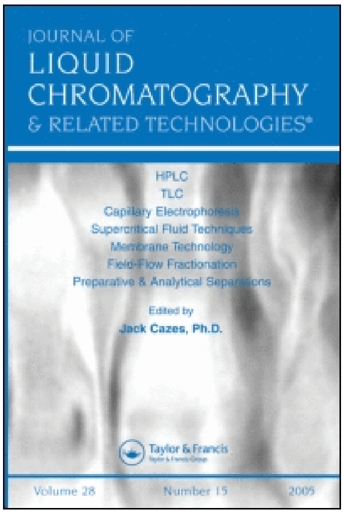

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Gaudin, Karen , Chaminade, Pierre , Ferrier, Danielle and Baillet, Arlette(2000) 'IMPROVEMENT OF EVAPORATIVE LIGHT SCATTERING DETECTION OF CERAMIDES USING TRIETHYLAMINE AND FORMIC ACID IN NON-AQUEOUS REVERSED PHASE LIQUID CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 23: 3, 387 — 397

To link to this Article: DOI: 10.1081/JLC-100101458
URL: http://dx.doi.org/10.1081/JLC-100101458

J. LIQ. CHROM. & REL. TECHNOL., 23(3), 387–397 (2000)

IMPROVEMENT OF EVAPORATIVE LIGHT SCATTERING DETECTION OF CERAMIDES USING TRIETHYLAMINE AND FORMIC ACID IN NON-AQUEOUS REVERSED PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

The use of Porous Graphitized Carbon (PGC) stationary phase has enabled to emphasize the efficiency of the couple triethylamine (TEA) and formic acid (HCOOH) as a response enhancer for the Evaporative Light Scattering Detection (ELSD) of ceramides in Non-Aqueous Reversed Phase (NARP) Liquid Chromatography. 0.1% v/v of TEA and a stoechiometric amount of HCOOH greatly increase the intensity of the detection response whatever the nature of the eluent and the mobile phase composition was. This increase was not proportional to the amount of the TEA and HCOOH added as the response remained stable above 0.1 % v/v (7 mM).

INTRODUCTION

In high performance liquid chromatography, UV detection is not a suitable technique for detection of underivatized ceramides because of the low UV detectability due to the weak chromophore of ceramides at 200 nm.[1] However, derivation of ceramides is possible which allows an efficient detection in UV[2,3] or in fluorescence.[4] But derivation is a time consuming factor in view of routine analysis. The compatibility between the detector and the eluent is another important factor in the choice of detection for ceramides. Ceramides are soluble only in organic solvents and the large range in polarity of these molecules requires the use of gradient elution. As ELSD fulfills all these requirements, it was then selected. Furthermore, this detection is commonly used for direct detection of lipids.[5-9]

The principle of the detector consists of the nebulization of the column effluent by a gas stream. The eluting solvent is then vaporized, leaving the non volatile components of the sample as a cloud of particles. These particles are detected by measuring the amount of light scattered when the cloud crossed a light beam. The intensity of the detection response depends on the particle sizes which determine the mechanism of light scatted.[10] The particle sizes depend on the droplet homogeneity generated by the nebulizer. This homogeneity is linked with the properties of the liquid (surface tension, density, and viscosity), the flow-rates of the gas and liquid stream, and the solute concentration.[11,12] The value of the mobile phase flow-rate is stated by the column dimension and the gas flow-rate ensues from the mobile phase flow.[13] Thus, the main factor to take into account for improving the response of the ELSD is the nature of the eluent.

The use of salts to improve lipids detectability and column lifetime has already been reported. [8,14] However, these salts were added in the aqueous part of the mobile phase and were thus not tested in NARP chromatography. In a previous article,[15] an important increase in solute detectability was noted when using TEA and a stoechiometric amount of HCOOH in non-aqueous mobile phase using octadecyl silica (ODS) stationary phase. TEA was initially added in the mobile phase to improve the peak shape by suppressing the solute-free silanol interactions that may occur. HCOOH was added in stoechiometric amount to avoid extreme pH values and the subsequent hydrolysis of the chromatographic support that may occur even with trace amounts of water. However, using either methanol/propanol or acetonitrile/propanol mobile phases, it was noticed that, in our specific case:

> The retention times of ceramides were not altered by the addition of TEA/HCOOH, suggesting that the increase of the peak response observed with TEA/HCOOH was not due to a change of peak geometry consecutively of the suppression of silanol interaction; the peak heights were signifiantly increased; this increase was not proportional to the amount of TEA/HCOOH added as the response increased between 0.01 to 0.1% and remained stable above 0.1% v/v.

From these primary results, it was decided to use a PGC stationary phase[12,13] since its stability enables us to study separately the influence of TEA or HCOOH without any risk for the packing.[18] It was also decided to study the applicability of this response modifier in various solvents, first to ensure this technique was applicable in NARP chromatography and second to appreciate if the response improvement depends also on the organic solvents used.

This study constitutes generalization of the use of TEA/HCOOH as a ELSD response enhancer in NARP chromatography.

EXPERIMENTAL

Standards and Solvents

N-Palmitoyl-D-sphingosine (C16:0S), N-Stearoyl-D-sphingosin (C18:0S), N-Palmitoyl-DL-dihydrosphingosine (C16:0D), ceramide type III, and ceramide type IV were all purchased from Sigma (St. Quentin Fallavier, France). Ceramide III (C18:0P) was a generous gift of Cosmoferm (Delft, Netherlands). These ceramides (C16:0S, C18:0S, C16:0D, and C18:0P) were prepared together in methanol at concentrations approximately about 0.03 mg/mL for C18:0S and C16:0S, 0.05 mg/mL for C18:0P and C16:0D. Type III and type IV were prepared at 1 mg/mL in MeOH.

All of the following solvents are HPLC-grade: acetonitrile (ACN), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), Toluene, Ethyl Acetate, methanol (MeOH), and tetrahydrofuran (THF) were purchased from Fisher Scientific (Elancourt, France), whereas propanol-1 (PrOH) and Acetone are from Prolabo (Nogent sur Marne, France). Trifluoro acetic acid (TFA) was stored in sealed ampoules at 4°C (Sigma, St. Quentin Fallavier, France). TEA and HCOOH were purchased as analytical reagents (Prolabo, Nogent sur Marne, France).

Apparatus

Chromatographic measurements were carried out with a Thermo Separation Products P1000 XR gradient pump with a TSP SCM1000 (Thermo Separation Products, San Jose, California, USA), vacuum membrane degasser connected with a Kontron autosampler 360 (Bio-Tek Kontron Instruments, Milan, Italy) equipped with a 5 μL sample loop injection valve. Detection was performed with a Cunow DDL 11 ELSD (Eurosep, Cergy, France) where the drift tube temperature was set at 35°C and nitrogen pressure at 1 bar. The chromatograms were recorded with a PC-integrator KromaSystem 2000 1.60 (Kontron Instruments, Milan, Italy).

The flow-rate was set at 0.4 mL.min^{-1}. The column, hypercarb (5 µm), 100 x 2.1 mm I.D. (Hypersil, Runcorn, UK) was thermostated at 50°C with a Jetstream 2 temperature controller (Thermotecnics Products Gmbh, Austria).

RESULTS AND DISCUSSION

This study was realized with a mixture of four ceramides: C16:0S, C16:0D, C18:0S, and C18:0P. These ceramides were selected in the same range of hydrophoby (i.e. they have similar hydrocarbonated volume), in order to work with isocratic mobile phases. Although a minimum set of ceramides was selected to weaken the possibility of a coelution phenomenon, these four compounds provide different structural variations encountered in ceramides: the variation of two methylenes, the presence of a carbon double bound in C16:0S and C18:0S in the amine moiety, the presence of a further hydroxyl group in C18:0P in the phytosphingosine moiety. This variability was introduced to see if there was a variation of the response coefficients from the chemical structure of these compounds, although the response coefficient should remain the same for similar solute density.[19]

Solvent Selection

On PGC packing material, ACN and MeOH were not strong enough for ceramide elution, thus they were used as weak solvents. In order to select stronger solvents, binary gradients were performed from 100 % of ACN or MeOH to 100 % of a potential stronger solvent in 15 min and the final composition of the gradient was left running for 1 hour. CH_2Cl_2, $CHCl_3$, THF, Toluene, Acetone, Ethyl Acetate, and Propanol were tested. These solvents were representative of different groups of selectivity and were miscible with either ACN or MeOH. This selection was also guided by the solvent used on ODS for ceramide elution.[15] Acetone, Ethyl Acetate, and Propanol were not strong enough to elute ceramides regardless of the choice of weak solvent. The other solvents can be classified in order of the increase of their elution strength for ceramides as follows: THF, CH_2Cl_2, $CHCl_3$ and Toluene. Therefore, the influence on the peak response was examined only for these four solvents and their combinations with the weak solvents (ACN or MeOH). The binary gradients using MeOH led to shorter retention times than the corresponding gradients using ACN.

Selection of Response Modifiers

Table 1 describes the influence of TEA, HCOOH or TEA:HCOOH 1:1 (mol:mol) added at 0.1% v/v in the mobile phase concerning both retention times and response intensity. Results in this table were obtained from chromatograms recorded with the four strongest solvents associated with ACN

Table 1

Influence of Various Modifiers on Retention and Detection

Modifiers	Modifier Amount	Relative Retention[1]	Relative Peak Height[2]	Background Noise (mV)[3]
Without Modifier	---	1.00	1.00	1.0
Triethylamine (TEA)	0.1% v/v	1.01 ± 1.3 10^{-3}	0.96 ± 0.21	1.0
Formic Acid (HCOOH)	0.1% v/v	0.98 ± 3.2 10^{-3}	0.66 ± 0.07	1.0
TEA:HCOOH 1:1 mol:mol	0.1% v/v	0;99 ± 7.5 10^{-3}	25.00 ± 29	1.0
Trifluoracetic Acid (TFA)	0.1% v/v	0.90 ± 0.04	0.59 ± 0.24	10.0
TFA:TFA 1:1 mol:mol	0.1% v/v	not available	not available	100.0

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[1] Mean value of t_R with modifier divided by Mean value of t_R without modifier. (Mean value obtained from various conditions of mobile phases and various ceramides) ± standard deviation of the relative retention values. [2] Mean value of peak heights with modifier divided by Mean value of peak heights without modifier. (Mean value obtained from various conditions of mobile phases and various ceramides) ± the standard deviation of the relative peak heights. [3] For a detector full scale of 1000 mV.

or MeOH. It was observed that neither TEA nor HCOOH alone improved the ELSD response since there was rather a slight decrease of peak heights with response modifier than with free mobile phases. The background noise is of the same order of magnitude for those two response modifiers at about 1 mV (the full range of the detector is 1000 mV) and similar levels are encountered with pure solvents. Those two response modifiers also did not influence the retention since retention times are not significantly altered. The association of TEA with HCOOH led to a significant increase in peak height without modification of neither background noise nor retention times. The fact that the background noise is not altered by the TEA/HCOOH mixture, suggests that this response modifier is sufficiently soluble in organic solvents and volatile enough to be usable with ELSD.

As Trifluoroacetic acid (TFA) is widely used on PGC to modify the electronic interactions due to this stationary phase,[20,21] this acid was tested. TFA is generally used in hydroorganic mobile phases. The addition of this organic modifier was selected to replace HCOOH and to try to improve peak shape when using chlorinated solvents. Irregular peak shapes due to the use of chlorinated solvents have already been reported.[16]

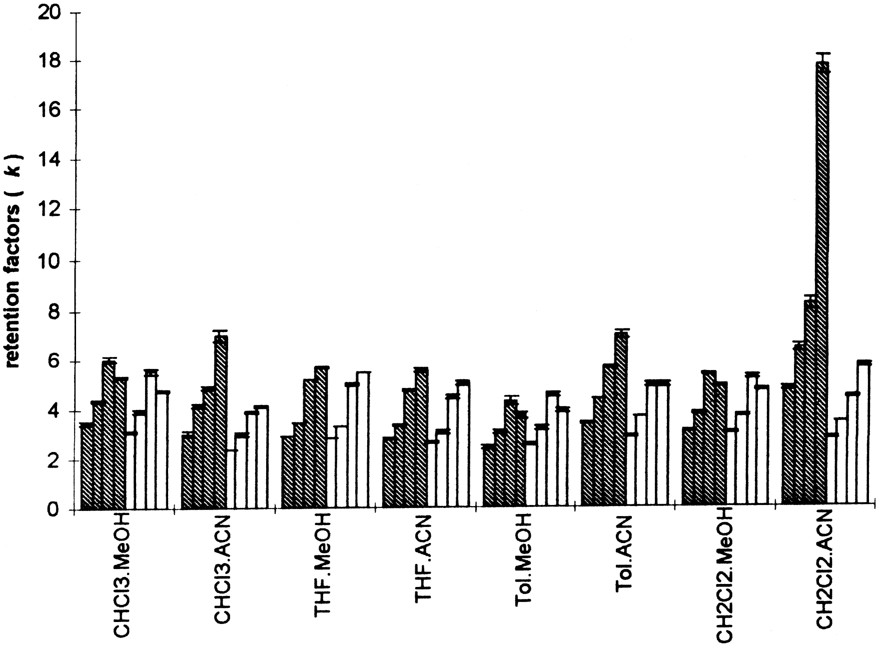

Figure 1. The retention factors (mean value of 3 measurements) for the four ceramides in each mobile phase composition. Hatched boxes represent the mobile phases without any response modifiers and the whites boxes the mobile phases with 0.1% of TEA and a stoechiometric amount of HCOOH. In each mobile phase condition, the retention factors of the selected ceramides are represented in the following order: C16:0S, C16:0D, C18:0S, and C18:0P.

Table 2

Compositions of Isocratic Mobile Phases

Strong Solvent	Weak Solvent ACN	 MeOH
THF	90 / 10	81 / 19
CH_2Cl_2	90 / 10	65 / 35
$CHCl_3$	73 / 27	55 / 45
Toluene	52 / 48	32 / 68

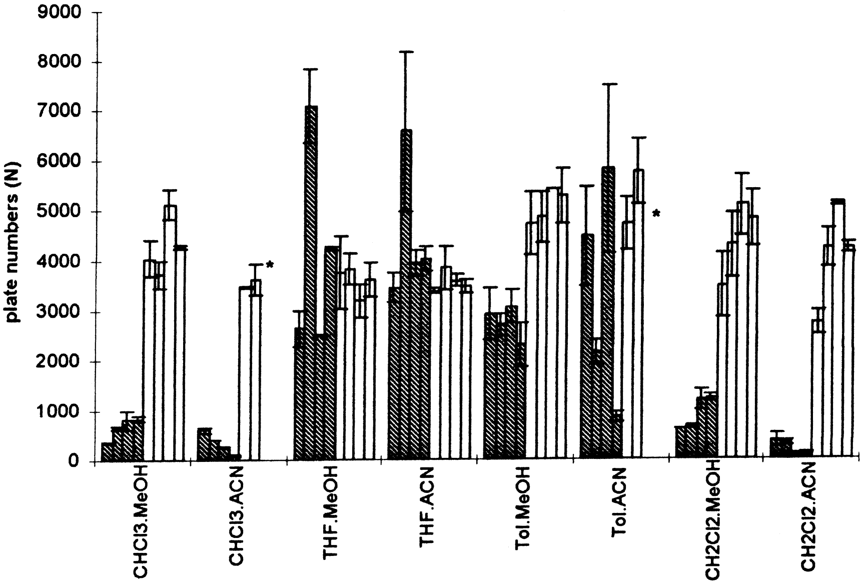

Figure 2. The plate numbers (mean value of 3 measurements) for the four ceramides in each mobile phase composition. Hatched boxes represent the mobile phases without any response modifiers and the whites boxes the mobile phases with 0.1% of TEA and a stoechiometric amount of HCOOH. In each mobile phase condition, the plate numbers of the selected ceramides are represented in the following order: C16:0S, C16:0D, C18:0S and C18:0P. *Calculation of the plate numbers of compounds C18:0P and C18:0S not possible because of an incomplete resolution.

When TFA was added at 0.1% v/v in the mobile phase, it induced an important background noise with a decrease of the peak response. Moreover it led to a 10% decrease in retention time values. The background noise was amplified when TFA was associated with TEA. These results eliminated TFA as a candidate for enhancing the response.

Therefore, the comparison of the peak response was made only between mobile phases without and with 0.1% v/v (7 mM) of TEA and a stoechiometric amount of HCOOH. This percentage of response modifier was selected since the increase of the amount of response modifier until 0.5% v/v (35 mM) displayed no significant increase of the intensity of the peak response compared to that measured with 0.1% v/v (from 0.1 to 0.5% the peak heights were increased about 10% which corresponds more or less to the precision of the detector response). And, under 0.1% v/v of TEA/HCOOH the response was significantly smaller. Therefore a minimum amount of response modifier (0.1% v/v, i.e., 7 mM) was necessary to reach the maximum increase of the response.

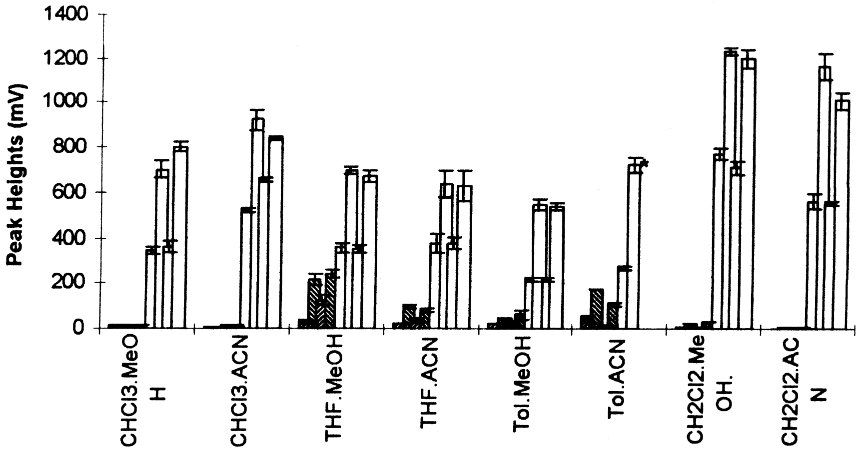

Figure 3. The peak heights (mean value of 3 measurements) for the four ceramides in each mobile phase composition. Hatched boxes represent the mobile phases without any response modifiers and the whites boxes the mobile phases with 0.1% of TEA and a stoechiometric amount of HCOOH. In each mobile phase condition, the peak heights of the selected ceramides are represented in the following order: C16:0S, C16:0D, C18:0S, and C18:0P. *Condition of coelution.

Improvement of the Response with Various Solvents

In order to study the variation of the response due to the mobile phase composition without any change in peak height due to the variation of retention time, the 8 possible combinations of isocratic mobile phases were selected as to be isoeluotropic. Thus in these conditions, the height of the peaks can be comparable. The compositions of the tested mobile phases were experimentally defined in order to obtain retention factors (*k*) of the 4 selected ceramides ranging between 2 and 6. The different mobile phase compositions are summarized in Table 2. Testing the response modifiers in different mobile phase composition had the advantage of allowing the generalization of this phenomenon in NARP chromatography.

The obtained chromatographic profiles were nearly identical, except in the case of Toluene/ACN with response modifier mobile phase where the peaks of C18:0S and C18:0P totally overlapped.

The comparison of the retention factors and the plate numbers calculated in presence and absence of the couple TEA/HCOOH in each mobile phase composition displayed the weak influence of this response modifier on chromatographic behavior. Figure 1 shows that the retention was slightly decreased by the use of TEA/HCOOH modifier, especially in the cases of

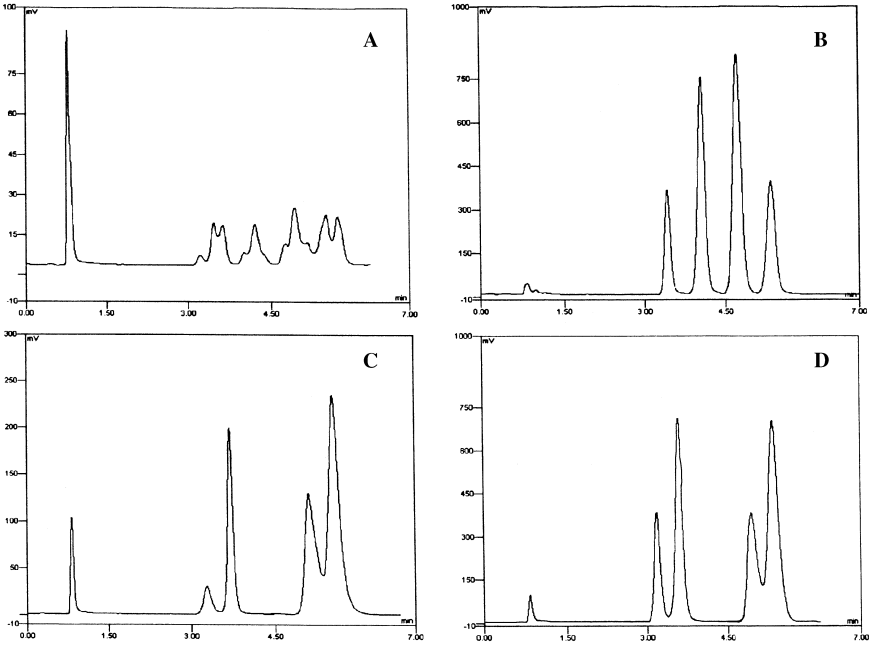

Figure 4. Chromatogram (A) in the isocratic conditions of $CHCl_3$-MeOH (55:45) without response modifier and chromatogram (B) when TEA/HCOOH was used. In this condition, the order of the elution of the four ceramides were C16:0S, C16:0D, C18:0P, and C18:0S. Chromatogram (C) in the isocratic conditions of THF-MeOH (81:19) without response modifier and chromatogram (D) when TEA/HCOOH was used. In this condition, the order of the elution of the four ceramides were C16:0S, C16:0D, C18:0S, and C18:0P.

mobile phases with chlorinated solvents. In absence of TEA/HCOOH, very deformed peaks were obtained with chlorinated solvent (e.g. see chromatograms on Figure 4A). The values of plate number (Figure 2) show an improvement of the column efficiency with TEA/HCOOH when chlorinated solvents were employed, whereas in other conditions the difference was less pronounced.

Without the couple TEA/HCOOH, the best response was obtained with THF and Toluene regardless of the choice of the weak solvent (Figure 3 and 4). In the cases of chlorinated solvents, very irregular peak shapes and tailing peaks were obtained which led to very small values of peak heights. In each mobile phase, the addition of TEA/HCOOH led to an undeniable improvement of the response. There was no significant difference of the peak heights between mobile phases with MeOH and ACN, although these two solvents solubilized ceramides very differently. The increase of response by TEA/HCOOH was of the most important for chlorinated mobile phases. However, in the cases of chlorinated solvents, this improvement concerned also the peak shapes. Thus, we cannot assert that the addition of TEA/HCOOH did not induce any chromatographic phenomenon. However, this specific behavior could also be due to a heterogeneous particle size formation during the nebulization which leads to irregular peak shapes. This phenomenon is thought to occur when chlorinated solvents were used as shown in figure 4A.

The relative peak height variations are not significant and were rather a consequence of a partial overlapping since the four ceramides were injected in mixture, or else a consequence of the change of the position of the peak on the chromatograms since inversion of the elution order of the compounds occurred. Therefore, a relationship between the relative response and the chemical structure of the compounds was not underscored. Thus our conclusion is more in favor of the homogeneity of the response coefficient, which is in agreement with the "universal" nature of the ELSD.[22]

In conclusion, this study using PGC has shown that the improvement of the ELSD response also observed on ODS for ceramides, is due to the presence of the both, TEA and HCOOH. From the fact that the modification of the peak geometry was observed in the cases of chlorinated mobile phases on PGC, we cannot exclude that there was non influence of the couple TEA/HCOOH on the stationary phase. However, this response modifier has an incontestable influence on the detection. Moreover, this response modifier was compatible with various solvent combinations. From these results, we can advocate the use of the couple TEA/HCOOH as a response enhancer for the ELSD in NARP using either PGC or ODS.

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Received May 10, 1999
Accepted June 15, 1999
Manuscript 5072