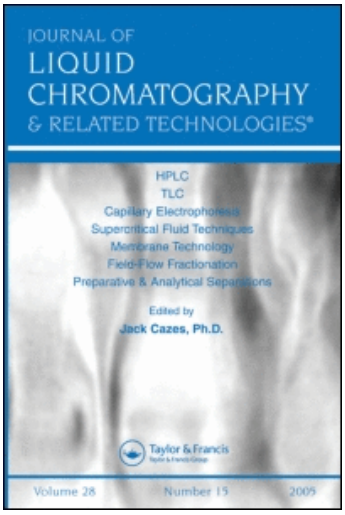


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Characterization of Mammalian Metallothionein Isoforms by Capillary Zone Electrophoresis With Diode Array Detection Using Tris-Borate Buffer

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**To cite this Article** Virtanen, V. and Bordin, G.(1998) 'Characterization of Mammalian Metallothionein Isoforms by Capillary Zone Electrophoresis With Diode Array Detection Using Tris-Borate Buffer', Journal of Liquid Chromatography & Related Technologies, 21: 20, 3087 — 3098

**To link to this Article: DOI:** 10.1080/10826079808001259

**URL:** http://dx.doi.org/10.1080/10826079808001259

J. LIQ. CHROM. & REL. TECHNOL., 21(20), 3087–3098 (1998)

# CHARACTERIZATION OF MAMMALIAN METALLOTHIONEIN ISOFORMS BY CAPILLARY ZONE ELECTROPHORESIS WITH DIODE ARRAY DETECTION USING TRIS-BORATE BUFFER

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## ABSTRACT

Four mammalian metallothioneins (MTs), rabbit liver MT (RL MT), rabbit liver MT-1 (RL MT-1), and rabbit liver MT-2 (RL MT-2) and horse kidney MT (HK MT), were subjected to capillary zone electrophoresis (CZE) with on-line diode array detection (DAD) by using tris-borate buffer at neutral or nearly neutral conditions thus, giving information about the putative isoforms in conditions also found in tissues. The MTs were found to exhibit a different polymorphism. Rabbit liver MT exhibited 3 main peaks and horse kidney 5 main peaks. Isoform abundance between batches was found to vary noticeably. Differences in electrophoretic behaviour were observed. As expected, the 2-charged main peak of RL MT-2 showed highest electrophoretic mobility. Main peak of RL MT-1 and two of rabbit liver MT had lower electrophoretic mobilities corresponding to single charge.

Two later migrating horse kidney MT peaks had nearly similar electrophoretic behaviour as the single charged rabbit liver MT peaks. Interestingly, horse kidney MT exhibited also peaks with lower electrophoteric mobilities and different behaviour than the other peaks observed. The ultra violet (UV) spectra obtained by using diode array detection (DAD) showed clear difference in the structure of these peaks compared to other peaks observed. These peaks showed no, or nearly no, absorbance at wavelengths 225 and 250 nm that corresponds to cation-thiol bondage, Zn-thiol, and Cd-thiol, respectively. We can, therefore, assume that horse kidney MT sample contains components that are in apothionein form are only partially saturated with metals. The use of diode array detection appears to be a good tool for a quick identification of apothioneins and partially metal-saturated metallothioneins.



## INTRODUCTION

Metallothioneins (MTs) belong to a class of low molecular mass proteins (6-7 kDa), characterised by a high cysteine content (~ 30%), and the lack of aromatic amino acids. MT is a protein induced by various stimuli such as metals, stress, and some drugs. MT has the ability to bind with high affinity and stoichiometry $d^{10}$ metals such as zinc, cadmium, copper, and mercury. The characteristics and properties of these molecules have been subject to several monographies.[1-4] Functions proposed for MTs include the homeostasis of some essential metals (Zn, Cu) and detoxification of harmful (Cd, Pb) and excessive essential metals.[5] MT isoforms arise from genetic polymorphism which is found in many species. Considering their electrophoretic properties, two major isoforms of MT have been identified in mammals, MT-1 and MT-2, named after their order of elution in anion-exchange chromatography. They have a single charge difference at neutral pH due to certain amino acid substitutions.[6] Furthermore, many animal species generate various sub-isoforms of MT-1 and MT-2, therefore displaying significant microheterogeneity. From the relatively few detailed data of MT isoform expression, it seems that some of them are of minor abundance and expression compared to others.[7-8]

The primary role of different MT isoform and sub-isoform may well be distinctive but until they can be routinely analysed, their expression and their function will remain difficult to establish. Progress in this field is largely dependent on efficient separation of each isoMT and sub-isoMT and, therefore, on the development of analytical techniques providing a high degree of resolution. Recently, CZE using both uncoated and surface modified, coated, capillaries showed promising results.[9-15] In our previous paper we introduced the use of CZE with tris-borate buffer at neutral or nearly neutral pH.[14]

Separation of isoforms at neutral or nearly neutral pH is of interest in order to study their properties under original conditions. Under these conditions the metals are still bound to molecules unlike at acidic pH values where the metals are dissociated. In this paper we study the use of diode array detection (DAD) to obtain more information about the separated putative isoforms. The use of DAD gives possibility of obtaining a UV spectrum of the separated peaks thus enabling the possibility to further characterise the isoforms and better understanding of their behaviour.

## EXPERIMENTAL

### CZE Instrumentation and Methods



Capillary zone electrophoresis (CZE) was performed on a P/ACE System instrument (Beckman, Fullerton, CA, USA). Data were collected and processed either with a System Gold software or P/ACE Station software (Beckman). Fused-silica capillaries of 50 μm I.D. and 375 μm O.D.s containing polyimide cladding were obtained from Beckman. Overall capillary length was 57 cm with an on-line detection window at 50 cm. The capillary was housed in a cartridge which allowed liquid cooling to maintain constant capillary temperature at a chosen value during the run. The capillaries were prepared by flushing them for 10 min each with I M HCl, 1 M sodium hydroxide solution and bidistilled water. The capillaries were then rinsed with buffer solution for 5 min.

The running method included the use of normal polarity at 11 kV for the running voltage, with a voltage ramping time of 0.17 min. Pressure injection mode (0.5 psi) for 5 s was used unless otherwise stated. The separation was established at 17°C unless otherwise stated. Separated components were detected at 200 nm, or a UV spectrum of each individual component was established by using a diode array detector (Beckman, Fullerton, CA, USA).

### Chemicals

All chemicals for buffer solutions were of research grade (Merck, Darmstadt, Germany or Fluka, Buchs, Switzerland). All buffers were prepared with doubly distilled water and were degassed in an ultrasonic bath. The pH of the buffers was adjusted by adding either 0.1 M sodium hydroxide or HCl to the solutions. In the case of additions of MeOH the pH was adjusted after addition of MeOH to the buffer solution. The buffers were filtered using a 0.2 μm filter (Gelman Sciences, Ann Arbor, MI, USA). Metallothionein samples, rabbit liver MT (MT-1 and MT-2), rabbit liver MT-1, rabbit liver MT-2 and horse kidney

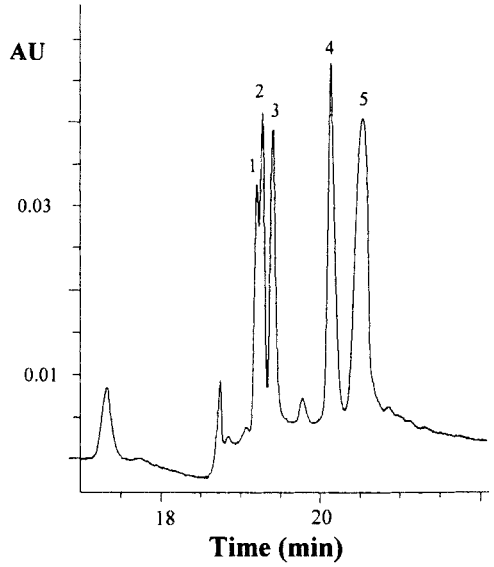


**Figure 1.** Electropherogram of horse kidney metallothionein sample separated using tris-borate buffer (110 mM - 110 mM) at pH 6.90. Normal polarity was used with 11 kV for the running voltage. Pressure injection for 5 s was used. Temperature was 17 °C. Separated components were detected at 200 nm.



MT were purchased from Sigma (ST. Louis, MO, USA). Different batches of the various MT samples were used and compared. Metallothionein samples were dissolved in bidistilled water at a final concentration of 1.0 mg/mL in order to enhance resolution by stacking. Metallothionein samples were prepared weekly and stored at 4°C and an aliquot was taken daily from this stock solution.

## RESULTS AND DISCUSSION

### CZE Separation of Metallothionein Samples

The four metallothionein samples used exhibited a different polymorphism as shown in Figure 1 and 2. Horse kidney exhibited 5 major peaks when separated using tris-borate buffer at pH 6.90 (Figure 1). In our previous paper we observed that HK exhibited 3 major peaks and 2 minor ones.[14] This difference in peak abundance is due to the use of different batch of sample. The earlier observed minor peaks, observed when using batch 82H9590, are the

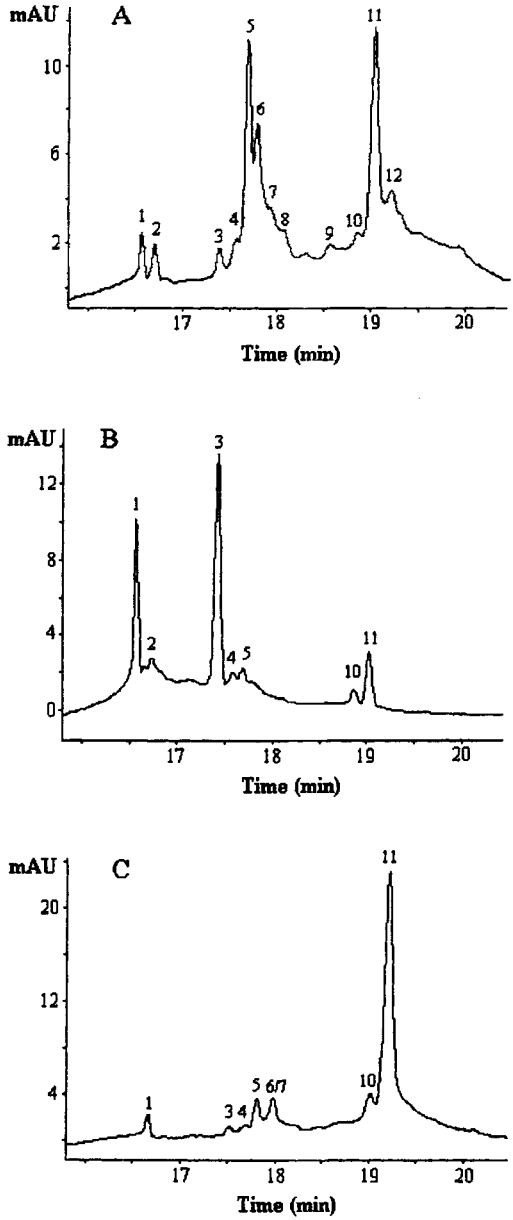


**Figure 2.** Electropherograms of rabbit liver MT (A), rabbit liver MT-1 (B) and rabbit liver MT-2 ( C) samples obtained by using tris-borate buffer (110 mM - 110 mM) at pH 6.40. Other conditions as in Figure 1.

same peaks that have now greater abundance in batch 73H9544. This difference is logical since the batches are made from different animals and differences between individuals in generating MTs are probable.

In our previous paper we observed that in order to obtain a good separation of MT, samples from different species required small adjustments of the running conditions.[14] Electropherograms of rabbit liver MT, rabbit liver MT-1, and rabbit liver MT-2 separated by using tris-borate buffer at pH 6.40 are shown in Figure 2. Rabbit liver MT (Figure 2A) exhibited 3 major peaks and several smaller ones. Rabbit liver MT-1 (Figure 2B) exhibited 2 major peaks and 5 smaller ones. Rabbit liver MT-2 (Figure 2C) exhibited one major peak and 6 smaller ones. As the rabbit liver MT sample should theoretically be a mixture of both rabbit liver MT-1 and MT-2, it is likely to observe all the peaks of MT-1 and MT-2 in the electropherogram obtained from rabbit liver MT. In order to identify with certainty which peak in rabbit liver MT is also found in rabbit liver MT-1 and MT-2 samples, additions of MT-1 and MT-2 to rabbit liver MT sample were made (data not shown).



It was noticed that peak labelled 1 was observed in all three samples being major peak in MT-1 sample. Peak 2 was found in rabbit liver MT and very little in MT-1 sample but not in MT-2 sample. Peak 3, that was the major peak found in MT-1 sample, is also found in other samples. In MT-2 this is likely to be residue left after cleaning procedure. Interestingly, this peak is found in rabbit liver MT in quite small abundance. Other peaks found in rabbit liver MT migrating in the area of MT-1 peak, namely peaks 4-8, are found only in quite small abundance in MT-1 (batch 94H9504) and MT-2 samples (batch 13H95481).

We tested also another MT-1 sample (batch 57H9518, data not shown) and in that one the peaks 5 and 6 were found in 2-3-fold abundance compared to batch 94H9504, but still the peaks observed were relatively small. This indicates, like in case of horse kidney MT, that batches prepared from different animals are prone to variation in isoform abundance. Peak 9 was observed only in rabbit liver MT. Peaks labelled 10 and 11 were found in all samples, except when the other rabbit liver MT-1 sample (batch 57H9518) was used, the peak 10 was not noticed. Peak 11 is main peak in MT-2 and is also found as a main peak in rabbit liver MT and as a residue also in MT-1 samples. Peak 12 is found in rabbit liver MT and as a small peak in the other MT-1 sample (batch 57H9518, data not shown).

## UV Spectra of the Main Peaks

Typical UV spectra of MT main peaks are shown in Figure 3. Horse kidney MT exhibited both types of scans. Peaks labelled 1-3 in Figure 1

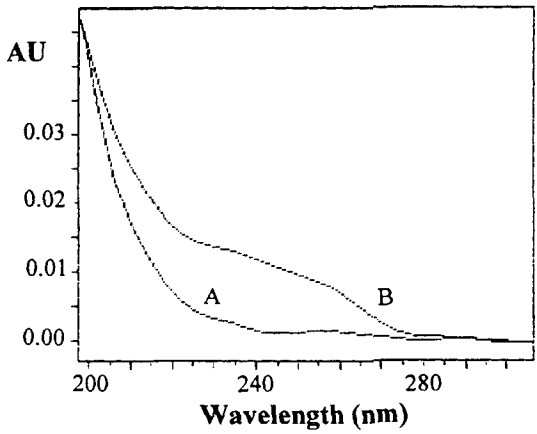


**Figure 3.** UV spectra of apothionein or only partially metal saturated isoform (A) and metallothionein (B) obtained by using photodiode array detection. Each line represents a scan between 190 and 300 nm. The scans depicted represent those obtained at the apex of each peak detected. Other conditions as in Figure 1 except pH for rabbit liver samples was 6.40.



exhibited type A UV spectra corresponding to compounds lacking totally or nearly totally cation-thiol bondage. Characteristic wavelengths for cation-thiol bond found in metallothioneins are 225 nm for Zn-thiol and 250 nm for Cd-thiol.[2,16]. This may indicate that the three peaks of horse kidney MT labelled 1-3 in Figure 1 are in apothionein form or in only partially metal-saturated form. The two last peaks of horse kidney MT labelled 4 and 5 in Figure 1 give type B UV spectra which is typical for metallothioneins containing zinc and cadmium.

In our previous paper,[14] we supposed that rabbit liver MT, the main peak labelled 5 in Figure 2A, was probably a modified isoform since it was not found or was found in very small abundance in rabbit liver MT-1 and MT-2. Theoretically, all peaks found in the electropherogram of MT should be attributable to either MT-1 or MT-2. We also concluded, that the definite identification of the observed peak 5 as a true metallothionein isoform awaits further characterisation. In this study, by the use of diode array detection, rabbit liver MT main peak labelled 5, as well as peaks labelled 6 and 11 in Figure 2A, were found to give type B UV spectra, thus corresponding to metallothioneins containing zinc and cadmium bound to thiol groups. This, together with the observation that between batches there are differences in abundance of peaks, could indicate that peaks 3-6 and maybe even peaks 7 and 8 correspond to MT-1 type compound with very minor differences either in their amino acid sequences or metal amounts bound. This difference in metal amount could be in the total

amount or in the ratio Zn/Cd bound to the compound. The identification of the differences awaits further characterisation. Rabbit liver MT-1, main peaks labelled 1 and 3 in Figure 2B, gave also type B UV spectra as well as rabbit liver MT-2 main peak labelled 11 in Figure 2C. Interestingly, no peaks observed in rabbit liver samples gave type A UV spectra.

## Characteristics of Electrophoretic Mobilities

Effects of buffer pH, buffer concentration, and buffer composition on electrophoretic mobilities of horse kidney MT, peaks labelled 2,4 and 5 in Figure 1, are shown in Figure 4. Peak 2 was chosen to model the behaviour of peaks 1-3 since they all gave similar UV spectra and peak 2 was found as a major peak in both batches tested. The electrophoretic mobility of all peaks was increasing slowly with increasing buffer pH (Figure 4A), but still a difference in behaviour of peak 2 was observed compared to peaks 4 and 5. The electrophoretic mobility was clearly lower for peak 2 than for peaks 4 and 5. As the peak 2 was found to be either in apothionein form or containing very little metals, its electrophoretic behaviour is likely to be affected differently than metal-saturated metallothionein isoforms as pH changes. This could indicate differences in charge or in charge distribution. Interesting phenomenon was observed when buffer concentration was varied (Figure 4B). The electrophoretic mobility of peak 2 stayed nearly constant as the concentration of the tris-borate buffer increased from 50 mM- 50 mM to 150 mM - 150 mM. Peaks labelled 4 and 5 in Figure 1 showed tendency of decreasing electrophoretic mobility with increasing buffer concentration. This phenomenon noticed for peaks 4 and 5 is well known in the literature.[17-18] The change of buffer composition affected, only slightly, the electrophoretic mobility of peak 2 (Figure 4C). The tendency for peaks 4 and 5 is similar to peak 2 up till buffer concentration of 110 mM tris - 110 mM borate. When borate concentration remained at 110 mM and tris concentration was decreased, a slight increase in electrophoretic mobilities was observed.

This effect found is interesting and more studies should be done to characterise this behaviour. The effect of several buffer anions and cations on electrophoretic mobility have been earlier studied.[19-21] One reason for this effect observed could be that borate interacts with the silica surface of the capillary. This alters the double layer which affects zeta potential and the electroosmotic flow.[22] Changes in zeta potential affect electrophoretic mobilities of the analytes.

Rabbit liver MT, peaks labelled 5 and 11 in Figure 2A, were chosen for electrophoretic studies. The tendency of these peaks is very similar to those of horse kidney, peaks 4 and 5 (Figure 4A), when buffer pH was varied. The one charged isoform, MT-1, labelled as peak 5 in Figure 2, had very similar

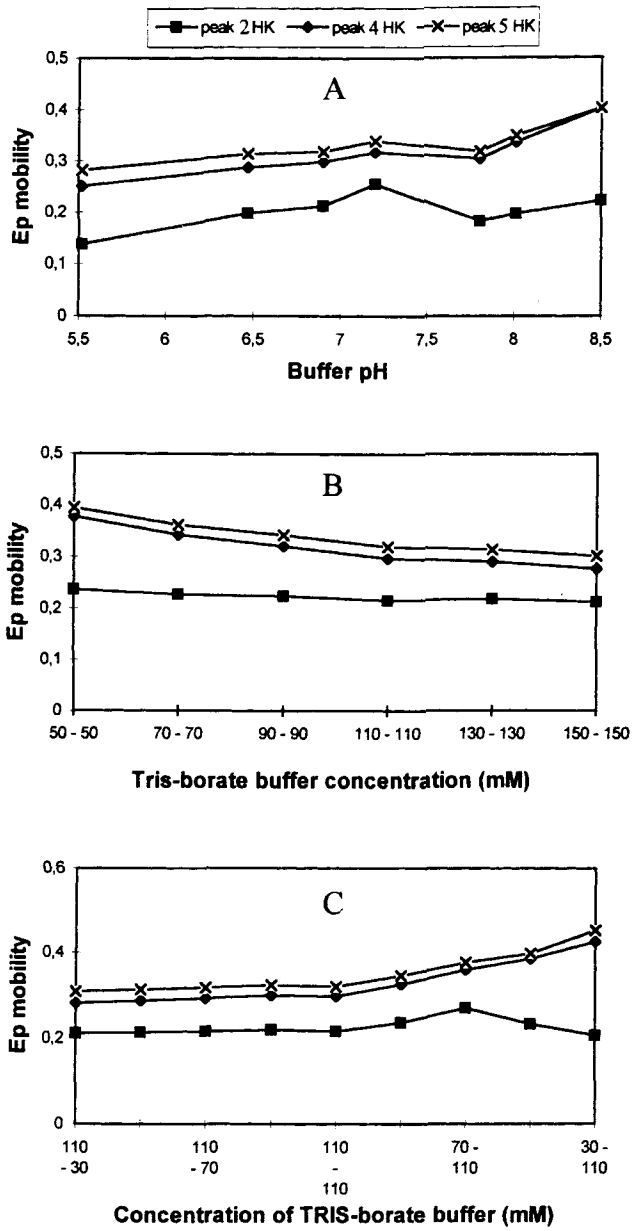


**Figure 4.** Effect of buffer pH (in A), buffer concentration (in B) and buffer composition (in C) on electrophoretic mobilities (x $10^8 m^2/Vs$) of horse kidney main peaks. Other conditions as in Figure 1.

electrophoretic mobility as horse kidney, peaks 4 and 5, and the 2-charged isoform, MT-2, labelled as peak 11 in Figure 2, had logically higher mobility of 0.12 to 0.20 units depending on the pH used. The tendencies of these peaks were also similar as horse kidney peaks 4 and 5, when the effect of buffer concentration was studied, decreasing electrophoretic mobility with increasing ionic strength. Also here MT-1 had nearly similar mobility as horse kidney, peaks 4 and 5 (Figure 4B) and MT-2 had similarly higher mobility as in the case of effect of the buffer pH. Buffer composition affected the electrophoretic mobilities of rabbit liver MTs similarly as for horse kidney MT, peaks 4 and 5 in Figure 4C.

## CONCLUSIONS

Four mammalian metallothioneins (MTs), rabbit liver MT (RL MT), rabbit liver MT-1 (RL MT-1), and rabbit liver MT-2 (RL MT-2) and horse kidney MT (HK MT), were subjected to capillary zone electrophoresis (CZE) with on-line diode array detection (DAD). Separation of isoforms was obtained by using tris-borate buffer at neutral or nearly neutral conditions thus giving information about the putative isoforms in conditions also found in tissues. The MTs are found to exhibit a different polymorphism.



Differences in electrophoretic behaviour were observed. As expected, the two charged main peaks of RL MT-2, also found as one of the main peaks in RL MT, showed the highest electrophoretic mobility. Main peak of RL MT-1 and two of rabbit liver MT had lower electrophoretic mobilities corresponding to single charge. It was also noticed that the batches are prone to variation in isoform abundance. Unlike what we stated in our previous paper,[14] we now found that the rabbit liver peak labelled 5 in Figure 2A, is indeed found in MT-1 and MT-2 samples in small amounts, and that the abundance of this peak varies noticeably between batches. In our previous studies, we happened to have batches where this putative isoform was not present in a detectable amount. The UV spectra of the main peaks of rabbit liver MT and those of rabbit liver MT-1 were typical for metallothioneins, containing shoulders for Zn-thiol and Cd-thiol bondage at 225 nm and 250 nm, respectively.

The two later migrating horse kidney MT peaks had nearly similar electrophoretic behaviour as the supposed single charged rabbit liver MT peaks. Interestingly, horse kidney MT also exhibited peaks with lower electrophoteric mobilities and different behaviour, thus differing from the other peaks observed. The ultra violet spectra obtained by using diode array detection showed clear difference in the structure of these peaks compared to other peaks observed. These peaks showed no or nearly no absorbance at wavelengths 225 and 250 nm that correspond to cation-thiol bondage, Zn-thiol and Cd-thiol, respectively. We can, therefore, assume, that horse kidney MT sample contains components that

are in apothionein form or only partially saturated with metals. The identification of these peaks as true metallothionein isoforms awaits further characterisation. It was noticed, as with rabbit liver samples, that the batches are prone to variation in isoform abundance. The use of diode array detection appears to be a good tool for a quick identification of apothioneins and partially metal-saturated metallothioneins.

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Received February 8, 1998
Accepted April 29, 1998
Manuscript 4735