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# On-line solid-phase extraction–capillary electrophoresis for enhanced detection sensitivity and selectivity: application to the analysis of metallothionein isoforms in sheep fetal liver

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**Abstract**

A simple and rapid capillary electrophoresis (CE) method for analysis of metallothionein (MT) isoforms is described. A modified two-step solvent extraction procedure was used in combination with CE and an on-line solid-phase preconcentration device for sensitive and reproducible detection of MT isoforms in sheep fetal liver. Preparation of twenty samples was practicable within a working day with subsequent automated overnight analysis by solid-phase extraction (SPE)–CE. A commercially available divinylbenzene-based reversed-phase resin was found to be most suitable for the SPE device because it is resistant to extremes of pH and can be adequately regenerated between analyses. Each SPE device was readily constructed from commonly available materials and was used for the reproducible separation of over 100 biological samples before replacement. Precision of analysis within or between sample batches was $<10\%$ and usually $<5\%$ while detection limits were at least 28 ng/ml for standards and 272 ng/ml for biological samples. This would indicate a detection limit of about 0.5 μg/g wet weight of tissue. Recovery of MT from tissue cytosols by solvent extraction was measured using radiolabeled MT and was found to be just over 50% increasing to almost 70% by addition of NaCl to the homogenisation buffer. The combined solvent extraction and SPE–CE methodology was applied to the analysis of MT in sheep fetal liver and the results compared favorably with those obtained by high-resolution chromatography. MT-1 levels were 2 to 4-times higher than those of MT-2 and both isoforms decreased from day 89 to day 136 of gestation. These results were compared with MT levels in fetal liver from sheep embryos that had been perturbed by temporary transfer to an advanced uterine environment. Hepatic MT levels at day 136 of gestation were 3 to 8-times higher than in normal fetal liver and significant differences were observed with both isoforms. 

*Keywords:* Selectivity; Solid-phase extraction; Metallothioneins

## 1. Introduction

Many mammals have multiple linked and metal-inducible metallothionein (MT) genes, some of which are functional and code for a family of metal-binding protein isoforms [1,2]. Although the cysteine content (32%) and location within these isoforms is highly conserved, differences between the remaining amino acids may be as low as 2% or as high as 40%. Thus the protein isoforms can have quite similar or quite distinct charge and/or hydrophobicity. From the relatively few detailed studies of MT isoform expression, it is clear that some are of minor abundance and expression compared to others [3,4]. They may show inducer or cell specific induction [5]

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and, for example, MT-3 and MT-4 genes are expressed almost exclusively in brain and squamous epithelial tissue, respectively [6,7]. The primary role of different MT isoforms may well be distinct but until they can be rapidly and routinely analysed, their expression and hence their function will remain difficult to establish.

Both sample preparation and analysis of MT isoforms can be straightforward, if time consuming, using various forms of chromatography [8–11]. This approach may not be very practicable for their rapid and routine analysis and so alternative methods have been evaluated. The utility of capillary electrophoresis (CE) based techniques for the rapid separation of MT isoforms has recently been demonstrated in several different laboratories [12–18]. Although these techniques offer excellent separation and resolution within minutes or even seconds and require only very small sample volumes, the relatively poor sensitivity of UV detection has been a major obstacle to the acceptance of CE as a routine analytical tool for many biological applications.

On-column techniques for analyte concentration, such as stacking, field amplification and transient isotachophoresis (ITP), yield some sensitivity enhancement but are limited by the amount of sample that can be loaded into a capillary. Improvements in both sensitivity and selectivity using automated on-line pre-concentration techniques such as ITP–CE [19–21] have been evaluated but this type of modification usually requires some technical adaptation of laboratory constructed equipment. The concept of using an on-line solid-phase extraction (SPE) device for micro-separation techniques was first demonstrated by Kasicka and Prusik [22], who used their immunoaffinity concentrator on-line with capillary ITP. Guzman et al. [23] applied the same idea to CE, where an immobilized antibody concentrator was attached to the inlet end of the analytical capillary. Sample was pressurized through the extractor and the bound analytes eluted and separated. An integrated SPE capillary system was available commercially for a limited period (AccuSep C/PRP, Waters Chromatography, Watford, UK) and more readily constructed, inexpensive alternative designs have been published [24–26]. Membrane and various other types of extraction/affinity systems have been developed [27,28] and a review of these techniques has been published [29]. In addition to increasing the sensitivity of CE, a great advantage of many of these techniques is that they also enhance specificity for the analyte of interest. This selectivity may be partial in the case of hydrophobic SPE material or very specific, as in the case of immunoaffinity or other affinity devices. Problems associated with hydrophobic SPE devices at least in part relate to the nature of the solid-phase material used, since the common silica bonded reversed-phase polymers are sensitive to low, and particularly high pH solutions. Hence, it has proved difficult to reproducibly regenerate capillary and SPE material after separation of biological samples. We have therefore screened several pH-insensitive reversed-phase media and report the utility of one such material for general-purpose on-line SPE–CE.

To demonstrate that this method is robust and that it can be used repeatedly to separate biological samples with adequate precision, we have applied the SPE–CE technique to the analysis of sheep fetal liver samples and by summation of isoform levels, have compared the results with data for total MT obtained using a more established independent technique, namely high-resolution size exclusion chromatography (HRSEC). Sheep fetal liver Zn and MT levels have been shown to be very high early in gestation with a rapid subsequent decline up to and beyond birth at about 140 days post-conception [30,31]. Evidence for the pattern of mRNA levels is more contradictory with one study demonstrating a high correlation between hepatic Zn and MT-1a mRNA [31] and another showing peak MT-1a mRNA levels between 130 and 135 days gestation, with comparatively little of this mRNA at days 100 and 140 [32]. The levels of MT-1b, MT-1c and MT-2 mRNA were also measured in the latter study and showed a similar trend to MT-1a mRNA. We have used the SPE–CE technique to study MT-1 and MT-2 protein concentration in the developing sheep fetal liver and also to investigate the effect of temporary embryo transfer to an advanced uterine environment on fetal hepatic MT isoform levels. Previous studies have shown that such treatment and other manipulation of embryos prior to permanent transfer increases fetal size by about 40% at day 37 of gestation, as compared to that obtained by direct transfer of embryos to synchronized ewes [33]. The

initiating factor and mechanism of this phenomenon is currently the subject of a major study [34] from which material for the present work was obtained. High levels of maternal progesterone in asynchronous ewe recipients have been implicated but the mechanism which sustains enhanced fetal growth is unknown.

## 2. Experimental

### *2.1. Animals*

Scottish Blackface ewes were used in this study and details of embryo generation and transfer have been published [34]. Briefly, superovulation was induced in ewes and the embryos were fertilized by intrauterine insemination via laparoscopy. Embryos were recovered three days later and transferred laparoscopically to synchronous (Day 3) permanent recipients or asynchronous (Day 6) temporary recipients for three days. Embryos recovered from the latter ewes were then transferred to Day 6 permanent recipients and are referred to as perturbed. Tissue samples, were obtained from the recovered fetuses at various time intervals from day 35 to 136 of gestation. For the present work, samples of liver at time points of 89, 110, 125 and 135 or 136 were kindly made available by participants in the fetal growth study. Tissue was snap-frozen in liquid nitrogen and stored at −80°C until required.

### *2.2. Sample extraction*

Using 5 ml polypropylene tubes, 400 mg of thawed liver was homogenized (33%, w/v) with ice-cold 100 m*M* Tris–HCl buffer pH 8.0 and centrifuged at 15 000 *g* for 30 min (4°C). While vortexing, 700 μl of ice-cold ethanol–acetonitrile (1:3) were slowly added to 700 μl of the supernatant followed by intermittent vortexing to ensure thorough mixing. The sample was centrifuged at 10 000 *g* for 10 min (4°C) to remove precipitated proteins. Further ethanol–acetonitrile solvent (3.2 ml) was then added to 0.8 ml of the supernatant, the tubes were capped and the contents mixed thoroughly. The samples were centrifuged at 10 000 *g* for 10 min (4°C) and the supernatant discarded. The pellet was re-suspended in 2×200 μl of ice-cold 100 m*M* Tris buffer pH 8.0, using sonication to achieve homogeneous re-suspension, and the solution transferred to a microcentrifuge tube before re-centrifuging, this time at 4°C. Leaving the pellet in place, the caps were pierced with a syringe needle and the samples were heated for 2 min using a heating block at 100°C. The samples were immediately and rapidly cooled on ice and the precipitate was pelleted by further centrifugation at 4°C. The supernatant was used for analysis and liver samples of well-characterized MT status, obtained from a Zn-injected Scottish Blackface ewe, were used as quality controls in each extraction sample batch.

### *2.3. Instrumentation*

A P/ACE 2050 system (Beckman Instruments, High Wycombe, UK) was used for all separations and capillary cartridges were fitted with polyimide-coated fused-silica capillaries [57 cm total length (50 cm from inlet to detector)×75 μm I.D., 375 μm O.D.: Composite Metal Services, Hallow, UK]. The temperature of the capillary was maintained at 25°C by means of circulating coolant and detection was made by monitoring UV absorption at 200 nm.

### *2.4. Extractor construction*

The construction of the extractor was similar to that described previously [26] in which a 5 mm length of polyethylene tubing (280 μm I.D., 610 μm O.D.; ''Intramedic'' PE 10, Clay Adams division of Becton Dickinson, NJ, USA), hereafter referred to as the extractor tube, was widened with a metal wire (310 μm O.D.) and fitted over the end of a short piece of uncoated fused-silica capillary (approx. 3 cm×75 μm I.D., 375 μm O.D.). A polysulfone filter (0.2 μm HT-200 Tuffryn membrane filter, Gelman Sciences, Northampton, UK) was cut in small squares which fitted the approx. size of the I.D. of the extractor tube and then pushed into the extractor tube with a metal wire to form a frit against the end of the silica capillary. Rezorian material (Rezorian A 161 cartridges, Supelco, Poole, UK) was suspended in ethanol and drawn into the extractor tube with a syringe attached to the free end of the silica capillary using 0.05 cc/m PVC connecting tubing (0.25 mm

Orange/Blue, Anachem, Luton, UK). When the extractor tube was 2/3 full, a further filter square was inserted to form another frit and the open end of the extractor tube was fitted over the end of the separation capillary [57 cm (inlet to window, 50 cm)×75 μm I.D.]. Care was taken to leave enough room in the extractor tube for the insertion of the capillary without unduly compressing the Rezorian material. Ethanol was then flushed through the extractor tube to check for abnormal flow restriction. Using the Beckman cartridges, the short capillary section was trimmed so that the protruding in- and outlet ends, with the extractor tube fitted, were of equal length and the extractor tube was then inspected under a microscope (Fig. 1). The cartridge was inserted into the P/ACE unit as normal with no further modifications necessary.

### 2.5. Extractor washing and separation conditions

Preliminary studies in our laboratory have shown that the water quality is crucial when using preconcentration before separation. Therefore the water used in this study was of highest quality obtained from a Milli-RO 6 primary system in combination with a Milli-Q Plus 185 secondary treatment system. Alternatively, less pure water can be prepared to the required purity by passing it through a hydrophobic SPE device such as a Rezorian cartridge. Reagents used were of highest available grade (AristaR grade, BDH, Poole UK). Before a series of runs the concentrator was conditioned by purging for 1 min with acetonitrile followed by 1 min with water. Each run started with a pre-rinse using run buffer (20 m*M* sodium phosphate, pH 7.0) under high pressure followed by sample or standard injection for 1–8 min under low or high pressure. Unbound analyte and sample solution was removed by high-pressure purging with run buffer for 1 min. The elution of analytes from the extractor was made by low-pressure injection of 50% acetonitrile in 5 m*M* sodium phosphate, pH 2.5, for 0.3 min. Separation was performed by applying a potential of 20 kV (anode at inlet and cathode at outlet) until analysis was completed. The following wash procedure (each stage, 1 min at high-pressure) was found to be critical in maintaining extractor performance and inter-sample reproducibility: (1) 1% Triton X-100; (2) 100% acetonitrile; (3) 0.1 *M* NaOH; (4) 50% acetonitrile in 5 m*M* phosphate buffer, pH 2.5; (5) 100% acetonitrile and (6) water.

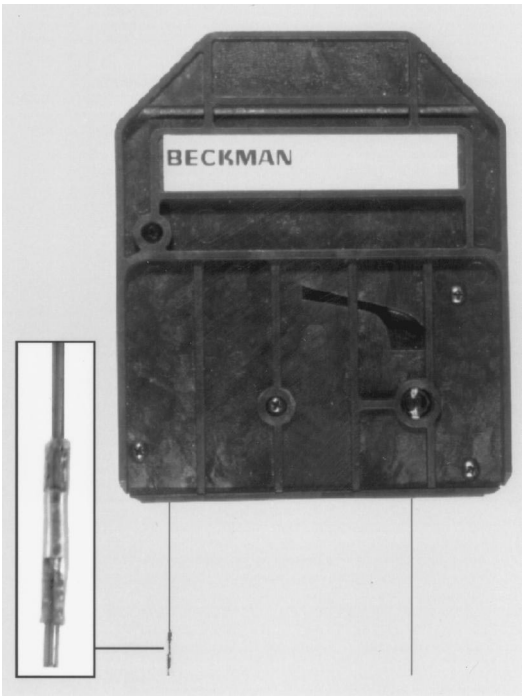


Fig. 1. SPE device installed on the inlet capillary of a Beckman P/ACE capillary cartridge. A magnified view of the device is presented inset and shows the polypropylene extractor tube containing Rezorian material and plugged at both ends by capillaries.

This procedure was able to regenerate the extractor even when loaded with large amounts of protein. The cycle was then repeated for analysis of further samples and the whole process was automated through software control (System Gold, Beckman Instruments). The samples were solubilized in 0–75 m*M* sodium phosphate and injected for 1 to 8 min under high or low pressure.

### 2.6. HRSEC of fetal sheep livers

The liver samples were homogenized and centrifuged as described in Section 2.2. The supernatants were then transferred to microcentrifuge tubes and stored at −20°C until analysis. Before analysis, the samples were thawed and centrifuged at 16 000 *g*. 200 μl of the supernatant was injected on a Superose

12 HR 10/30 column (Pharmacia Biotech, Alleroed, Denmark) and eluted with 100 m*M* Tris–HCl+100 m*M* NaCl pH 8.6 at a flow-rate of 0.5 ml/min. From 9 to 53 min, fractions of 1 ml were collected and analysed for Zn using flame atomic absorption spectrophotometry. The chromatography was monitored by a PC controlling the FPLC-system. The MT content was calculated on the basis of 1 mol MT binds 7 moles of Zn.

## 3. Results

### *3.1. Extraction efficiency*

The extraction efficiency for MT using the two-step solvent method was measured in liver samples of widely different endogenous MT content (approximately 35 or 1000 μg MT/g) by spiking the sample cytosols with a radiolabeled tracer (purified sheep MT-1 labeled with [$^{125}$I]Bolton and Hunter reagent [35]). Recovery of MT in the extract was 51.7±2.7% and there was no significant difference between livers with high or low MT content ($P=0.1166$). The variations in extraction efficiency from day-to-day were investigated and found not to vary significantly ($P=0.1648$).

### *3.2. SPE-adsorption optimisation*

The efficiency of SPE depends on the relative affinity of the analyte for the solid and the liquid phase. As a way of manipulating the equilibrium in favor of adsorption onto the solid-phase, we decided to study the effect of increasing ionic strength of the sample buffer on the affinity of MT for the Rezorian material. A very marked increase in adsorption efficiency was demonstrated for low- and high-pressure injection of a purified MT sample when the buffer concentration was raised from 0 (water) to 25 m*M* phosphate, pH 7.0 (Table 1). There was no further improvement in raising the buffer concentration up to a maximum of 75 m*M*. However, the adsorption precision was observed to increase with high buffer concentration at low-pressure injections, but decrease at high-pressure injections. High-pressure injection resulted in a higher overall loading of the extractor than low-pressure injection. However, the relative difference in adsorbed MT after low- and high-pressure injection for a constant time (approx. ×8) was less than the relative difference in the applied pressure (×36). For these reasons all standards and samples were routinely dissolved in 75 m*M* phosphate pH 7.0 and injected at low pressure.

### *3.3. Relationship between corrected absorbance peak area and loading time or concentration of MT-1 standard*

The relationship between loading time (0–10 min) at low pressure and peak area/migration time was investigated and found to be linear (Fig. 2A). To evaluate the SPE capacity, a more concentrated solution of sheep MT-1 (670 μg/ml) was injected for 4 min at low pressure. The resulting peak exceeded the maximum absorbance scale of the detector and was taken as evidence of sufficiency of the SPE device when injecting for 4 min. The standard curve of sheep MT-1 (5–50 μg/ml) was found to be linear (Fig. 2B). Since limited sheep MT-2 was available, the concentrations of MT-1 and -2 in sheep livers were determined from the MT-1

Table 1
The effect of phosphate buffer concentration and loading-pressure time on the efficiency of rabbit MT-2 (20 μg/ml) extraction by Rezorian reversed-phase material using on-line SPE–CE

| Buffer concentration (m*M*) | Low pressure (0.5 p.s.i. for 4 min) | High pressure (18 p.s.i. for 1 min) |
|---|---|---|
| 0 | 1.0±12.8 | 2.8±8.3 |
| 25 | 12.7±4.5 | 24.4±4.0 |
| 50 | 12.2±2.0 | 20.3±12.9 |
| 75 | 12.7±1.7 | 20.2±14.3 |

Data are corrected peak areas (area/migration time) relative to the values for low pressure loading (0.5 p.s.i. for 4 min) at 0 m*M* buffer concentration (water), and are the mean±R.S.D. of three replicates.

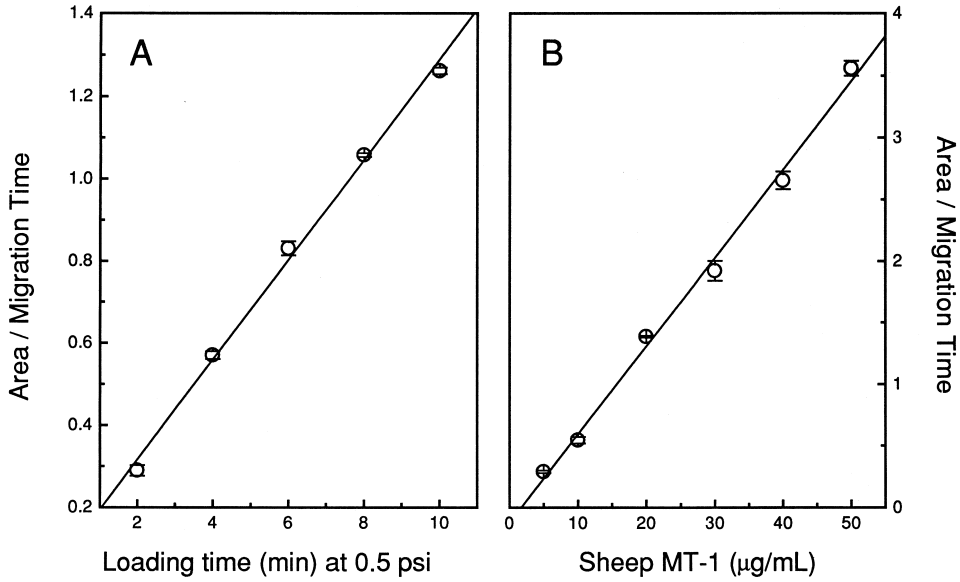


Fig. 2. (A) The relationship between the loading time of purified sheep MT-1 (17 μg/ml of 75 m*M* phosphate buffer, pH 7.0) through a Rezorian SPE device at 0.5 p.s.i. and the amount of protein separated on-line by CE. (B) Standard curve of sheep MT-1 using a constant loading time of 4 min at 0.5 p.s.i. Values are corrected 200 nm absorbance peak areas and error bars show standard deviations of duplicate data.

standard curve assuming the same extinction coefficient at 200 nm for both MT-1 and MT-2. In addition, at least two standards were measured before running each batch of liver samples. All samples were analysed in duplicate (Fig. 3) and the mean variation (R.S.D.) in corrected peak area between duplicate determinations was 4.6% ($n=25$). The detection limits were calculated using $S/N=2$. From the standard curve and standard addition curve, the detection limits for low-pressure 4 min injections, were found to be 28 ng sheep MT-1/ml for standards and 272 ng sheep MT-1/ml for liver samples.

### *3.4. MT content of liver samples*

The MT-1, MT-2 and total MT content of the livers were analysed using on-line SPE-CE and HRSEC, respectively. Both methods showed the same pattern in total MT content (Fig. 4). Calculation of HRSEC separated MT was based on analysis of fraction Zn levels but it is probable that the MT also bound some Cu. Hence, the HRSEC MT values presented in Fig. 4 are probably underestimates of the total MT. Lower variance in the HRSEC total MT data was largely due to better precision of Zn analysis by atomic absorption spectrophotometry as compared to UV absorbance detection at 200 nm for SPE–CE. Indeed, variance in the integrated UV absorbance peaks for MT separated by HRSEC and SPE–CE was similar.

As regards normal fetal liver, the MT content decreased during gestation from day 89 to day 136, whereas the MT content in the perturbed fetal liver decreased from day 89 to day 125 and then stabilized or increased slightly from day 125 to day 136. At day 136 the total concentration of MT was three- or eight-times higher in the perturbed fetus than in normal fertilized fetus, analysed with SPC–CE and HRSEC, respectively. The individual isoforms of sheep MT followed the same pattern as the total MT content for both normal and perturbed fetus (Fig. 5). However the MT-1 concentration was two- to four-times the MT-2 concentration during all time points investigated (Table 2). In addition the MT-1/MT-2 ratios at each time point were the same for both the perturbed and normal fetus. The MT-1/MT-2 ratio increased only at day 125 but only significantly

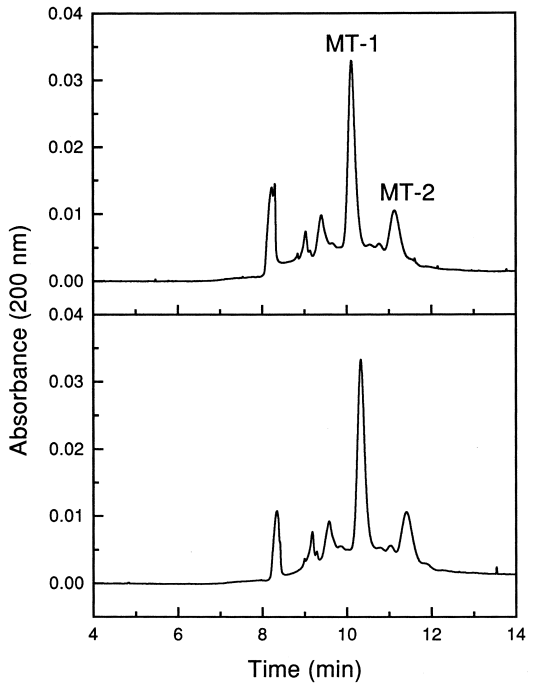


Fig. 3. SPE–CE electropherograms of a sheep fetal liver MT extract obtained using the two-step solvent extraction procedure. This example shows duplicate separations of a 10-fold diluted extract from a 110-day perturbed sheep fetus.

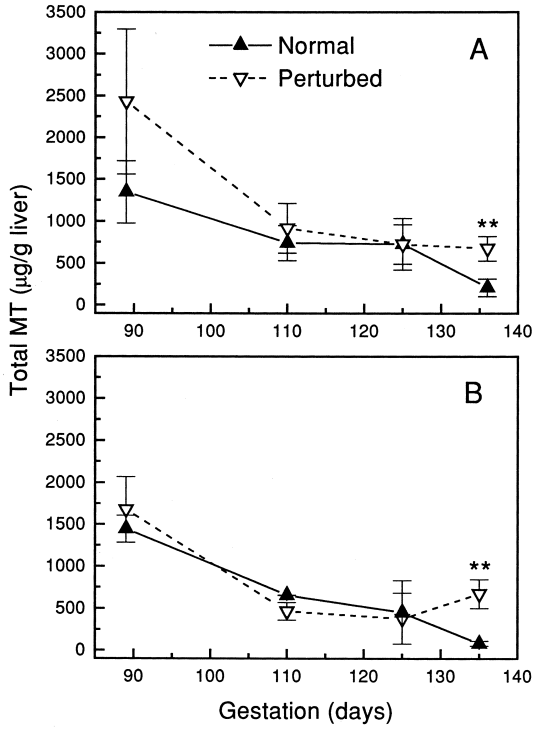


Fig. 4. Total MT levels in the liver of normal and perturbed sheep fetus at intervals during gestation. MT was measured by SPE–CE (summation of MT-1 and MT-2) (A) and from the Zn content of the MT fraction separated by HRSEC (B). Data-points are the mean of 3–4 replicates and error bars indicate standard deviations. The data were analysed by unpaired *t*-tests and ** indicates significance at $P<0.01$.

($P<0.05$) when both perturbed and normal ratios were pooled.

## 4. Discussion

On-line SPE–CE is a relatively novel technique which improves some of the sensitivity and specificity limitations of standard CE methods without eliminating their considerable practical benefits. Major advantages of the SPE devices described here and elsewhere [24,25] are that they can be easily constructed from readily available and inexpensive materials, they can be used in conjunction with most commercial CE systems and, depending on the choice of SPE material, can be re-used repeatedly for analysis of biological samples without deterioration in performance. In the present study, we have shown that a specific combination of reversed-phase material and regeneration system allows repeated reproducible and quantitative analysis of MT isoforms in liver extracts by on-line CE. In addition, we have modified and adapted a two-step solvent-based procedure for the rapid extraction of MT from liver samples for subsequent analysis by SPE–CE. In combination, these techniques facilitate the rapid analysis of MT isoforms at tissue concentrations $>0.5$ μg/g. At the expense of loading time and/or the SPE efficiency at higher loading pressures, detection limits can be improved still further.

Several divinylbenzene-based reversed-phase materials such as the irregularly shaped XAD resins (Serva Fine Biochemicals, Heidelburg, Germany) and spherical Rezorian material (Supelco UK, Poole, UK), were considered for use in the SPE device. At least partly due to the particle shape, larger size (mean diameter 120 μm) and larger pore size (120–175 Å), the performance of Rezorian material in the

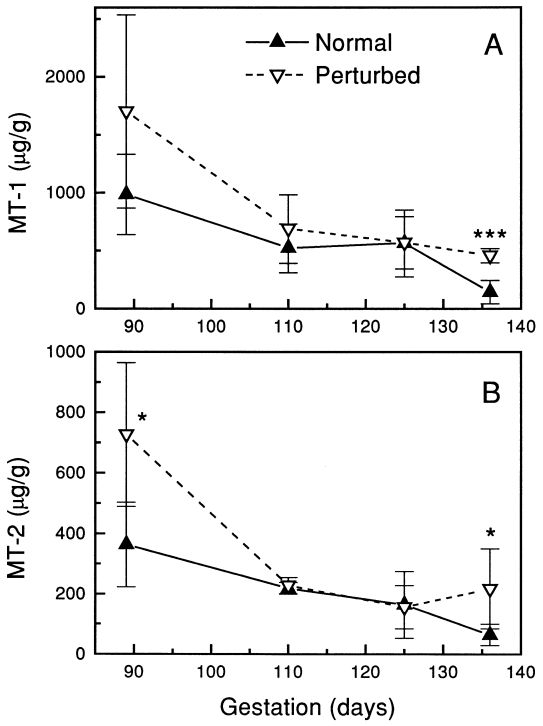


Fig. 5. Isoform levels for MT-1 (A) and MT-2 (B) in normal and perturbed sheep fetal liver measured by SPE–CE. Data-points are the mean of 3–4 replicates and error bars indicate standard deviations. The data were analysed by unpaired *t*-tests and * or *** indicate significance at $P<0.05$ or $P<0.001$, respectively.

SPE device in terms of the uniformity of liquid flow and also adsorption properties, was superior to the other resins evaluated. As found previously with a $C_{18}$ reversed-phase device, extraction of MT from a purified protein solution was linear at loading times of up to several minutes at 0.5 p.s.i. (1 p.s.i.= 6894.76 Pa). A standard curve generated by loading purified MT-1 at concentrations of 5–50 μg/ml and at 0.5 p.s.i. for 4 min was also linear. The relative standard deviations (R.S.D.s) of corrected peak areas at different loading times and concentrations for replicate analyses were generally <5%. During development of the loading procedure, it was noted that an appreciable amount of the loaded protein was not binding to the Rezorian material in the SPE device. This was detected by the appearance of a positive absorbance peak during the post-low pressure loading wash with electrolyte and therefore efforts were made to improve binding efficiency. As recommended by the manufacturer for off-line use of the Rezorian A-161 cartridges, binding efficiency is enhanced at lower flow-rates and therefore the relative recovery of MT isoforms (MT peak area/volume loaded) was higher at 0.5 p.s.i. than at 18 p.s.i. In RP-HPLC, association of a charged analyte with the reversed-phase material is manipulated by using ion-pairing agents or changing the salt and/or solvent concentration of the elution buffer. Our studies showed that by increasing the phosphate buffer concentration in the sample solution, a marked improvement in binding efficiency was achieved. This improvement was noted with both high- and low-pressure loading (Table 1) and a significant benefit of this phenomenon is that samples containing salts at even quite high concentrations, can be analysed by this technique. Not only is adsorption to the solid-phase increased, but salts are subsequently washed off to waste and do not therefore appear in the electropherogram or affect resolution by diminishing or abolishing stacking effects.

Table 2
The ratio of MT-1/MT-2 in normal and perturbed fetal liver at intervals during gestation

| Gestation (days) | Normal | Perturbed | Combined data |
|---|---|---|---|
| 89 | 2.8±0.3 (4) | 2.3±0.5 (3) | 2.6±0.4 (7) |
| 110 | 2.3±0.8 (3) | 2.9±1.1 (5) | 2.7±1.0 (8) |
| 125 | 4.0±1.7 (3) | 3.8±0.8 (4) | 3.9±1.1 (7) |
| 136 | 2.4±1.0 (4) | 2.6±1.3 (3) | 2.5±1.0 (7) |

Isoforms measured by SPE–CE using 200 nm absorbance peak area were corrected for differences in migration velocity by division with migration time. Values are the mean±S.D. for the number of replicates in parentheses.

After loading samples with a high protein content through Rezorian material, attempts at complete regeneration without deterioration in SPE performance using only 0.1 *M* NaOH were unsuccessful. Cleaning with an anionic detergent like sodium dodecyl sulfate (SDS) increased the electroosmotic flow (EOF) during on-line SPE–CE, resulting in lower resolution of the separated components. This effect was probably due to the adherence of some SDS molecules to the extractor, even after solvent washing, thus increasing the negative charge of the capillary-extractor internal surface. Cleaning with the neutral surfactant Triton X-100 was found to be very efficient in regenerating the Rezorian material and

did not adversely affect protein separation or resolution. After analyzing biological samples, this detergent washing step was done first to ensure the best chance of removing Rezorian-bound detergent during subsequent solvent and alkali washing steps. With the improved regeneration procedure, use of the SPE device without deterioration could be extended for the analysis of at least 100 biological samples.

The rapid batch preparation of MT for analysis by chromatographic and electrophoretic techniques is problematic due to the difficulty in obtaining a relatively pure extract using a simple and practical approach. Thus, the use of size-exclusion chromatography (SEC), even on a small scale, was not seriously considered. MT isoforms are relatively heat stable and so treatment of homogenate or cytosol samples at 60–100°C for a limited period is often used to remove contaminating proteins and/or particles [9,11]. MT in the samples can then be concentrated by molecular filtration while removing low-molecular-mass ($M_r$) components in the filtrate. However, in our experience, ultrafiltration using 1000 or 3000 molecular-mass cut-off filtration units is slow, expensive and does not result in adequate removal of contaminants. Heat treatment on its own does not remove low $M_r$ components and does not concentrate the sample, which for reasons of CE detection sensitivity, is usually necessary. We have however, used heat treatment at a later stage in our extraction procedure to further reduce the level of contaminating protein. MTs are also relatively resistant to denaturation by polar solvents and both acetone and ethanol have been used for selective removal of protein contaminants in bulk and batch MT separations [36–38]. Studies of MT recovery using different solvents for extraction suggest that ethanol may give a better yield than acetone [39]. We have found an ethanol–acetonitrile solvent combination to be very effective at denaturing contaminating proteins and unlike acetone, it does not attack some common laboratory plasticware. Concentrations of 80–90% ethanol–acetonitrile are sufficient to precipitate but not denature MTs and the resulting reconstituted protein gives good separations by various CE techniques [40]. It should be noted, however, that all supernatant solution must be completely removed from the pellet obtained after centrifugation of the sample containing 90% solvent. Failure to do this will affect the efficiency with which MT is adsorbed by the Rezorian material in the SPE device.

For quantitative work, MT recovery and intra- and inter-batch precision were evaluated using a radiolabeled MT-1 tracer. When spiking the liver cytosol sample prior to the addition of solvent, 52% of tracer activity was recovered in the final heat-treated extract. The precision of recovery was around 3% and the endogenous level of MT in the liver samples did not significantly affect the MT recovery or precision. Nevertheless, further attempts have been made to improve the MT recovery and we have evaluated the effect of adding solvent at −20°C followed by sample incubation at the same temperature for 1 h. The use of low temperature during solvent addition helps to protect certain vulnerable proteins and enzymes from denaturation or inactivation. In our experience, solvent addition at −20°C and +20°C, with or without subsequent incubation, made no difference to the recovery or the CE separation of MT isoforms. Therefore, in contrast to other published methods, we have not used solvent chilled to −20°C or incubated the solvent–sample mixture at low temperature. We have however used ice-cooled solvent as a general precaution.

Increasing the NaCl content of the homogenisation buffer markedly increased MT recovery from 52% to approximately 70% at 500 m*M* NaCl. Further increases in NaCl did not yield corresponding improvements in recovery. We therefore propose that to maximize recovery of MT, the homogenisation buffer of 100 m*M* Tris–HCl should contain 500 m*M* NaCl. For SPE–CE, the MT precipitated by addition of ethanol–acetonitrile to 90% should be re-dissolved in 75 m*M* phosphate pH 7.0 in order to take advantage of the enhanced loading efficiency afforded by a higher buffer concentration, as discussed above. A quality control of known MT isoform content, or a sample spiked with a known amount of purified MT isoform should be subjected to the same extraction procedures as unknown samples. In the present work, replicates of a sheep liver sample of known MT content were used as quality controls and the data were corrected for inter-batch variation in MT recovery.

The solvent extraction technique in combination with SPE–CE was applied to the study of MT

isoform expression in sheep fetal liver during the latter part of gestation and the results for total MT obtained were compared with data from analysis of the liver cytosol samples by an independent method, namely HRSEC. The results from both the SPE–CE and HRSEC methods demonstrate a decrease in total MT from day 89 to 136 of gestation which is in agreement with the trend in Zn and total MT levels recorded elsewhere at similar time points throughout the latter part of fetal development [30,31]. In the perturbed fetus, total MT measured by SPE–CE and HRSEC, was found to be three- and eight-fold higher, respectively, than that of normal fetal liver at day 135/6. This effect was highly significant ($P<$ 0.01) with both techniques and may indicate a divergence in hepatic metabolism of the perturbed and normal fetus between day 125 and 136. Many factors produced as a consequence of this physiological change could be responsible for up-regulating MT expression and further attempts will be made to identify likely candidates. Both MT-1 and MT-2 charge forms showed a similar pattern to the total protein and the level for each MT isoform in the perturbed animal liver was significantly elevated at day 135. There was also a tendency for liver MT levels of perturbed animals to be higher at 89 days gestation and this difference reached statistical significance ($P<0.05$) in the case of MT-2. The MT-1/MT-2 ratio for normal and perturbed livers was consistently 2.4–2.9 at days 89, 110 and 136 but was significantly increased at day 125 (Table 2). The predominance of MT-1 has been observed previously in CE separations of adult sheep liver MT [26] and partly reflects the multi-isoform composition of the MT-1 charge form [41]. MT-1 genes are thought to be more responsive to stress factors than MT-2 genes and so the increase in MT-1 at day 125 may indicate a stress-induced stimulation of MT is occurring at around this time.

In conclusion, the method developed in the present study, to separate and quantify MT isoforms, has proved to have some distinct advantages of speed, sensitivity, selectivity and reproducibility which have been lacking from alternative chromatographic-based techniques. Many tissues contain basal levels of MT which are higher than the detection limit quoted here (0.5 μg/g tissue) and this method should be applicable to isoform analysis in most species. The application of this technique to the analysis of human samples would be a particularly useful development and is currently being evaluated.

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