criteria of trace element status

1.3.1. Copper

Low intakes of available Cu by ruminants are usually accompanied by an exponential decline in hepatic Cu reserves. Under severe Cu depletion, total liver copper declines by ± 50 % every 25 to 50 days (Bremmer & Mills, 1981). While the rate of release of hepatic Cu is normally sufficient to maintain plasma Cu within the normal range until liver Cu falls below 30 mg Cu/kg DM (dry matter), exceptions to this has been noted (Mills, 1987).

Copper is distributed evenly throughout the liver. It does not accumulate in the fetal liver of sheep to the same extent as in the fetal liver of cattle. Levels increase towards the end of gestation, and are equal to dams levels on a dry weight basis (Puls 1994).

Liver Cu concentrations are undoubtly a 'storage criterion' that indicates the state of depletion rather than deficiency (Suttle, 1986). There is no experimental basis for selecting a particular threshold for liver Cu concentration below which the performance and health of grazing livestock are likely to be impaired. There are probably wide bands (0.08 to 0.32 mmol Cu/kg liver DM), which can coincide with the marginally deficient state. Generally, Cu concentrations of below 20 – 30 mg Cu / kg liver DM and of < 0.5 mg/l plasma are used to indicate a Cu deficiency (Van Ryssen, 1997).

Low levels of Cu in the livers of cattle are the result of a primary deficiency, when the diet is inadequate, or a secondary (conditioned) deficiency when the dietary intake is sufficient, but the utilization of the copper is impeded, for example by the interaction of molybdenum and sulphate (Ehret *et al.*, 1975).

Copper concentrations in organs that do not store the element may be more helpful, decreasing only when the phase of dysfunction is approached. (Suttle, 1986; Van Ryssen, 1997).

An increase in the Cu concentrations in the kidney cortex above 0.4 mmol/kg DM indicates the likelihood of toxicity because values are normally kept within the narrow range of 0.2 to 0.3 mmol/kg DM (Suttle, 1986; Wooliams et al., 1986). It is thus possible that concentrations below 0.2 mmol/kg DM in the kidney may be a more reliable indicator of dysfunction or disorder than some arbitrary selected liver Cu concentration (Suttle, 1986).

The short half-life of the principal cupro-protein in blood plasma, caeruloplasmin, makes it a good indicator of acute Cu deficiency, but the many reports of hypocuraemic animals with no impairment of health or growth show that hypocupreamia generally indicates marginal deficiency and not dysfunction (Suttle, 1986).

The concentration of superoxide dismutase in the erythrocytes might give information on the state of dysfunction because it continued to show a linear decline after plasma Cu had reached a minimal concentration during chronic Cu deficiency in sheep and cattle (Suttle & McMurray, 1983; Suttle, 1986).

1.3.2 Cobalt

Cobalt is an essential trace element in the diet of ruminants. It is required for the synthesis of vitamin B_{12} (cobalamin) by ruminal microorganisms. Vitamin B_{12} constitutes the essential functional core of enzymes involved in propionate utilization and methyltransfer processes (Mills, 1987).

The concentration of Co in the livers of sheep and cattle is sufficiently responsive to changes in Co intake to assist in diagnosing disorders in the field, provided that care is taken during analysis. The assay of Co in liver by AAS is susceptible to interference from high Fe concentration (Puls 1994).

The liver usually has the highest concentrations of cobalt and is the principal storage organ for vitamin B_{12} (Smith, 1987).

Under experimental conditions, the relationship between Co and vitamin B_{12} in sheep liver has been studied (Andrews *et al.* 1959; Andrews *et al.* 1960). They found that Co concentrations reflect vitamin B_{12} concentrations only within limits and have limited diagnostic value (Andrews *et al.*, 1959; Andrews *et al.* 1960). The Co concentration in tissues is not of much value to indicate the vitamin B_{12} status of an animal (Van Ryssen, 1997). Studies of liver Vitamin B_{12} concentrations of animals in various stages of Co deficiency and of the proportion of the total liver Co present as the vitamin indicate that vitamin B_{12} concentrations are a more sensitive and reliable criterion of adequacy. The relationship between liver Co and liver vitamin B_{12} is curvilinear with a wide scatter at high liver Co concentrations, but the two correlate linearly and well over the lower liver Co concentrations, which have diagnostic significance (Underwood & Suttle, 1999).

The concentration of Vitamin B_{12} in liver and plasma is useful but include inactive analogues of cobalamin. Most probably, liver vitamin B_{12}, like serum vitamin B_{12} in the sheep, shows a storage type of response to Co supply and that neither is a sensitive index of deficiency (Suttle, 1983).

McMurray *et al.* (1986) suggested that the use of MMA increases the precision of diagnosing Co deficiency in ruminants, compared with the use of vitamin B_{12} analyses alone. Diagnosis is best made by the determination of both plasma B_{12} and MMA. Serum B_{12} levels drop below adequate some weeks before MMA levels increase (Puls 1994). A functional dimension can be introduced by reference either to the impaired metabolism of methylmalonic acid (MMA) (Gawthorne, 1968; Suttle, 1986) which is due to a deficiency of the adenosylcobalamin coenzyme which assists the isomerisation of MMA, or to whole body function as reflected by growth rate. McMurry *et al.* (1986) have presented more extensive information that confirms the likelihood of abnormally high MMA concentrations in plasma increases when serum vitamin B_{12} values fall below 220 pmol/l. Furthermore, MMA increased before clinical signs of disease, like a reduction in body weight and photosensitization, were seen.

1.3.3. Selenium

The common method of assessing Se status has been the analysis of whole blood since the principal Se-containing constituent of blood is the selenoenzyme, glutathione peroxidase (GPX), present in the erythrocytes. Whole blood Se and erythrocyte glutathione peroxidase concentrations are normally highly correlated and can serve the same diagnostic purpose (Anderson *et al.*, 1979; Suttle, 1986). Selenium concentrations and GPX activities in tissues, such as muscle and liver, reflect dietary Se supply and may correlate well with concentrations in blood, depending on the duration of deprivation. Most of the Se in liver is in the storage form, GPX1, reductions in concentration thus reflect depletion more than deficiency (Underwood & Suttle, 1999).

The range of Se concentrations in whole blood and liver, which is useful to indicate Se status of animals, is much wider than that of the commonly used Se concentration in serum or plasma. The GPX activity in erythrocytes is useful, but the analytical technique is difficult to standardize among laboratories (Van Ryssen, 1997).

In the study of Van Ryssen *et al.* (1998) the liver showed the greatest reaction of all the tissues that have been investigated (whole blood, plasma, hart, liver, kidneys and muscle). It was concluded that changes in the bio-availability of Se was best reflected in the liver (Van Ryssen *et al.*, 1998).

The simple threshold Se values can not be used universally because adequacy will depend firstly, upon the extent to which vitamin E substitutes for Se in providing protection from oxidant stress, and secondly, upon the severity of that stress (Suttle, 1986).

Fetal levels are similar to adult levels on a dry weight basis (Puls 1994).

1.3.4 Zinc

Most Zn tissue concentrations are of little value in predicting the Zn status of animals (Van Ryssen, 1997; Underwood & Suttle, 1999). At a severe deficiency the Zn concentration in plasma will be < 0.4 mg Zn / l (Van Ryssen, 1997). Fetal liver Zn is normally 40-80 ppm on a wet weight basis. Levels seem to decrease from 100-200 ppm at 80-90 days to 30-60 ppm on a wet weight basis at 130-140 days gestation (Puls 1994).

However, many factors other than the Zn intake can affect the plasma Zn concentration (stress, physiological stage of animal, infection and injuries). Alkaline phosphatase activity in plasma can also be used to indicate a Zn deficiency, but its activity is also affected by other factors (Van Ryssen, 1997).

The capacity to store Zn in any of its organs other than bones is extremely limited so that animals do not normally carry large reserves of zinc. Liver Zn is elevated by Cu excess (Puls 1994). As the deficient state develops, there is usually a small decline in the Zn concentration of most soft tissues, but the fall can be greater in pancreas and porcine liver (Underwood & Suttle, 1999).

Maternal hepatic Zn may be an insensitive measure of Zn stores available for delivery to peripheral tissues. Either hepatic or plasma metallothionein has been suggested to better reflect Zn status than liver or plasma Zn levels and may be a better measure of Zn status through gestation (Graham *et al.*, 1994).

1.3.5 Manganese

Lambs are born with moderate liver stores from 1.0-2.0 ppm Mn on a wet weight basis. Manganese homeostasis may break down with prolonged excessive dietary intake leading to very high liver levels (Puls 1994).

Manganese deficiency is uncommon under farm conditions although an Mn deficient diet usually results in a lowering of the limited amount stored in the body, located mainly in the bones and to a smaller extent in the liver. The manganese concentrations in blood, bones, hair and liver decline only slightly in animals deprived of manganese. Liver manganese is frequently measured, because it is the richest of the tissues in manganese, but it showed no significant change in concentration, whereas heart and lung both showed significant depletion from far lower maximal concentrations. Plasma Mn fell significantly from 2.74 – 1.85 ng/ml in animals deprived of manganese (Underwood & Suttle, 1999).

The liver levels of Mn are useful but not exactly reliable indicators of Mn deficiency, except when the deficiency is severe (Underwood, 1966; Ehret *et al.*, 1975). The concentrations in the liver are higher than most other tissues and can be increased or decreased with varying Mn intake (Ehret *et al.*, 1975).

No reliable tissue analysis exists to indicate the Mn status of an animal (Van Ryssen, 1997).

1.3.6 Biochemical values used to assess the trace element nutrition of sheep

Table 1.3.1 Biochemical values used to assess the trace element nutrition of sheep [1] (Caple & McDonald, 1983)

Mineral	Indicator	Nutritional status		
		Deficient	Marginal	Adequate
Cu	Plasma ceruloplasmin (U/l)	<5	5 to 40	40 to 90
	Plasma Cu (mgCu/l)	<0.3	0.3-0.5	0.5-1.3
	Erythrocyte Cu superoxide dismutase (U/g Hb)	<200	200 to 450	>550 lambs >450 mature
	Liver Cu (mgCu/kg dry weight)	<10	10 to 50	50 to 700
Se	Blood gluthathione peroxidase (µmole NaDPH oxidized/min./gHb)	<20 (illthrift) <30 (myopathy)[2]	<50	Up to 550
	Blood Se (mg/l)	<0.02	0.02 to 0.05	>0.05
	Liver Se (mg/kg wet weight)	<0.2	0.2 to 0.5	>0.5
Co	Serum / plasma vitamin B12 (µg/l)[3]	<0.50	0.5 to 1.0	1.0 to 10.0
	Liver vitamin B12 (mg/kg wet weight)	<0.1	0.1 to 0.2	>0.2

[1] Usually 5 to 10 sheep in each flock are sampled

[2] Where sheep have adequate vitamin E i.e. plasma levels >1mg/l

[3] Sheep with established rumen function

1.4. Trace element dispersion within a liver

Trace elements are not homogeneously dispersed throughout the liver. The lack of homogeneous dispersion of minerals within specific lobes as well as amongst the various lobes of bovine livers has been observed by Henderickx & Van der Heyde (1961).

In the study done by Theron *et al.* (1974), analysis of variance of the trace element concentrations showed that the minerals were not homogeneously dispersed within the caudate lobe of the liver. In all cases, except Mn, significant improvement in dispersion was obtained after homogenization. The anomalous behaviour of Mn may be attributed to the fact that the concentrations present approached the limit of detection by the method. A degree of homogenization appears to take place during storage. It is advantageous to store livers for a couple of weeks before analysing them since homogenization on a routine basis is not practical (Theron *et al.*, 1974).

Chapman *et al.* (1963) found significant variations in Cu concentrations when they compared different sample sites of entire bovine livers.
They evaluated the liver biopsy technique for mineral nutrition studies with beef cattle and found that the range of differences in Cu and Fe concentrations in liver biopsies attributable to site of biopsy were relatively small.

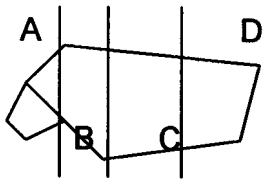

Figure 1.4.1 Diagram of the area of the liver from which biopsies were taken (Chapman *et al.*, 1963)

The average Fe concentrations for the four sites ranged from 186 mg/kg (site A) to 203 mg/kg (site D). The differences between the four sites were not statistically significant.

The differences for average value for Cu, ranged from 223 to 258 mg/kg. Liver and biopsy site significantly affected the Cu concentration in the biopsies. Cattle that are not deficient in Cu will usually have 150 to 250 mg/kg of Cu in their liver, as compared to 10 to 25 mg/kg for Cu-deficient cattle. When these differences are compared to the range of differences between biopsy sites it is quite evident that the latter differences, though statistically significant, were quite small as compared to those of cattle that are not deficient and those that are deficient in Cu (Chapman *et al.*, 1963).

Van Eijk *et al.* (1974) measured the Fe concentration in human liver specimens. In 9 cases, in which the liver tissue was obtained via obduction, they investigated the possibility of differences in Fe content of the left, right or center pieces of the liver. The results are given in Table 1.4.1 below. As can be seen the differences in one liver are very small and are nearly within the standard error of the method. Therefore, the part of the liver from were tissue is obtained has a negligible influence on the results of the Fe concentration (Van Eijk *et al.*, 1974).

Table 1.4.1 The deviation of iron values in different parts of the liver (The data are given in mmoles Fe/100 g dry weight) (Van Eijk *et al.*, 1974)

Liver	**Left**	**Centre**	**Right**
1	1.49	1.34	1.71
2	2.6	2.52	2.61
3	1.99	2.39	2.11
4	1.68	1.82	1.89
5	0.57	0.68	0.71
6	1.41	1.58	1.71
7	1	0.58	0.32
8	3.45	2.17	1.8
9	1.08	1.15	1.36
Mean value	**1.7**	**1.58**	**1.58**

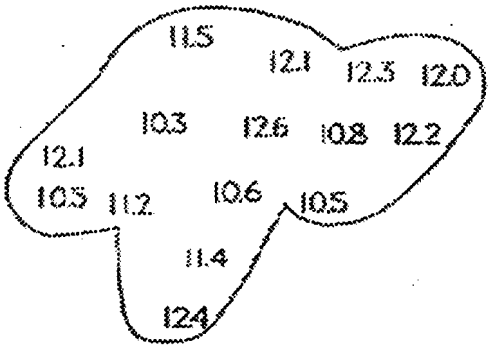

Fig. 1.4.2 Distribution of copper throughout the liver of an 8 weeks old pig (Hogan *et al.*, 1971)

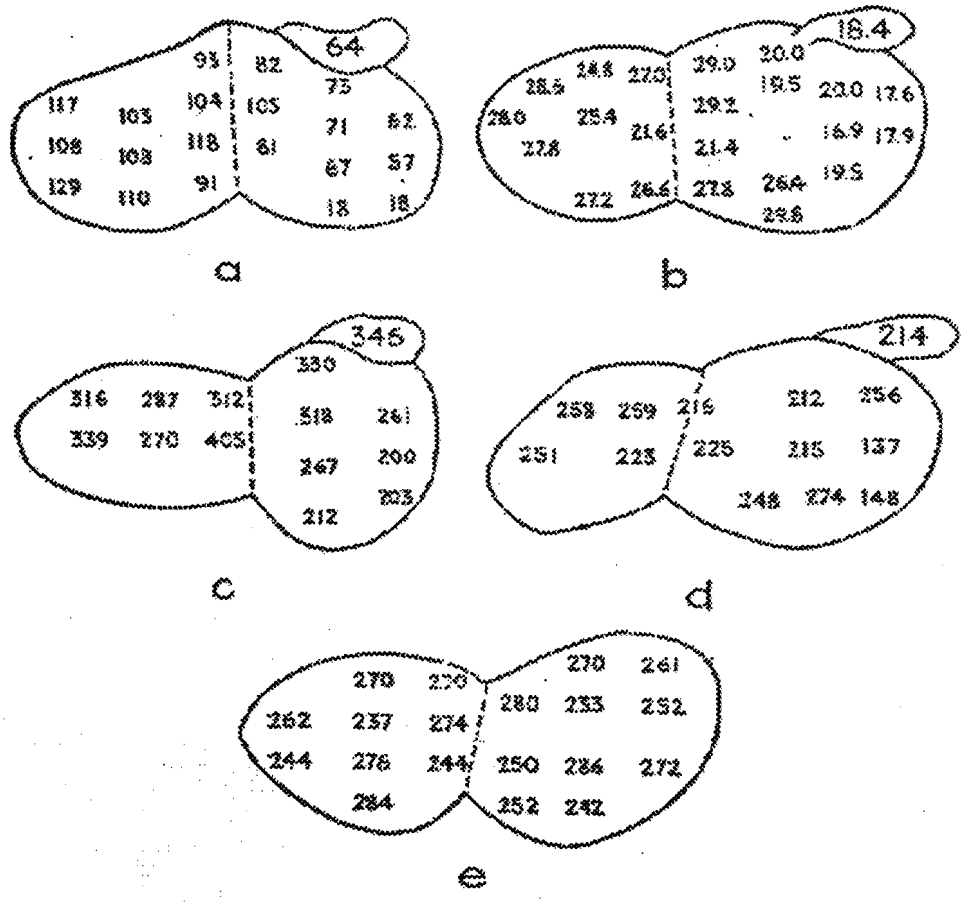

Fig. 1.4.3 Distribution of copper throughout the liver in relation to the positions of the caudal, dorsal (right-hand side), and ventral (left-hand side) lobes for: new-born lambs (a) and (b), four-tooth ewes (c) and (d), and an aged ewe (e) (Hogan *et al.*, 1971)

In the livers of pigs either given a Cu supplement there was no significant variation in Cu concentration according to the sight from which the sample was taken (Figure 1.4.2).

In two newborn lambs examined, there was a statistically significant trend towards increasing concentrations in samples between the dorsal (right) lobe to the ventral (left) lobe. Cu levels for samples taken from different parts of the livers of mature sheep are apparently more uniform than those from newborn animals.

Lamb (a): There is a highly significant trend from left to right. Mean values (for each set of vertical figures) across the diagram were 60, 57, 72, 102, 107 and 118 mg/kg.

Lamb (b): Although the copper figures are much lower than those for lamb (a), there is again a significant trend. Mean values were 18, 19, 22, 27, 25, 26, 28 and 28 mg/kg.

Sheep (c) and (d): Although in each case trends appear to exist, these are not statistically significant. Means were respectively 297 ± 60 mg/kg and 225 ± 36 mg/kg.

Aged ewes (e): There was no tendency for Cu values to increase from one side of the liver to the other. Mean values were 262, 258, 261, 269 and 253 mg/kg (Fig.1.4.3).

Table 1.4.2 Copper concentrations in samples taken at different depths from top surface (Hogan *et al.*, 1971)

Section	Sample	Cu (mg/kg)
A	1	47.3
	2	39.8
	3	55.8
B	4	42.3
	5	43.2
	6	44.2
	7	44.5
	8	49.2
C	9	48.3
	10	42.5
	11	43.2
	12	43.7
	13	38.2

Detailed examination did not reveal any serious variation in copper concentration according to the depth within the liver from which samples were taken.

It was found that, despite any variation in copper content that may be present, good agreement was obtained between the mean copper concentration for repeated biopsy samples of the same liver and that for replicated representative samples from the corresponding whole liver (Hogan *et al.*, 1971).

The distribution of copper within the livers of various animal species has been the subject of several investigations. No difference in copper concentration between the lobes of a pig liver was reported by Hogan *et al.* (1971); Cassidy & Eva (1958) and Osborn *et al.*, (1983). Although Osborn *et al.*, (1983) claims a much larger within-lobe variation.

In rats, the median and right lobes appear to contain a higher concentration of Cu than the omental and caudate lobes (Osborn *et al.*, 1983).

Young lambs appear to accumulate more Cu in their ventral (left) lobe than their dorsal lobe, but this disparity is absent in older sheep (Osborn *et al.*, 1983).

Bingley & Dufty (1958); Osborn *et al.* (1983) reported an uneven distribution of copper in the livers of neonatal calves, with the caudate lobe having the highest concentration.

In contrast to copper, the hepatic distribution of other trace elements and vitamins has not been studied in any detail. Osborn *et al.* (1983) examined the distribution of copper, vitamin B_{12} and zinc in the livers of sheep (Osborn *et al.*, 1983).

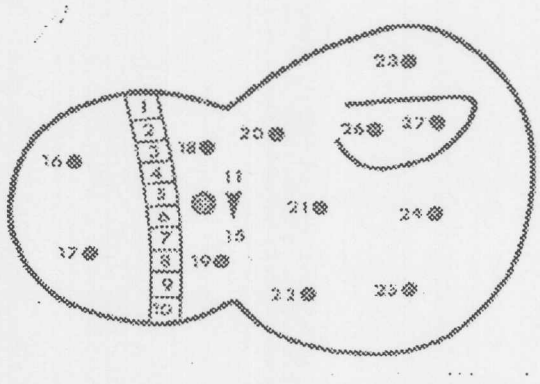

Fig.1.4.4 Diagram of a sheep liver showing the sites from which samples were taken for analysis (Osborn *et al.*, 1983)

Table 1.4.3 The mean concentrations of Cu, vitamin B_{12} and Zn in the six livers of sheep (Osborn *et al.*, 1983)

Mean (range) :g/g wet weight			
Liver	Cu	Vitamin B_{12}	Zn
1	38.5(34-47)	0.29(0.14-0.50)	47.3(38-62)
2	19.9(17-24)	0.54(0.44-0.64)	41(37-48)
3	41.3(37-50)	0.29(0.25-0.36)	44(41-81)
4	56.9(45-81)	0.70(0.56-0.89)	45.6(38-58)
5	49.5(44-63)	0.39(0.30-0.47)	43.6(39-53)
6	35.4(26-48)	0.40(0.35-0.55)	42.6(38-48)

The concentration of Cu and vitamin B_{12} showed no consistent ordering of values in the liver. Although the values for Zn did show a consistency in ordering, the extreme values were not significantly different from the mean (Osborn *et al.*, 1983).

The results of this study, done with one-year-old sheep, confirm the studies of Hogan *et al.* (1971) that the concentration of hepatic copper is uniform and that a biopsy sample should be representative of the whole liver. A similarly uniform distribution was found for vitamin B_{12} and Zn and thus reliable estimates of the animal's liver concentrations of these components from biopsy samples appears possible (Osborn *et al.*, 1983).

1.5. Formalin preservation

In the study done by Theron *et al.* (1974), random multiple samples were taken from the caudate lobe of a fresh liver and replicate determinations were carried out on them. Portions of the liver were preserved in formalin for 22 days, after which the analysis was repeated. The results of the analysis are given in Table 1.5.1. No statistically significant differences were noted for any of the five elements and it was concluded that formalin preservation up to 22 days did not affect the trace element levels (Theron *et al.*, 1974).

Table 1.5.1 Comparison of fresh and formalin preserved livers (Theron *et al.*, 1974)

		Cu mg/kg	Fe mg/kg	Mn mg/kg	Zn mg/kg	Mg mg/kg
Fresh	Mean	29.1	121	5.9	20.8	221
	SD	9.16	39.2	1.96	7.41	88.6
Preserved	Mean	27.53	143	5.3	26.8	178
	SD	1.80	23.2	0.76	10.39	11.62
Results	Variance Analysis	NS	NS	NS	NS	NS
	t	NS	NS	NS	NS	NS

NS = Not Significant at 95 % limit of confidence.

Sullivan *et al.* (1993) studied the effect of formalin fixation on the concentration of Se in pig liver. Sometimes only formalin fixed tissue is available for testing and established normals for Se in formalin-fixed liver have not been available. Analysis of fresh, frozen, and formalin-fixed matched liver samples was done to provide these data for comparisons.
The Se concentrations were measured in the fresh, frozen and formalin-fixed cubes of liver (Table 1.5.2).

Table 1.5.2 Mean (s.e.) concentrations (mg/kg) of selenium in fresh, frozen and formalin-fixed porcine liver 0-28 days after collection (n = 12 pigs/treatment group) (Sullivan *et al.*, 1993)

No. of days after collection						
Tissues	0	2	7	14	21	28
Fresh/ frozen	0.550 (0.034)	0.513 (0.073)	0.539 (0.109)	0.553 (0.037)	0.538 (0.026)	0.573 (0.037)
Formalin-fixed		0.453 (0.077)	0.476 (0.071)	0.459 (0.034)	0.505 (0.035)	0.475 (0.035)

Eleven treatments were defined to include Se in fresh liver on day zero (one treatment) and in frozen and formalin-fixed liver on days 2, 7, 14, 21 and 28 (ten treatments). Testing of the contrasts between treatments revealed no statistically significant difference between values for Se in fresh and frozen liver, but the contrast of combined values for Se in fresh and frozen liver versus values for Se in formalin-fixed liver were highly significant (Sullivan *et al.*, 1993).

Comparison of the regression lines revealed that the concentration of Se in formalin-fixed liver was 86% of that in fresh/frozen liver across days 0-28. A slight positive increase in Se (0.001115 ppm/day) was present for both treatments over the test period (Sullivan *et al.*, 1993).

$$\text{Fresh Se} = \frac{\text{Formalin-fixed Se} - (\text{days fixed} * 0.001115 \text{ mg/kg/day})}{0.86}$$

The increase in Se in stored frozen and formalin-fixed liver, as reflected by the positive slope of the regression equation, is probably caused by dehydration from freeze-drying and formalin fixation, respectively. With loss of water, the reduced mass of the tissue cause a relative increase in Se, although total Se remains the same.

The significantly lower intercept concentration of Se in formalin-fixed tissue on day zero is attributed to leaching of Se into the formalin solution early in fixation. Similar effects from fixation have been observed with Cu and Zn in formalin-fixed liver (Sullivan *et al.*, 1993).

Dehydration of tissue samples by freezing or formalin fixation and leaching of Se from fixed tissue must be considered in interpreting data from stored samples. The calculated regression equations take those effects into consideration and provide for easy extrapolation from the Se concentration determined in formalin-fixed liver to the expected concentration in fresh liver (Sullivan *et al.*, 1993).

Table 1.5.3 Liver copper analysis on goats with neurological and other diseases (Summers *et al.*, 1980)

			Liver copper in mg/kg (wet wt.)	
Animal no.	Pathological diagnosis	Age	Fresh	Fixed
4	VLG	4 mo.	8.5	7.0
5	VLG	2mo.	2.3	1.8
6	VLG	2mo.	9	5.7
9	Urolithiasis	6mo.	34.5	22.6
10	Coccidiosis	2mo.	2.4	3.6
11	Neonatal Sudden Death	3wk.	20.2	12.8

Table 1.5.4 Effects of fixation of liver tissue in formalin solution on weight and copper analysis (Summers *et al.*, 1980)

Procedure	Results	% Change
Weight of 1 g of liver tissue:		
(a) Fresh	1.00 g	
(b) Fixed for 7 days	1.14 g	+14 %
(c) Fixed for 14 days	1.18 g	+18 %
Cu analysis of 1 g of liver tissue		
(a) Fresh	40.60 ppm	
(b) Fixed for 7 days	37.00 ppm	-9 %
(c) Fixed for 14 days	32.80 ppm	-19 %

Summers *et al.* (1980) investigated the consequences of performing Cu analysis on tissue specimens preserved in formalin. In almost all cases it was observed that levels were lower in the preserved tissues, whether from goats with neurological disease or otherwise. The repeatability of the assay was within 5% (data not shown), which was considered to be acceptable. The explanation for the lowered values following preservation were two-fold; firstly, imbibition of fluid by the tissue in preservative and secondly, loss of Cu into the solution. When specimens of the preserved tissue are taken for analysis, the weight of the imbibed fluid will have an effect of lowering the concentration of the assayed element per unit weight of tissue. While the tissue is bathed in fixative some Cu will be lost into the fluid medium. The explanation for the single case where the preserved level is higher is not known; possible it resulted from Cu contamination in that particular batch of fixative (Summers *et al.*, 1980).

Boskey *et al.* (1982) undertook a study to determine which measurements of cellular and matrix components are unchanged by formalin fixation of bone and other hard tissues. The mineral composition of bone was not modified by brief (3 day) or more prolonged (11 day) exposure to neutral formalin (Boskey *et al.*, 1982).

It is essential, however, that the formalin be buffered at neutral pH, since mineral dissolution would be expected in non-buffered, acidic, formic acid-rich formalin solution. Although the phosphate-buffered aqueous solution might be expected to alter the mineral composition by the adsorption of excess phosphate, the phosphate apparently suppressed mineral dissolution by shifting the equilibrium between hydroxyapatite mineral and calcium and phosphate ions to the left, leaving the composition unchanged (Boskey *et al.*, 1982).

It is apparent, then, that mineral parameters measured in formalin-fixed tissues can be compared with those measured in fresh-frozen tissues (Boskey *et al.*, 1982).

This study has only dealt with tissues fixed, or stored, for a relatively short period of time. Prolonged (> 1year) exposure to formalin is known to alter even relatively stable lipid composition of such tissues (Deierkauf & Heslinga, 1961). Thus one must anticipate that biochemical studies on tissues left in formalin for such long periods of time are questionable (Boskey *et al.*, 1982).

Van Eijk *et al.* (1974) measured the Fe concentration in human liver specimens. Since liver tissues are often kept in formalin or Telly's solution before chemical analysis are performed, they investigated the influence of storage in saline, formalin, buffered formalin and Telly's solution for a period of 24 h. From the tables below we may conclude that during this period a substantial amount of Fe was lost. In Table 1.5.6 and Table 1.5.7, the analysis is given in 5 fold for one normal liver and for a liver of a patient with secondary hemochromatosis. For reliable data it is better to determine the Fe content directly after obtaining the tissue (Van Eijk *et al.*, 1974).

Table 1.5.5 The influence of the storage of liver tissue for 24 h in buffer or formalin on the iron content (Fe content in mmoles Fe/100g dry weight) (Van Eijk *et al.*, 1974)

Liver No. (different livers)	After 5 min 0.14 M NaCl	After 24 h 0.14 M NaCl	After 24 h 4% formalin	After 24 h 4% formalin pH 7	After 24 h in Telly's solution
1	0.65	0.37	0.48	0.29	0.55
2	0.63	0.46	0.46	0.33	0.24
3	2.58	2.30	2.48	1.84	2.59
4	2.16	2.00	2.51	1.67	2.56
5	1.57	1.30	1.07	1.52	1.19
6	1.51	1.51	1.45	1.27	1.40
7	3.45	2.89	3.24	2.30	3.16
8	1.08	0.96	1.13	0.78	1.07

Table 1.5.6 Influence of storage in different solutions on the iron content of liver (normal) tissue (All samples were taken from the same liver, iron values in mmoles Fe/100g dry weight) (Van Eijk *et al.*, 1974)

Normal Liver Sample	5 min in 0.14 M NaCl	24 h in 0.14 M NaCl	24h in formalin 4%	24h in formalin 4% pH 7, phosphate buffer	24h in formalin 4% pH 8, bicarbonate buffer	24h in Telly solution
1	2.05	1.49	2.57	1.30	2.34	2.08
2	2.00	1.25	2.46	1.81	2.70	2.20
3	2.32	1.42	2.18	1.56	2.34	2.02
4	2.31	1.57	2.39	1.83	2.32	2.05
5	2.10	1.22	2.28	1.70	2.52	2.51
Mean	2.16±0.15	1.39±0.15	2.38±0.15	1.64±0.21	2.44±0.17	2.17±0.2

Table 1.5.7 Influence of storage in different solutions on the iron content of liver (Secondary Hemochromatosis) tissue (All samples were taken from the same liver, iron values in mmoles Fe/100g dry weight) (Van Eijk *et al.*, 1974)

Secondary Hemochromatosis Sample	5 min in 0.14 M NaCl	24h in formalin 4%	24h in formalin 4% pH 8, bicarbonate buffer	24h in Telly solution
1	28.9	26.3	33.7	29.3
2	27.3	25.0	25.6	28.2
3	37.3	23.6	21.5	19.3
4	30.5	22.5	27.2	22.0
5	31.4	23.5	27.5	28.6
Mean Value	31.2±3.8	24.2±1.5	27.1±4.4	25.5±4.5

*Idiopathic hemochromatosis: Excessive Fe accumulation in the liver and other viscera and by fibrotic changes in the liver (Fairbanks *et al.*, 1971).

The best way to keep liver with the lowest lost of Fe is storage in buffered formalin (Lillie *et al.*, 1939). Storage in formalin buffer with bicarbonate gives the smallest loss of Fe. But on the other hand the loss of Fe in the medium is significant (Van Eijk *et al.*, 1974).

Brown *et al.* (1979) did a study concerning the estimation of Pb levels in human lung and vertebra, with particular reference to formalin fixation.

Table 1.5.8 Mean Pb concentration (µg/g) in human lung

Reference	Sex	No. of donors	µg/g wet	µg/g dry	µg/g ash
Barry (1975)	M	59	0.22	*0.98*	20
	F	36	0.22	*0.98*	20
Gibbs and Bogdanovic (1974)	?[1]	16	0.50[2]	3.05[2]	50[2]
	?[3]	4	0.36[2]	2.16[2]	36[2]
Gross *et al.* (1975)	M[4]	42	0.36	*1.17-1.60*[5]	23.9
Horiuchi *et al.* (1959)	M,F[6]	47	0.30	1.34	*27*
Ophus and Mulius (1977)	M[7]	146	*0.10*	0.45	*9.2*
	F[7]	98	*0.10*	0.43	*8.8*
Schroeder and Tipson (1968)	M, F	150	0.39	*1.74*	*35*[8]
Stringer *et al.* (1974)	M	41	*0.23*	1.04[9]	*21.2*
	F	25	*0.13*	0.57[9]	*11.6*
Tipson & Shafer (1964)	M, F	141	0.74	3.28	67[8]

Numbers in block type are based on data in the reference indicated; numbers in italics have been calculated using respective mean ash contents of wet and dry lung of 1.1 % and 4.9% from Tipson & Shafer (1964) (Brown *et al.*, 1979).

[1]right lungs only, 8 segments of each analysed separately

[2]median level

[3]left lungs only, 8 segments of each analysed separately

[4]all segments of both lungs sampled

[5]figures based on levels in ashed and in wet tissue

[6]multiple samples, anatomical location not specified

[7]tissue fixed in formalin; 10 samples from right apex
[8]the corresponding median level is 47 μg/g ash
[9]samples lyophilised to constant weight

There are three grounds on which the data of Ophus & Mylius (1977) are open to criticism. First, the data are presented as Pb levels in dry tissue, but 'dry' is not defined, although a common meaning is 24 h drying, or drying to constant weight, at 110 °C (Brown *et al.*, 1979).

In addition, dry weight is probably the least usual basis for expressing metal concentrations in human tissues, and this increases the difficulty of comparing the present results with data already in the literature.

Secondly, the selection of all samples from the apex of the right lung may not give results which are representative of the lungs as a whole, since there is evidence (Gibbs & Bogdanovic, 1974) that apical concentrations of several metals (including Pb) are higher (on an ash weight basis) than in other lung segments, and also that the ash content of "dry" lung varies between segments in no obviously systematic way (Brown *et al.*, 1979).

The third criticism is probably the most serious, and relates to the analysis of tissues that have been fixed and/or stored in formalin. With such samples, leaching out of the metal(s) of interest is a distinct possibility and, as shown in the table above, the present data are lower than those from several of the major studies in this area. In particular, the mean levels (particularly for males) found by Stringer *et al.* (1974) are higher than those of Ophus & Mylius (1977), although they are cited as agreeing because of the similar range of lead levels.

It should be noted that in Table 1.5.8 the conversions to other bases are only approximate, because of the different weight bases used by different authors to express lead concentrations (Brown *et al.*, 1979).

Table 1.5.9 Mean Pb concentration in human vertebra

Reference	Sex	No. of donors	µg/g wet	µg/g dry	µg/g ash
Grandjean (1975)	M F	81 38	*0.88* *0.69*	1.63 1.32	*4.65* *3.97*
Gross *et al.* (1975)	M	45	4.42	*8.2-10.2*[1]	29.1
Horiuchi *et al.* (1959)	M, F	36	5.28	*10.0*	*29.1*
Schroeder & Tipton (1968)	M, F	53	?	?	51[2]
Waterloo	M F	122 61	6.56 5.0	12.1 9.6	35.0 29.2

Numbers in block type are based on data in the reference indicated; numbers in italics have been calculated using respective mean ash contents of wet and dry vertebra of 18.92 % and 35.07% for males, and of 17.31 and 33.22% for females. These figures are based on vertebral samples from 122 males and 61 females analysed in the laboratory at Waterloo (*cf.* Table 1 in Forbes *et al.*, 1976; Brown *et al.*, 1979).

[1]figures based on levels in wet and in ashed tissue

[2]median level

A second case of a significant data set where there is a strong presumption of leaching of lead by formalin is in the vertebral lead levels reported by Grandjean (1975). As shown in the table above, the mean lead concentration in vertebra samples from 81 males and 38 females is less than one fifth that reported in several other studies (Brown *et al.*, 1979).

Grandjean's (1975) concentration basis is "dry" vertebra, described as transfer (from the formalin) to concentrated alcohol, rinsing with ether and drying to constant weight in desiccators. This procedure is presumably less rigorous than 110 °C, so that the relative contribution of the weight basis and leaching to the decreased lead levels are again unclear.

However, it may be significant that the two studies reporting lower lead levels refer to the residents of Scandinavian countries (Norway and Denmark, respectively) (Brown *et al.*, 1979).

The difficulties arising from the lead levels published by Ophus & Mylius (1977) in lung and by Grandjean (1975) in vertebra raise a number of matters relevant to all studies of trace element concentration in human tissues. First, the fixation of tissues in formalin, prior to trace element analysis, should be avoided because of the likelihood of loss by leaching. Secondly, meaningful comparisons among different studies are difficult to make unless standard conditions for the weight basis of expression of the results are used in preparing the tissues (Brown *et al.*, 1979).

1.6 Prolonged storage

In the study done by Theron *et al.* (1974) the caudate lobe of a liver were stored in formalin, random samples being removed after 6 weeks, and again after 6 months. In each case multiple determinations were carried out.

Table 1.6.1 Comparison of mineral levels after six weeks and six months (Theron *et al.*, 1974)

Storage Period	No. of determinations		Cu	Fe	Mg	Mn	Zn
6 weeks	30	Mean	31	74	159	3.3	39.4
		S.D.	2.5	9.4	10.1	0.41	8.5
6 months	30	Mean	31	78.2	154	3.7	33.9
		S.D.	4.5	10.9	22.2	0.45	3.1
		t	0.0	1.56	1.17	3.08	3.18

At the 95 % limit of confidence the statistical difference "t" is significant if t>1.96.

The concentrations of Cu, Fe and Mg were not affected by prolonged storage in formalin. Statistically significant differences in the levels of manganese and zinc were detected after 6 months (Theron *et al.*, 1974).

The investigations into the effect of prolonged storage in formalin on manganese concentrations revealed a statistically significant increase (P±0.05). This is, however, difficult to equate with the variations of individual animals. Groups from different farms sampled at the same time and therefore subjected to similar storage conditions showed extreme variation. This was well illustrated in a group from Farm 2 where the 19 animals had a mean of 3.65 mg/kg and varied from 2.0 to 9.2 mg/kg. Although this variation was extreme, the pattern of high and low within a group was constant. This was interpreted as indicating an inconsistent loss or gain of Mn due to storage in formalin and it was concluded that the various comparisons were valid (Ehret *et al.*, 1975).

Significant loss (P<0.05) loss of Zn content shown to occur during six weeks to six months storage in 10 % formalin (Ehret *et al.*, 1975).

Bratton *et al.* (1984) did a study in order to establish guidelines for using fixed tissue from Pb exposed cattle as a means of estimating the level of Pb present in liver and kidney at the time of natural death or euthanasia. Changes in the levels of Zn, Cu and Fe were also evaluated in these same tissues even though these elements were available to the calves only in recommended dietary amounts.

Metal concentrations in the 10 % phosphate buffered neutral formalin were measured prior to immersion of tissues and after 6 months of containing immersion tissues (Bratton *et al.* 1984).

Table 1.6.2 The mean results ± s.d. (in µg/dL) as found by Bratton et al. (1984)

	Pb	Fe	Cu	Zn
Before	< 10	< 10	< 10	3
After	< 10	16 ± 3	43 ± 3	24 ± 9

Tissue concentrations of Zn, Fe and Cu are shown in Table 1.6.3. Cu and Zn had generally low correlations between the frozen and fixed concentrations ($R^2 < 0.50$). Regression analysis did not appear useful in predicting values for these metals at the normal concentrations observed in this study. Copper levels tended to increase in the kidney when fixed and the trend was the same for both the wet weight and the dry weight analyses. The extensive washing process should have helped eliminate extraneous Cu contamination. However the kidney Cu levels increased in tissue after six months in fixative while liver Cu concentrations decreased (Bratton *et al.*, 1984).

The R^2 values for Fe (wet kidney 0.65, dry kidney 0.72, wet liver 0.63, and dry liver 0.76) indicate that this method of analysis might be useful in estimating and determining lower confidence bounds for frozen tissue Fe concentrations, given fixed tissue concentrations (Bratton *et al.*, 1984).

Table 1.6.3 Zinc, Fe and Cu concentrations (mg/kg, x ± s.e.) in frozen and formalin-fixed kidney and liver (n=24) (Bratton *et al.*, 1984)

		Wet weight		Dry weight	
Element	Tissue	Frozen	Formalin	Frozen	Formalin
Zn	Kidney	38.76±2.28	19.62±1.41	201.96±11.6	104.46±8.5
	Liver	73.10±5.02	26.69±2.49	303.15±22.3	135.19±14.7
Fe	Kidney	35.23±2.34	41.31±3.6	179.15±14.8	211.41±18.5
	Liver	62.88±12.95	66.23±9.4	107.13±18.2	294.2±40.4
Cu	Kidney	6.72±0.55	17.56±1.11	30.91±2.13	97.84±6.63
	Liver	143.07±7.85	85.67±6.56	555.38±29.6	403.32±31.2

Leaching of Cu appears to be a high probability as does contamination with Cu. Copper levels in kidney were three times greater in the fixed kidney as compared to the frozen kidney while Cu levels in liver were decreased. This suggests that if tissues high in Cu are immersion fixed with tissues lower in Cu, leaching and contamination present a problem. Leaching also appears to be a problem in Zn concentration of tissues (Bratton *et al.*, 1984).

Fixed tissue, especially liver, can be used to determine if Pb toxicosis is a valid diagnosis when the only specimen available is fixed tissue. The method of analysis (wet or dry weight) does not appear to matter, nor should there be concern about contamination or leaching of Pb in the samples. Similar conclusions cannot be drawn for Cu, Zn or Fe (Bratton et al., 1984).

The preferred specimens for Pb analysis from animals that have died are frozen liver and kidney. These could be routinely obtained, but often this is not done in cases of Pb toxicosis, due to the usual absence of lesions highly suggestive of Pb toxicosis at necropsy. The analysis of formalin-fixed tissues is therefore often warranted (Hamir *et al.*, 1995).

The analysis of formalin-fixed tissues for diagnostic purposes has been recommended for Se and Cu, and tissue levels of both these elements remains unchanged by formalin-fixation (Sullivan *et al.*, 1993; Thornburg *et al.*, 1990).

However, Summers *et al.* (1980) found Cu concentrations of liver decreased progressively when the tissue was fixed in formalin for 14 days. They attributed the decreased concentration to imbibition of formalin into the tissue and also to leaching of the element into the fixative.

One report indicates that Pb is resistant to contamination or leaching from liver when preserved in formalin together with other tissues. However, the same investigation revealed Pb levels in the formalin-fixed kidney to be slightly lower than levels in the frozen kidney (Bratton *et al.*, 1985).

Hamir *et al.* (1995) documented hepatic and renal Pb concentrations in raccoons that were given lead acetate *per os* and to compare these results to the concentration of Pb in corresponding formalin-fixed tissues at two time points (one month and 16 months of formalin fixation).

Table 1.6.4 Tissue lead concentrations in frozen and formalin-fixed liver and kidney of eight raccoons administered oral lead acetate (Hamir *et al.*, 1995)

			Liver lead concentration (mg/kg, wet weight)			Kidney lead concentration (mg/kg, wet weight)		
				Formalin-fixed			Formalin-fixed	
Group	Raccoon no.	Lead dose*	Frozen	1mo.	16mo.	Frozen	1mo.	16mo.
A	1	0	12.97	<10(>23)⁂	2.92(78)	12.97	<10(>23)	4.92(62)
	2	0	11.13	<10(>10)	3.04(73)	15.87	<10(>37)	0.34(98)
B	3	1	21.68	10(54)	8.1(63)	18.77	<10(>47)	2.18(88)
	4	1	24.58	10(59)	16.2(34)	18.77	<10(>47)	2.98(84)
C	5	2	39.09	10(74)	16.2(22)	30.38	12.5(59)	13.4(56)
	6	2	42	15(64)	32.0(24)	36.19	12.5(74)	22(39)
D	7	4	76.83	37.5(51)	51.8(33)	30.38	30(1)	17.6(42)
	8	4	33.29	15.0(55)	29.4(12)	42	15(64)	30.6(27)

*Lead acetate (mg/kg body weight) administered orally once a day (5 days/week) for 8 weeks.

⁂Figures in parentheses indicate percentage of lead leached into formalin or reduced due to imbibition.

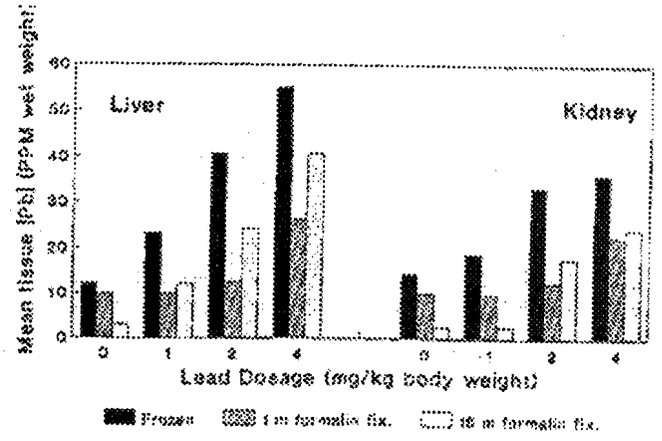

Figure 1.6.1 Mean Pb concentrations of frozen and formalin-fixed livers and kidneys of 8 raccoons administered oral Pb acetate (Hamir *et al.*, 1995)

Compared to the corresponding frozen tissue at one month and at 16 months, the Pb levels were lower in formalin-fixed kidney and liver (Table 1.6.4; Figure 1.6.1). At each level and for each comparison the P-value was less than 0.05. The observation contradicts a previously reported finding in which neither contamination nor leaching of Pb was observed from livers of calves given Pb acetate orally (Bratton *et al.*, 1985). The apparent conflicting results of these two studies may be explained by our assumption that there may be species variation in binding of Pb to tissues of different animals (Hamir *et al.*, 1995).

In more than 60 % of the instances (five out of eight), the Pb concentrations in formalin-fixed tissues at 16 months were higher than at one month (Figure 1.6.1). A likely explanation for this is that with prolonged formalin fixation, tissues appear to get dehydrated and, therefore, the concentration of tissue-bound elements (including Pb) may increase (Hamir *et al.*, 1995).

1.7. Use of glass and plastic bottles for storage

Table 1.7.1 Trace element content of materials that are frequently used for construction of animal accommodation and for sample collection (Mills & Williams *et al.*, 1971)

Approximate concentration (µg/g)	High >500	Plastics* Medium 200-500	Low 100-200
Polyethylene			
High density, white		Fe	Cr, Cd
High density, yellow	Zn, Cd		
High density, natural			Cr, Cu
Polyvinylchloride:			
Sheet, grey	Zn	Fe	Cd
Tube, clear	Cd, Cu	Sn	
Perspex:			
Clear			Cu
Black	Cr, Cu		Sn, Pb
Phenolic resin:			
Black	Mn, Zn	Cr, Fe	Cd
Stainless steels AlloysH	Cr to 20 %; Mn to 2%; Mo to 3.5%; Se to 0.6%; Pb to 0.34%		
Aluminium alloysI	Cu to 8%; Zn to 13%		

* Adapted from Scott (1970); Mills & Williams *et al.* (1971)

H British Standards Institution (1950, 1955)

I British Standards Institution (1963).

Table 1.7.1 illustrates the nature of trace element inclusions in plastic materials and in stainless steels frequently used as expedients to overcome this problem of contamination.

Under many circumstances the assumption is correct that these elements are sufficiently tightly bound in the matrix of the structural material to present no hazard. But this situation does not necessarily apply under humid conditions where food material or excreta can remain in contact with the plastic or metal, initiate attack thereon and act subsequently as a source of contamination (Mills & Williams *et al.*, 1971).

The solubility of metal components of commonly used plastics is normally low, but appreciable quantities of metal may be brought into solution if the plastic remains in contact with alkaline solutions such as urine from which free ammonia is being generated. Thus, ammoniac solutions have become heavily contaminated with copper after a brief period of contact with clear polyvinylchloride tubing (Mills & Williams *et al.*, 1971).

The nature of metallic inclusions may differ in products having the same designation but produced by different manufacturers. Accordingly, when working with elements for which the quantitative requirement is small such as Se, Co and Cr, care has to be taken over the selection of structural materials and the choice should be based upon at least a semi-quantitative assessment of trace element content (Mills & Williams *et al.*, 1971).

During the study of Theron *et al.* (1974) portions of a liver were formalin-preserved in plastic and glass bottles for one year. After this period, analysis were performed on random non-homogenized multiple samples from each portion. No statistically significant differences were found for any of the metals, indicating that either type of bottle could be used (Theron *et al.*, 1974).

Potential sources of contamination include the environment, containers, collection apparatus, and, probably most important, the chemist! Some aspects of container materials have been studied and recommendations for cleaning these have been made. Plastics are highly recommended, with Teflon and polyethylene being the most favourable (Mertz, 1987).

1.8 Blenders used for homogenisation

In order to minimize the effects of the possible uneven distribution of trace elements in the liver, it is often necessary to homogenise the liver and take an analytical subsample. This subsampling can be very susceptible to contamination in that extensive homogenisation is often carried out in some type of blender open to the atmosphere (Mertz, 1987).

Commercial blenders with stainless-steel blades can be a very significant source of contamination of the metals contained in the blades. Stainless steel can contain 5 to 20% chromium, along with percentage amounts of nickel, cobalt and manganese, in addition of being a obvious source of iron. All of these metals can be abraded or dissolved from blades during a high speed blending process. The amount of chromium contamination introduced in blending a total diet composite homogenate has been shown to be directly proportional to the blending time (Mertz, 1987).

If the elements of interest are chromium, nickel, cobalt, manganese or iron, then alternate homogenization procedures using non-stainless steel blenders must be developed (Mertz, 1987).

1.9. Trace element analysis by atomic absorption spectrophotometry

Sample preparation, matrix interferences, flame stoichiometry and storage of solubilized samples are factors which can affect the accuracy and precision of trace metal analysis in bovine livers by atomic absorption spectrophotometry (Theron *et al.*, 1973).

Kahnke (1966), using a wet ashing method, recovered ± 96% of added zinc, copper, manganese and iron in formalinised human tissue but reported a total loss for added magnesium.

Copper determinations in the air-acetylene flame are apparently free from interferences except those, which result from large amounts of solids in solution (Slavin, 1965).

In a reducing air-acetylene flame, the absorbance for Mn is reduced by phosphate, perchlorate, iron, nickel and cobalt. In an oxidising flame these interferences are minimised.

The interferences by boron, fluoride and phosphate in the determination of manganese can be suppressed by lanthanum (Schmidt, 1970).

The determination of zinc is generally free from interferences (Slavin, 1965).

Wet ashing of samples yielded significantly higher values for magnesium and zinc than the dry ashing method, while the reverse was found for manganese. Copper and iron concentrations were unaffected by the method of liquefaction (Theron *et al.*, 1973).

1.10. Moisture content of fresh and formalinised livers

1.10.1 Dispersion of moisture within a fresh liver

The possibility that moisture may not be homogeneously dispersed within a liver led to a comparison of moisture determinations, done on homogenized and non-homogenized samples taken from a fresh liver.

Table 1.10.1 Dispersion of moisture within a fresh liver (Theron *et al.*, 1974)

	Non-homogenized	Homogenized
Mean	63.3	65
s.d.	4.2	0.0

Variation in moisture content, which may be expected in fresh livers and hence the effect of this variation on mineral levels, is small when compared to proven biological variations. From this it may be concluded that trace element concentrations expressed on a wet basis are comparable for fresh livers.

1.10.2 Dispersion of moisture within a formalin-preserved liver

The dispersion of moisture within an individual liver after storage in formalin was investigated in the same manner as for the fresh liver. Table 1.10.3 indicates that the irregular dispersion of moisture within a formalinised liver is not significant. By comparing Table 1.10.2 with Table 1.10.1 it may seem that moisture apparently becomes more homogeneously dispersed on storage.

Table 1.10.2 Dispersion of moisture within a formalin-preserved liver (Theron *et al.*, 1974)

	Non-homogenized	Homogenized
Mean	71.0	75.5
s.d.	1.07	0.87
F = 1.53 F (5%) = 3.87		

Table 1.10.3 Moisture content of livers of various storage periods (Theron *et al.*, 1974)

Days stored	% Moisture
216	68.4
330	72.8
441	66.6
477	69.1
516	70
612	65.6
648	64.2
729	67.2
Mean	68.0
s.d.	2.53

Livers may be stored for up to two years without affecting moisture content significantly. In the light of the above study, it concluded that mineral levels expressed on a wet basis are directly comparable for fresh and stored livers (Theron *et al.*, 1974).

1.11. Evaluation of ethanol-based fixatives as a substitute for formalin

Fixation is necessary to preserve tissues in a state that maintains its original condition as closely as possible. Formalin has been used as a histology tissue fixative for over a century. Formalin fixes tissue by forming cross-links with the amino acid lysine, in proteins. This cross-linking action prevents cellular distortion and loss of chemical activity in the tissue, thus maintaining cellular antigenicity.

The use of formalin as a fixative in the histology laboratory is a potential danger to laboratory workers. Formalin results in common complaints such as eye, nose and throat irritations, and less commonly neurological disorders such as bronchitis and neoplasia.

A substitute of formalin is needed in a society that is becoming increasingly work safe conscious. Ethanol-based fixatives were compared with formalin for their ability to preserve tissue and cellular morphology, to retain antigenicity, and to facilitate the diagnosis of complex pathological specimens (Warmington *et al.*, 2000).Ethanol-based fixative F13 (60% ethanol, 20 % methanol and 7% polyethelene glycol) was statistically significantly different from formalin, providing superior preservation of tissue morphology. In 20 surgical cases, F13 and formalin were equally able to fix tissue to allow accurate diagnoses (Warmington *et al.*, 2000).

The effects of F13 on bacteria, bacterial spores, fungi, parasites and viruses in tissue are unknown. Formalin is known to render these pathogens non-viable yet research shows that ethanol-based fixatives do not have the same effect. If specimens are all treated as potentially infectious, as they currently should be, this problem should not cause infective risks (Warmington *et al.*, 2000). The cost effectiveness of F13 compared to formalin needs to be calculated. It is envisaged that the potential increase to worker safety and savings associated with the elimination of expensive fume extraction units necessary for formalin vapours should prove favorable for F13 introduction (Warmington *et al.*, 2000).

Chapter 2: Materials and methods

2.1 Introduction

The aim of this investigation was to determine if there was a difference in the Zn, Cu, Mn, Co and Se concentrations between fresh liver and liver preserved in formalin for one month, three months and six months.

2.2 Experimental Material

Thirty-six fresh livers were obtained from three different trials done by students of the Department of Animal and Wildlife Sciences at the University of Pretoria. Thirty-one of the 36 livers were collected from sheep (ovine) and five livers were obtained from impala.

2.3 Preparation

The livers were removed after slaughtering and were stored in a fridge until sampling. From each fresh liver a block was sampled out of the middle. This block was divided into 16 pieces, which was randomly assigned to four sample groups. Liver in each sample group weighed about 20 g to 30 g. These four sample groups were divided into the four treatments groups:

1. Treatment one (the control group) was oven dried immediately at 60 °C for 48 hours.
2. Treatment two was preserved for one month in an acid washed plastic (poli-ethylene) bottle with a screw top, containing four ml 10% buffered formalin for each one gram of liver.
3. Treatment three was preserved for three months in an acid washed plastic (poli-ethylene) bottle with a screw top, containing four ml 10% buffered formalin for each one gram of liver.
4. Treatment four was preserved for six months in an acid washed plastic (poli-ethylene) bottle with a screw top, containing four ml 10% buffered formalin for each one gram of liver.

Formalin

The formalin solution was prepared using Millonig 's buffered formalin solution method. The chemicals used were 428,6 g NaH_2PO_4, 87,0 g NaOH, 121,4 g $C_6H_{12}O_6$ (glucose) and 2,5 l H_2CO 40 % formalin. The dry chemicals were dissolved separately in 900 ml distilled water on a magnetic stirrer. The three solutions were added to the 2.5 l formalin in a 25 l container. The container was filled up to 25 ml with distilled water (Millonig 's buffered formalin solution method). The 40 % formaldehyde solution (AC-grade) that was used was obtained from Unilab® Saarchem (Pty) Ltd.

Table 2.1 Type analysis of 40 % formaldehyde solution (AC-grade)

	(w/v)
Assay (HCHO)	37 %
Methanol	7.5 %
Acidity (HCOOH)	0.02%
Fe	0.0002%
Cu	0.001%
Volume: 2.5 l	

Three ml of formalin from each sample was wet ashed (digested in acid) and three ml of formalin was centrifuged, from the same sample, to see if there was a difference between the results obtained through atomic absorption spectrophotometry. After no difference was found in the results, the mineral concentrations in formalin were determined using atomic absorption spectrophotometry after centrifugation of the formalin.

The volume of formalin was measured before the liver was added (four ml 10% buffered formalin for each one gram of liver) and again after the liver was removed from the formalin.

Sample preparation

The samples were removed after one month (Treatment two), three months (Treatment three) and six months (Treatment four) respectively, after which they were blotted dry and dried at 60 °C for 48 hours.

The dry samples were milled with a Merck miller (Kika Labortechnik; Typ: 220-240 V, 50/60 Hz, 180 W, 20 000 r/min).

Dry matter determination:

The fresh wet liver samples were dried at 60 °C for 48 h after which it was milled. Formalinised liver samples were removed from the bottle after varying periods of storage, placed on filter paper to remove excess formalin and dried at 60 °C for 48 h.

A dry matter (DM) determination at 100 ° C for 24 h was also done on all of the samples. One g of each liver sample was dried in an oven at 100 ° C for 24 h.

2.4 Laboratory analyses:

After the samples were milled they were wet ashed. HNO_3 and $HCLO_4$ were used in the wet acid digestion process.

The Cu, Mn and Co concentrations were obtained using the Varian Spectr-AA-50 (single beam) flame atomic absorption spectrometer after wet acid digestion.

The Se concentrations were determined using the hybrid generator method on a perkin-Elmer (Norwalk, Connecticut, USA) A.A. (atomic absorption spectrophotometer, model 2380). HNO_3, $HCLO_4$ and HCl were used during the digestion process of the samples.

Zinc concentrations were determined using the perkin-Elmer (Norwalk, Connecticut, USA) A.A. (atomic absorption spectrophotometer, model 2380) after wet ashing and dilution of the samples.

Wet ashing (acid digestion)

The samples were wet ashed before Cu, Co and Mn determination was done by using the Varian Spectr-AA-50 (single beam) flame atomic absorption spectrometer.

One g of each liver sample was digested in duplicate. With each rack of samples, which are digested, a beef liver control (0.1g) (National Institute of standards and technology, 1577b; Gaithersburg, MD) was included. Three ml of formalin, from the respective formalin containers in which each of the liver samples were preserved, were acid digested.

While weighing in the samples, the block was switched on to warm up for approximately one hour before starting the digestion process. Twenty-five ml of HNO_3 was added to every tube before putting them on the block for 10 minutes after which it was removed and cooled down for five min. before adding 10 ml $HCLO_4$ and placing it on the block for a further 20 min.
The samples were cooled for 30 min. before diluting each test tube to 25 ml with distilled water. The samples were stored in acid washed glass bottles with screw tops until analysis.

Copper, cobalt and manganese determination by using the Varian Spectr-AA-50 flame atomic absorption spectrometer.

The Varian Spectr-AA-50 (single beam) flame atomic absorption spectrometer combines minimal user setup and fast sample throughput. The instruments are controlled via a built-in keyboard and display. SpectrAA-50 features include an automatic monochromator, slit width and gas selection. Two fixed hollow cathode lamp positions with a manual selection lever is also included as well as an universal Mark 7 spray chamber/nebulizer with Mark VI-A burner.

The SpectrAA-50 was set according to the users guide. Lamp currents and slit-widths were substantially the same as recommended. A wavelength of 345.4 nm for Co, 327.4 nm vir Cu and 403.1 nm for Mn were used. An acetylene, air and nitrous oxide fuel mixture were used.

Flame stoichiometry was optimised for each of the elements by calibrating with the standards described in the manufacturer's manual. A 'reslope' standard was chosen by highlighting the chosen standard and pressing the 'reslope' button.

The established flow rates were noted and used throughout the investigation.

Selenium and zinc determination

The Se concentrations were determined using the hybrid generator method on a perkin-Elmer (Norwalk, Connecticut, USA) A.A. (atomic absorption spectrophotometer, model 2380).

Zinc concentrations were also determined on a perkin-Elmer (Norwalk, Conneticut, VSA) A.A. (atomic absorption spectrophothotometer, model 2380) after wet ashing and dilution of the samples.

All the glassware that was used for Se determination was left overnight in an acid bath (5% HNO_3-solution) and rinsed with distilled water after it has been thoroughly washed. After drying the glassware, it was kept separately and used only for Se determination.

One ml Se standard (20, 50, 100 and 200 mg/kg) and 0.1 g of the liver sample was weighed into acid-washed tubes. With each rack of samples, which are digested, a beef liver control (0.1g) (National Institute of standards and technology, 1577b; Gaithersburg, MD) was included.

Five ml of the digestion acid mixture (one: four $HClO_4$:HNO_3) was added into every test tube and the test tubes were placed on the programmable digestion block in the Varian extraction cabinet (Mulgrave, Australia) (model VGA-77). The block is programmed so that the digestion process will carry on for 16 hours. The program was as follows:

Four hours at room temperature

One hour from room temperature to 100 ° C

One hour at 100 ° C
One hour from 100 ° C to 180 ° C
Six hours at 180 ° C
Two hours from 180 ° C to 130 ° C
One hour at 130 ° C

Hereafter the rack of test tubes was removed from the block and it was cooled down to room temperature. 2,5 ml of 20 % HCl was added to every test tube and the rack of test tubes was placed back on the block for another 40 minutes (conversion of Se VI to Se IV). During these 40 min., the AA was set according to the users guide. A wavelength of 196 mm and amperage of eight mA was used.

An amount of 0.6 g Na (BH_4) was mixed with 100 ml 0.5 % NaOH-solution and poured into the Na (BH_4) bottle of the AA after it has been rinsed well with distilled water. Thereafter a 100 ml of 20 % HCl (6.4M) was poured into the other bottle of the hybrid generator. The rack with test tubes was removed from the block and cooled down to room temperature. The volume in the test tubes were made up to 20 ml with 20 % HCl for the standards and 10% HCl for the samples. The damp system was calibrated to deliver four ml per minute Na (BH_4) and eight ml per minute HCl. The carrier gas (nitrogen or Argon) had a flow rate of 400 ml per minute. After the AA has been set with the standards, the concentrations of the known controls was read and if the values of the controls corresponded with the known value, it was assumed that the wet ashing was done correctly and that the AA was set correctly. Thereafter the samples' concentrations were read.

2.5 Statistical analysis

The data were analysed statistically using the Proc GLM of the SAS® System (Edition 6) (SAS Institute, Inc., North Carolina, 1990).
The GLM procedure was used to show the differences between treatment one and the other treatments. The references that have been made regarding significance were based on testing against the $P < 0.05$ level. There was a significant time effect.

Chapter 3: Results

Manganese, Zn, Co, Cu and Se concentrations were determined in both fresh liver samples and after the preservation in formalin for one month, three months and six months respectively.
The results are shown in Table 3.1.1 and graphically in Figure 3.1.1-3.1.5.

3.1 Manganese, Zn, Co, Cu and Se concentrations in liver samples

Table 3.1.1 Mean (±s.e.) for Mn, Zn, Co, Cu (mg/kg) and Se (ng/g) concentration in livers after preservation in formalin over different time periods (DM basis; n=36)

Treatments	Mn (mg/kg DM)	Zn (mg/kg DM)	Co (mg/kg DM)	Cu (mg/kg DM)	Se (ng/g)
Fresh liver	$12.5^{a} \pm 0.306$	$168^{a} \pm 8.13$	$3.12^{a} \pm 0.163$	$181^{a} \pm 18.675$	$1363^{a} \pm 99.6$
Formalin 1 month	$9.6^{b} \pm 0.243$	$170^{a} \pm 8.04$	$3.12^{a} \pm 0.163$	$180^{a} \pm 18.420$	$1344^{a} \pm 75.6$
Formalin 3 months	$8.0b \pm 0.212$	$203^{b} \pm 10.34$	$3.71^{b} \pm 0.198$	$172^{b} \pm 18.518$	$1386^{a} \pm 81.6$
Formalin 6 months	$8.7^{b} \pm 0.288$	$177^{a} \pm 5.71$	$2.56^{b} \pm 0.075$	$149^{b} \pm 14.498$	$1319^{a} \pm 75.8$

^{a,b,c} Means (time 1, 3 and 6 months) in the same column bearing different superscripts differ significantly ($P < 0.05$) compared to time 0 (n=36).

3.1.1 Manganese

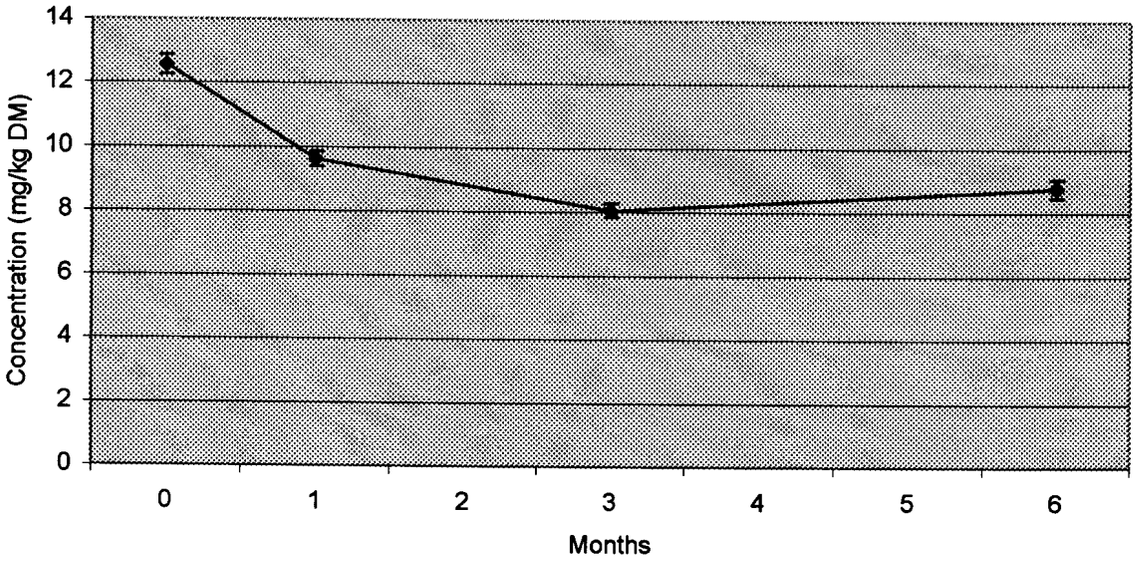

Figure 3.1.1 Mean manganese concentration (mg/kg DM) in livers preserved in formalin for different periods of time (Vertical bars represent standard error; n=36)

There was a significant decrease ($P<0.05$) in the Mn concentration from the control group to liver preserved in formalin for all the time periods (one month, three months and six months).

3.1.2 Zinc

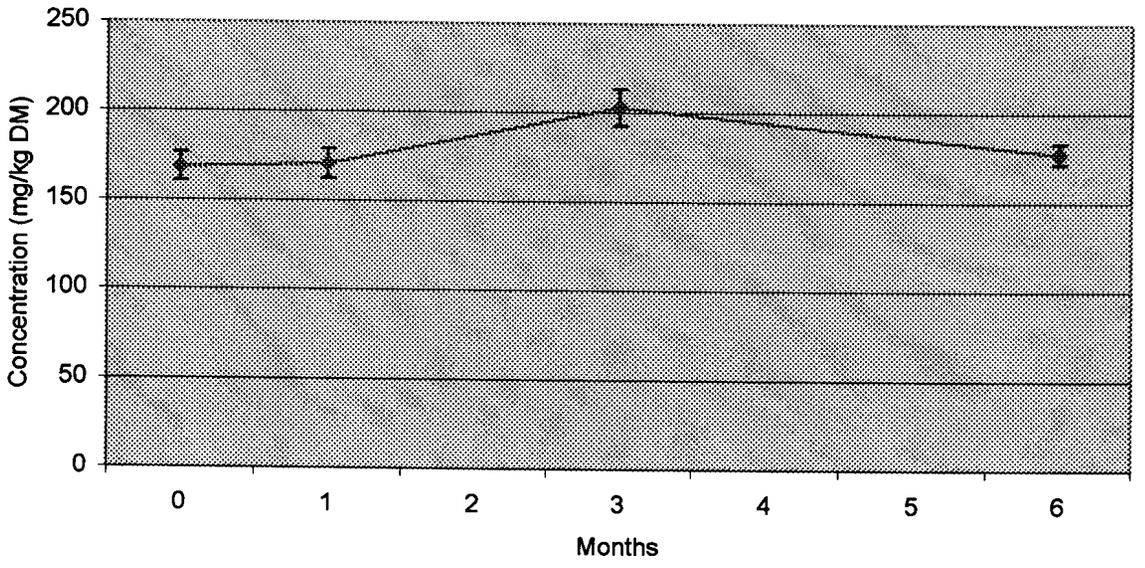

Figure 3.1.2 Mean zinc concentration (mg/kg DM) in livers preserved in formalin for different periods of time (Vertical bars represent standard error; n=36)

There was a significant increase in the Zn concentration from the control group to liver preserved in formalin for three months. Liver preserved in formalin for six months had a significantly higher Zn concentration compared to the control.

The Zn concentration in the liver that was preserved in formalin for three months was significantly higher than the other treatments.

3.1.3 Cobalt

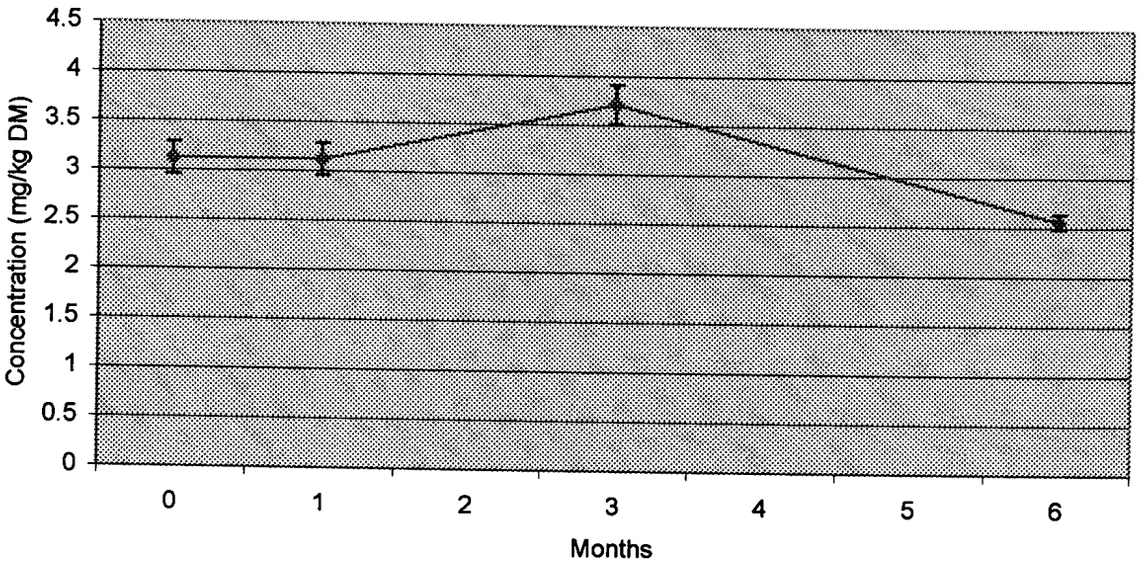

Figure 3.1.3 Mean cobalt concentration (mg/kg DM) in livers preserved in formalin for different periods of time (Vertical bars represent standard error; n=36)

The concentration of Co in liver preserved in formalin for three months was significantly higher than the control.
There was a decrease in the Co concentration from the control group to liver preserved in formalin for six months.

3.1.4 Selenium

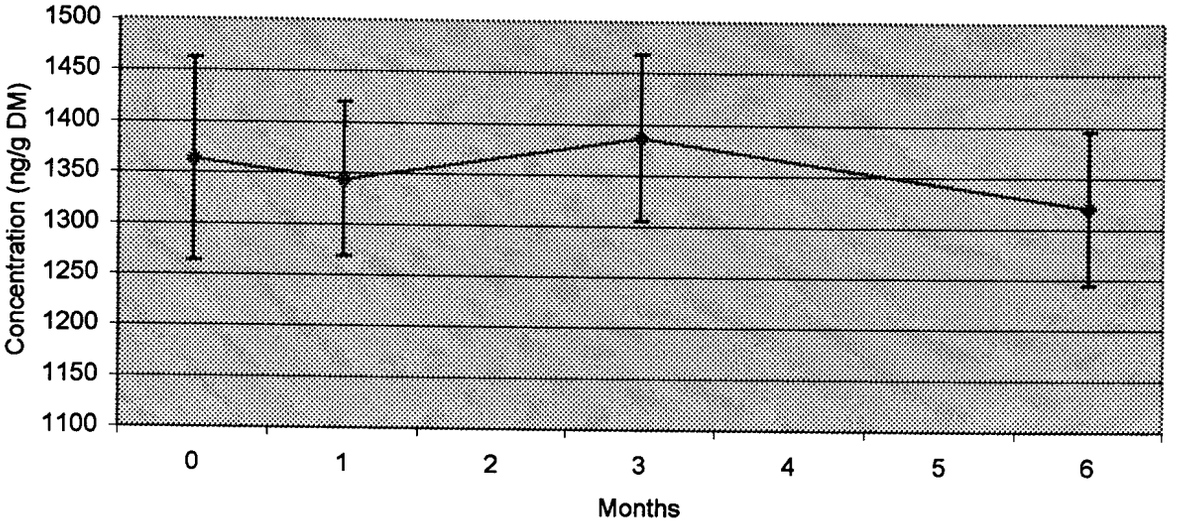

Figure 3.1.4 Mean selenium concentration (ng/g DM) in livers preserved in formalin for different periods of time (Vertical bars represent standard error; n=36)

Means of the Se concentration for every treatment was not significant different from the control.

3.1.5 Copper

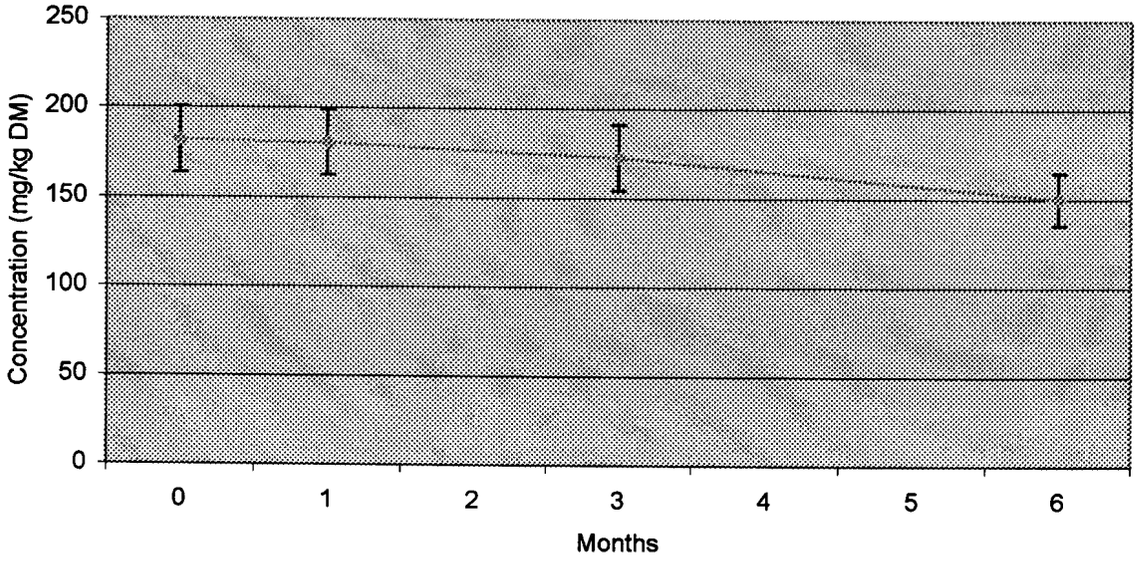

Figure 3.1.5 Mean copper concentration (mg/kg DM) in livers preserved in formalin for different periods of time (Vertical bars represent standard error; n=36)

There was a significant decrease in the Cu concentration from the control group to liver that was preserved in formalin for three months.
A decrease was also seen in the Cu concentration from the control to liver preserved in formalin for six months.

3.2 Manganese, Zn, Co, Cu and Se concentrations in formalin

Table 3.2.1 Determination of Se concentration in formalin that was wet ashed and formalin that was centrifuged from the same sample

Sample no.	Amount analysed	Concentration in formalin that was wet ashed (digested)	Concentration in formalin that was centrifuged
194C	3 ml	1.1	1.0
194C	3 ml	1.1	1.1
142C	3 ml	0.9	0.9
142C	3 ml	1.0	0.9
214C	3 ml	1.1	1.0
214C	3 ml	1.1	1.0
156C	3 ml	1.0	1.0
156C	3 ml	0.8	1.1
200C	3 ml	1.2	1.0
200C	3 ml	1.1	1.2
135C	3 ml	1.4	1.6
135C	3 ml	1.6	1.3
139C	3 ml	1.0	1.1
139C	3 ml	1.1	1.0

No difference was seen in the Se concentration between formalin that was wet ashed (digested) and formalin that was centrifuged. Afterwards, the analysis (readings) was done directly on formalin that was centrifuged.

Table 3.2.2 Mean (±s.e.) for manganese, zinc, cobalt, copper (mg/l) and selenium (ng/ml) concentration in formalin after preservation of livers over different time periods (n=36)

Treatment	Mn (mg/l)	Zn(mg/l)	Co(mg/l)	Cu (mg/l)	Se (ng/ml)
Pure formalin	0.053 ± 0^{a}	0.023 ± 0^{a}	0.003 ± 0^{a}	0.023 ± 0^{a}	0.000 ± 0^{a}
Formalin 1 month	$0.129^{b}\pm0.0033$	$0.4309^{b}\pm0.039$	$0.032^{b}\pm0.00997$	$0.449^{b}\pm0.0538$	$0.419^{b}\pm0.026$
Formalin 3 months	$0.128^{b}\pm0.0058$	$0.4926^{b}\pm0.042$	$0.016^{b}\pm0.00093$	$0.764^{b}\pm0.08075$	$0.408^{b}\pm0.034$
Formalin 6 months	$0.117^{b}\pm0.0035$	$0.4994^{b}\pm0.044$	$0.011^{b}\pm0.00053$	$0.906^{b}\pm0.09963$	$0.467^{b}\pm0.033$

^{a,b,c} Means (time 1, 3 and 6 months) in the same column bearing different superscripts differ significantly ($P< 0.05$) compared to time 0 (n=36).

3.2.1 Manganese

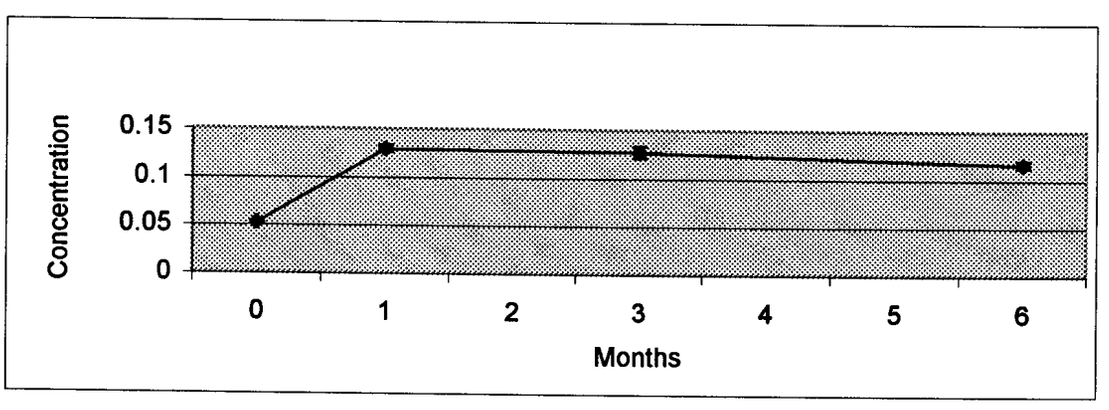

Figure 3.2.1 Mean Manganese concentration (mg/l) in formalin after one month, three months and six months of preservation of liver in formalin (Vertical bars represent standard error; n=36)

There was a significant increase ($P<0.0001$) in the Mn concentration from the control (pure formalin) to formalin in which liver was preserved for all the time periods (one month, three months and six months).

3.2.2 Zinc

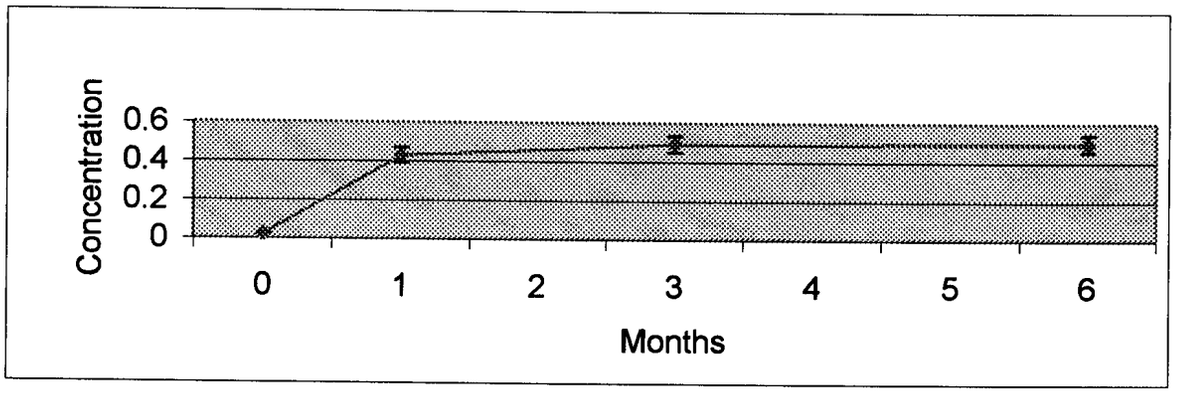

Figure 3.2.2 Mean Zn concentration (mg/l) in formalin after one month, three months and six months of preservation of liver in formalin (Vertical bars represent standard error; n=36)

There was a significant increase ($P<0.0001$) in the Zn concentration from the control (pure formalin) to formalin in which liver was preserved for all the time periods (one month, three months and six months).

3.2.3 Selenium

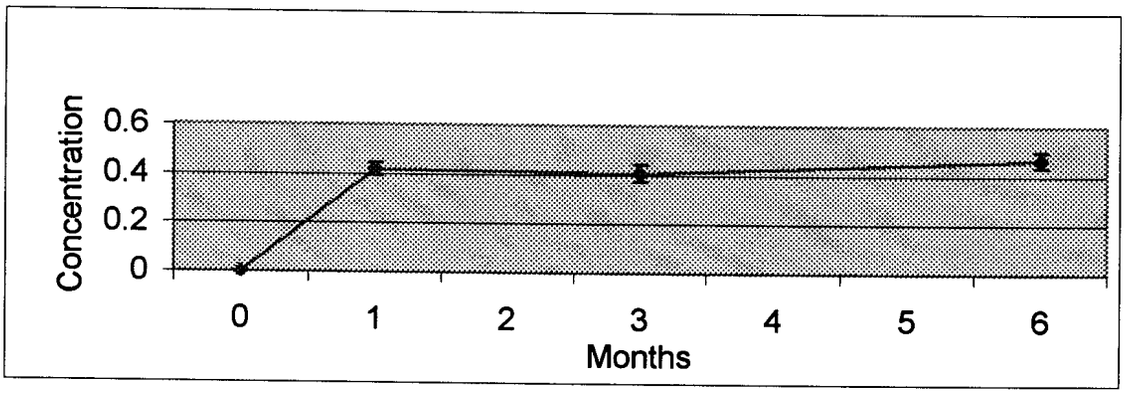

Figure 3.2.3 Mean Se (ng/ml) concentration in formalin after one month, three months and six months of preservation of liver in formalin (Vertical bars represent standard error; n=36)

There was a significant increase ($P<0.0001$) in the Se concentration from the control (pure formalin) to formalin in which liver was preserved for all the time periods (one month, three months and six months).

3.2.4 Cobalt

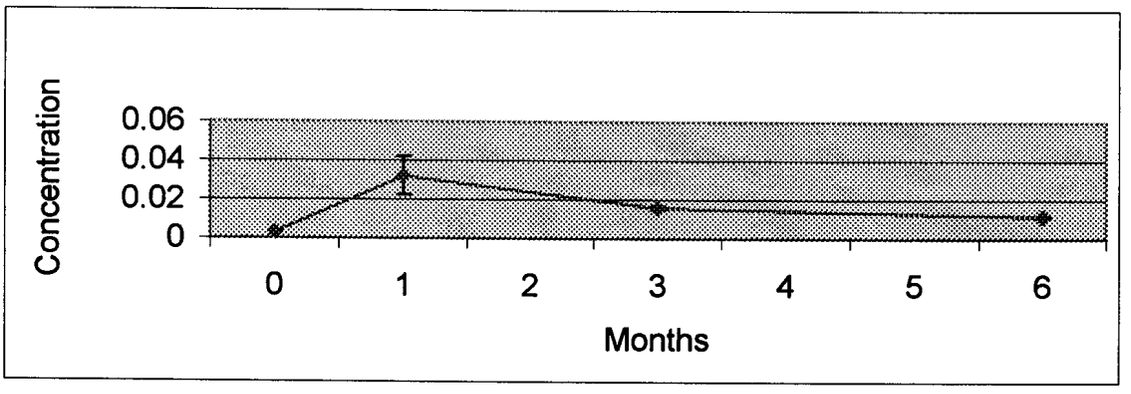

Figure 3.2.4 Mean Co concentration (mg/l) in formalin after one month, three months and six months of preservation of liver in formalin (Vertical bars represent standard error; n=36)

There was a significant increase ($P<0.05$) in the Co concentration from the control (pure formalin) to formalin in which liver was preserved for all the time periods (one month, three months and six months).

3.2.5 Copper

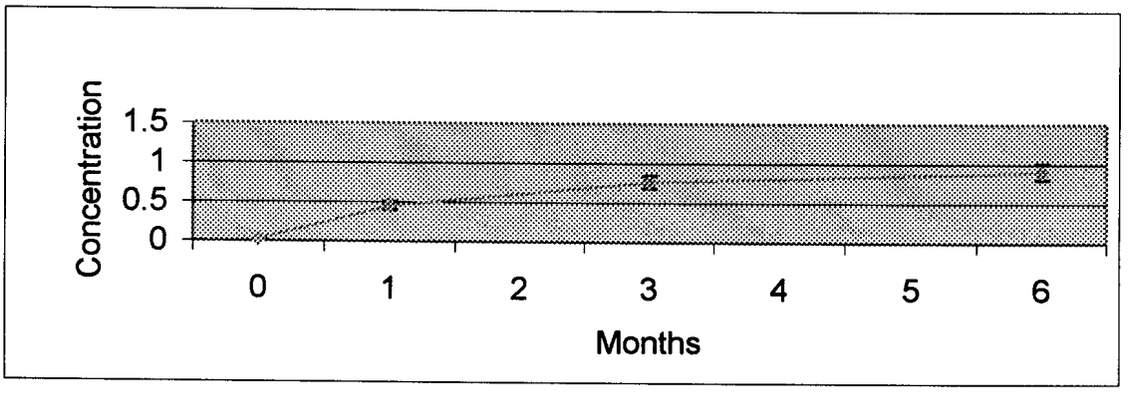

Figure 3.2.5 Mean Cu concentration (mg/l) in formalin after one month, three months and six months of preservation of liver in formalin (Vertical bars represent standard error; n=36)

There was a significant increase (P<0.0001) in the Cu concentration from the control (pure formalin) to formalin in which liver was preserved for all the time periods (one month, three months and six months).

3.3 Dry matter % in formalinised and fresh liver

Table 3.3.1 Mean (±s.e.) for dry matter % in fresh and formalinised liver over different time periods

Treatments	Dry matter (%)
Fresh liver	90.739±0.396
Liver in formalin for 1 month	92.118±0.379
Liver in formalin for 3 months	92.118±0.379
Liver in formalin for 6 months	92.118±0.379
a,b,c Means (time 1, 3 and 6 months) in the same column bearing different superscripts differ significantly (P< 0.05) compared to time 0 (n=36).	

Liver preserved in formalin for all the time periods (one, three and six months) had a significantly higher dry matter % than fresh liver (P<0.05). No difference was seen in the dry matter % between liver preserved in formalin for one, three and six months.

Dry matter % in liver is normally ± 70 %. The dry matter %, which was statistically analysed to determine if there was a difference between fresh and formalinised liver, was done on the samples after they were dried for a second time at 100 ° C. The dry matter % at 100 ° C was ± 90 %; this is the values represented in Table 3.3.1.

The concentrations of Zn and Se were not significantly different between fresh liver and liver preserved in formalin for six months. The Mn, Co and Cu concentrations were lower ($P < 0.05$) after six months of preservation. Theron *et al.* (1974) found that the concentrations of Cu, Fe and Mg were not affected by storage in formalin for 22 days, but detected statistically significant differences in the levels of Mn and Zn after six months.

No significant differences were found between the means of the four treatment groups for Se during this trail.

Table 4.1 Mineral concentrations in sheep liver (mg/kg DM) (Puls 1994).

	Co (mg/kg DM)	Cu (mg/kg DM)	Mn (mg/kg DM)	Se (mg/kg DM)	Zn (mg/kg DM)
Deficient	<0.0175	1.75-14	3.5-7.35	0.035-3.5	70-105
Marginal	0.0175-0.0595	17.5-70		0.525-0.875	
Adequate	0.0875-0.2975	87.5-350	7-15.4	0.875-5.25	105-262.5
High	0.2975-30.45	350-1750	3.5-10.5	7-35	350-1400
Toxic	17.5-1050	875-3500	17.5-1330	52.5-105	>1400

Wet weight. * 3.5-4.0 = approximate dry weight for most tissues

According to Puls (1994) the Co concentrations found during this trial for the four treatments (3.12, 3.12, 3.71, 2.56 mg/kg DM) could be categorized as being 'High'. The Cu concentrations (181, 180, 172, 149 mg/kg DM) found for the four treatments could be categorized as being 'Adequate'. Manganese concentrations (12.5, 9.6, 8.0, 8.7 mg/kg DM) during this study were 'High' while Zn concentrations for the four treatments (168, 170, 203, 177 mg/kg DM) could be categorized as being 'Adequate'. The Se concentrations found during this trial were 'Adequate' (1.363, 1.344, 1.386, 1.319 mg/kg).

Chapter 4: Discussion

When the effect of liver preservation was measured, no significant differences were found in the Cu, Co, Zn and Se concentrations between fresh liver and liver preserved in formalin for one month. There was, however, a significant decrease in the Mn concentration between fresh liver and liver preserved in formalin for one month.

The study done by Theron *et al.* (1974) was consistent with the results found in this trial for Cu and Zn, but not for Mn. Theron *et al.* (1974) took random, multiple samples from the caudate lobe of a fresh liver, as soon as possible after slaughtering, and carried out replicate determinations on them. Further portions of the liver were preserved in formalin for 22 days, after which the analysis was repeated. No statistically significant differences were noted for Cu, Fe, Mn, Zn and Mg concentrations. Theron *et al.* (1974) concluded that formalin preservation up to 22 days did not affect the trace element levels of the liver.

In the current study, the concentrations of Zn and Co were significantly ($P<0.05$) higher after three months of preservation of liver in formalin in comparison to these specific minerals in fresh liver.
The Mn and Cu concentrations were significantly ($P<0.05$) lower, after three months of preservation of liver in formalin, than the concentrations in fresh liver.

The mean concentration of the minerals (Cu, Co, Zn, Se and Mn) in liver preserved in formalin for three months was higher than the means of the other treatments. The discrepancy could be due to contamination although the standards and controls, which were analysed with each batch of samples, had the correct value.

The difference in the mineral concentrations between fresh liver and liver stored in formalin was negligible and would therefore not have any effect on the interpretation of the relative mineral concentration i.e. if a mineral is toxic or deficient.

No data could be found for formalin, used to preserve liver, which was wet ashed (acid digested) and analysed for minerals using AAS. During this trial AAS was done on the formalin, and it was found that the Mn, Zn, Cu, Co and Se concentration increased significantly ($P<0.05$) from pure formalin to formalin in which liver was preserved for al the time periods (one month, three months and six months). The concentrations of the minerals in the formalin probably were higher due to leaching of the minerals or evaporation of formalin. Another possibility is that formalin's composition differs between batches, and that it influenced the mineral concentrations in the formalin.

The formalin solution that was used for preservation of the livers during this trial was prepared using the method of Millonig's buffered formalin solution. A 40 % formaldehyde solution was used to make up the formalin solution that was used during this study. It was chemically pure, but contained 0.001% Cu per 2.5 l (w/v), which could have lead to contamination of the liver that was preserved in the formalin.

Summers *et al.* (1980) investigated the consequences of performing Cu analysis on tissue specimens preserved in formalin. In almost all cases, it was observed that levels were lower in the preserved tissues. The explanation for the lowered values following preservation was two-fold; firstly, imbibition of fluid by the tissue in the preservative and secondly, loss of Cu into the solution. It was found that the weight of the imbibed fluid would have an effect of lowering the concentration of the assayed element per unit weight of preserved tissue (Summers *et al.*, 1980).

The difference in the concentration between fresh liver and liver stored in formalin was small therefore it would not have any effect on the interpretation, i.e. if a mineral concentration is toxic or deficient, of the relative mineral concentration.

Theron *et al.* (1974) investigated the possibility that moisture may not be homogeneously dispersed within a liver. A comparison of moisture determinations was done on homogenized and non-homogenized samples taken from a fresh liver. The mean for the non-homogenized liver was 63.3 % with an s.d. of 4.2 while the mean for the homogenized sample was 65 % with an s.d. of zero within a fresh liver (Theron *et al.*, 1974).

The dispersion of moisture within an individual liver after storage in formalin was investigated in the same manner as for the fresh liver. The irregular dispersion of moisture within a formalinised liver is not significant. Moisture apparently becomes more homogeneously dispersed on storage. The mean for the non-homogenized liver was 71.0 % with an s.d. of 1.07 while the mean for the homogenized sample was 75.5 % with an s.d. of 0.87 within a formalin-preserved liver ($F = 1.53$ $F(5\%) = 3.87$) (Theron *et al.*, 1974).

Theron *et al.* (1974) found that livers may be stored for up to two years without affecting moisture content significantly and concluded that mineral levels expressed on a wet basis are directly comparable for fresh and stored livers (Theron *et al.*, 1974). During this trial, liver preserved in formalin (for one, three and six months) had a significantly higher DM % than fresh liver ($P<0.05$), probably due to dehydration due to formalin fixation. No difference was seen in the dry matter % between liver preserved in formalin for one, three and six months. Although it is preferred that mineral concentrations should be expressed on a dry matter basis, it would probably not influence the interpretation of the mineral concentrations, if the mineral concentrations were expressed on a wet basis. It is recommended that the expression of mineral concentrations should be standardised for easier comparison between studies.

During this trial liver preserved in formalin for all the time periods had a significantly higher dry matter % than fresh liver ($P<0.05$) probably due to dehydration because of formalin fixation. Although it is preferred that mineral concentrations are expressed on a dry matter basis, it would not affect the interpretation of the mineral concentrations if the mineral concentrations were expressed on a wet basis. The results of diagnostic tests, for example liver mineral levels, can only be interpreted using established reference criteria that relate these mineral levels, or the mineral status of the animals to production responses, when they are supplemented with a specific trace element (Grace & Clark, 1991).

Chapter 5: Conclusions

No statistically significant differences were noted for Cu, Co, Se and Zn concentrations for liver that was preserved in formalin for one month. It was concluded that formalin preservation up to one month did not affect the trace element levels except for Mn. The decrease in the Mn concentration was propably due to leaching.

The concentrations of Zn and Co were significantly ($P<0.05$) higher while the concentrations of Mn and Cu were significantly ($P<0.05$) lower, than in fresh liver, after three months of preservation of liver in formalin.

The concentrations of Zn and Se were not significantly ($P<0.05$) different between the fresh liver and liver that was preserved in formalin for six months. The Mn, Co and Cu concentrations were statistically lower after six months preservation of liver in formalin.

Liver concentrations of Se remained unchanged by formalin-fixation, over all the time periods, the analysis of formalin-fixed liver for diagnostic purposes can be recommended for Se.

The Mn concentration decreased significantly after one month, three months as well as after six months of formalin preservation of liver compared to fresh liver.

The difference in the mineral concentrations between fresh liver and liver stored in formalin was small. It would not have any effect on the interpretation (if a mineral is toxic or deficient) of the relative mineral concentration.

In this trial formalin was also analysed using AAS for all the minerals over the three time periods (one month, three months and six months).

There was a significant ($P<0.05$) increase in all the mineral (Cu, Co, Zn, Mn and Se) concentrations from pure formalin to formalin in which liver was stored over all the time periods (one month, three months and six months). From the analysis of the formalin it would appear as if evaporation or leaching has taken place, which would both lead to an increase of the mineral concentrations in formalin. The formalin used to preserve the liver contained Cu before it was used for preservation; this also has to be taken into consideration.

Liver preserved in formalin for all the time periods (one, three and six months) had a significantly higher dry matter % than fresh liver ($P<0.05$) probably due to dehydration because of formalin fixation. No difference was seen in the dry matter % between liver preserved in formalin for one, three and six months. Although it is preferred that mineral concentrations are expressed on a dry matter basis, it would not affect the interpretation of the mineral concentrations if the mineral concentrations were expressed on a wet basis.

Recommendations

1) The dehydration of tissue samples by freezing or formalin fixation and the leaching of minerals from fixed tissue must be considered in interpreting data from stored samples. Regression equations must be calculated to take those effects into consideration and provide for easy extrapolation from the mineral concentration determined in formalin-fixed liver to the expected concentration in fresh liver.

2) The formalin used for preservation must be buffered since mineral dissolution would be expected in non-buffered, acidic, formic acid-rich formalin solution.

3) Standard conditions for the weight basis of expression of the results must be used in preparing the tissues so that meaningful comparisons among different studies can be made. Results should rather be expressed on a dry weight basis.

4) Tissues with a high concentration of a certain mineral should not be stored in formalin with tissues with a lower concentration in the same mineral, because leaching and contamination can present a problem.

5) Alternative preservation solutions must be investigated. The cost effectiveness of F13, and other alternatives, must be compared to formalin.

6) Each new mixture of formalin should be analysed for the presence of the minerals that are being analysed and the samples' concentrations should be corrected accordingly.