DOKUZ EYLUL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE UTILIZATION OF PORPHYRINS IN QUANTITATIVE DETERMINATION OF SOME TRANSITION METALS BY THIN LAYER CHROMATOGRAPHY

by

İpek KAYNAK ÇAVDAR

January, 2009

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We have read the thesis entitled **"THE UTILIZATION OF PORPHYRINS IN QUANTITATIVE DETERMINATION OF SOME TRANSITION METALS BY THIN LAYER CHROMATOGRAPHY"** completed by **İPEK KAYNAK ÇAVDAR** under supervision of **PROF. DR. MELEK MERDİVAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Supervisor

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(Jury Member) (Jury Member)

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Prof. Dr Cahit HELVACI

Director

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İpek KAYNAK ÇAVDAR

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ABSTRACT

In this work, high performance thin layer chromatography has been used to quantify metal ions in various analytical medium. The 5, 10, 15, 20-tetrakis(bromohydroxyphenyl) porphyrin as chelating agent and the Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(IV) and Pd(II)-TBHPP chelates were synthesized and their structural and chromatographic behaviours were determined using IR, NMR, absorption and fluorescence spectrometry and thin layer chromatography. In quantification, using solid phase extraction C18 cartridge metal ions were enriched and retain metal chelates were eluted with tetrahydrofuran. The chelates were separated on HPTLC silica gel plate using aceton:chloroform (2:8, v:v) as mobile phase. Only, the TBHPP complexes with Zn(II), Cu(II), Co(II) and Hg(II) migrate differently from each other. Linear working range of TBHPP complexes with Zn(II), Cu(II), Co(II) and Hg(II) were 3.6-30, 1.2-30, 0.6-30, 3.6-60 ng μL^{-1}, respectively. LOD of TBHPP complexes with Zn(II), Cu(II), Co(II) and Hg(II) were 0.92 ng μL^{-1}, 1.21 ng μL^{-1}, 0.19 ng μL^{-1}, 0.90 ng μL^{-1}, respectively. Repeatibility for within- and between-day (RSD%) of TBHPP complexes with Zn(II), Cu(II), Co(II) and Hg(II) were 3.67 – 5.24, 2.66 – 3.51, 1.87 – 4.10, 3.09 – 4.51, respectively. The method was applied to the determination of Zn(II), Cu(II), Co(II) and Hg(II) in tap water, geothermal water, river water and dried tomato samples with good results.

Keywords: Porphyrin, Thin layer chromatography, Metal

BAZI GEÇİŞ METALLERİNİN İNCE TABAKA KROMATOGRAFİSİ İLE NİCEL TAYİNİNDE PORFİRİNLERİN KULLANILABİLİRLİĞİ

ÖZ

Bu çalışma, çeşitli analitik ortamlarda metal tayini için yüksek performanslı ince tabaka kromatografisi kullanılmasını içermektedir. Şelatlayıcı reaktif olarak 5,10,15,20-tetra(bromohidroksifenil)porfirin bileşiği ve Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(IV) ve Pd(II)-TBHPP şelatları sentezlendi ve IR, NMR, absorpsiyon ve floresans spektrometrisi ve ince tabaka kromatografisi kullanılarak yapısal ve kromatografik davranışları belirlendi. Nicel analiz için, C18 katı faz ekstraksiyonu kartuşları kullanılarak metal iyonları zenginleştirildi ve tetrahidrofuran ile geri alındı. Aseton:kloroform (2:8, v:v) hareketli faz kullanılarak HPTLC silika jel plakasında metal iyonları birbirinden ayrıldı. Sadece Zn(II), Cu(II), Co(II) ve Hg(II) iyonları birbirlerinden farklı göç ettiler ve ayrıldılar. Zn(II), Cu(II), Co(II) ve Hg(II)-TBHPP komplekslerinin doğrusal çalışma aralıkları sırasıyla 3.6-30, 1.2-30, 0.6-30, 3.6-60 ng μL^{-1} dir. Zn(II), Cu(II), Co(II) ve Hg(II)-TBHPP komplekslerinin gözlenebilme sınırları sırasıyla 0.92 ng μL^{-1}, 0.36 ng μL^{-1}, 0.19 ng μL^{-1}, 0.90 ng μL^{-1} dir. Zn(II), Cu(II), Co(II) ve Hg(II)-TBHPP komplekslerinin gün içi ve günler arası tekrarlanabilirlikleri sırasıyla %RSD olarak 3.67 – 5.24, 2.66 – 3.51, 1.87 – 4.10, 3.09 – 4.51 dir. Yöntem musluk suyu, jeotermal su, nehir suyu ve kurutulmuş domates örneklerine başarı ile uygulanmıştır.

Anahtar sözcükler: Porfirin, İnce tabaka kromatografisi, Metal

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CHAPTER ONE

INTRODUCTION

1.1 Preconcentration and Separation

Although great progress has been made in the development of highly selective and sensitive analytical methods, the analytical chemist is more and more called to deal with increasingly complex samples. Also, lower and lower detecton limits are required. Preconcentration or separation steps can therefore stil be necessary even in combination with highly selective and sensitive methods.

The chemical techniques used in preconcentration can in many cases provide analyte isolation as well as enrichment. Preconcentration cam imply extra advantages such as minimization of matrix effects and lowering of the limit of detection, can allow the application of simpler instrumentation in the final measurement, and may facilitate automated analyses. The most important preconcentration techniques for metal ions are liquid-liquid extraction and ion exchange. Other useful techniques are volatilization and coprecipitation.

The methods of analytical chemistry can be divided into two large gropus: (1) methods of separation and preconcentration of components, (2) methods of determination of components of the material to be analysed.

Separation is a process in which the components constituting the starting mixture are separated from each other. Preconcentration is a technique by which the ratio of concentration (or the amount) of trace components to the concentration (or the amount) of macrocomponent is increased. In separation, the components constituting the mixture may or may not differ in concentration from each other. In preconcentration, the components that have significantly different concentrations are treated.

Numerous methods are used for analytical preconcentration of trace elements. A large majority of them had previously been employed only for separation. Probably evaporation was first utilized for preconcentration; precipitation, extraction, electrochemical and other methods gained recognition at a later stage. Fire assay is one of the oldest methods of preconcentration.

By the nature of the separation methods used, they can be classified into 1) chemical and physico-chemical and 2) physical methods. With the first group may be classed extraction, sorption, precipitation and co-precipitation, partial dissolution of matrix, flotation, volatilization after chemical transformations, chemical transport reactions, fire assay, electrochemical methods and dialysis. The second group may include volatilization, crystallization, freezing out, filtration and gel filtration and ultracentrifugation.

The selection of a preconcentration method is dictated by (i) the practical problem being solved, the nature of the material to be analyzed, trace elements to be determined, the specified metrological parameters of the technique; (ii) the origin and previous history of the material to be analyzed; (iii) the combination of the selected method of preconcentration and subsequent method of determination of trace elements in a concentrate; (iv) the simplicity, the availability and the duration of the method; (v) the equipment available in the laboratory of the scientist and in those laboratories which will use the method; (vi) the specialization and qualification of the researcher developing the technique and of the analysts of the laboratories where this technique is to be employed; (vii) the need to ensure safe working conditions.

Solvent extraction is an effective and widely used method of preconcentration. It can be applied both for the removal of matrix and for the selective, group, or subsequent separation of trace elements. One of its variants extraction chromatography is an effective way of separating substances with almost similar properties and ensures high efficiency of preconcentration (Zolotov & Kuz'min, 1990).

Liquid-liquid extraction is a classical method for preconcentrating metal ions and/or matrix removal. Solid phase extraction is another approach that offers a number of important benefits. It reduces solvent usage and exposure, disposal costs and extraction time for sample preparation. Consequently, in recent years solid phase extraction has been successfully used for the separation and sensitive determination of metal ions (Camel, 2003).

1.2 Metals

Many elements occur in a sample in such minute amounts that, at the beginning of the development of instrumental analytical methods in the 19th century, it was impossible with the existing techniques to determine their concentration quantitatively, although their presence could be dedected qualitatively. To indicate such low and barely detectable concentrations the term ‘trace’ was used and the elements were referred to as trace elements (World Health Organization, 1996).

The number of determinations of metallic elements in environmental samples is already very high and is rapidly increasing. The samples being analysed include:

- water: industrial waste water, leachate from landfills, river water, sea and ocean water, rain water, drinking water etc.,
- aerosols (dust) collected from the atmosphere (both outdoors and indoors, e.g. in work places), dry deposition, fly ash from electrical power plants etc.,
- soil, sewage sludge and so on.

During the past decades, increasing attention has been focused on pollution of the natural environment. The abatement of pollution of the air, of surface, drinking and sea water and of the soil is an important and very complex task, requiring the cooperation of specialists from various disciplines including analytical chemistry.

For geological research, the determination of trace elements in both rocks and water samples is of great importance, both from an academic and an applied point of view.

These analyses are usually rather difficult owing to the complex nature of the samples and to the low concentrations involved. In addition, it is usually not safe to limit the scope of the analyses to one or a few elements; one should rather determine as many elements as possible using a multi-element analytical technique in order not to overlook important buy unexpected elements.

1.2.1 Their Importance of Trace Elements

Many elements occur in a sample in such minute amounts that, at the beginning of the development of instrumental analytical methods in the 19th century, it was impossible with the existing techniques to determnine their concentration quantitatively, although their presence could be detected qualitatively. To indicate such low and barely detectable concentrations the term “trace” was used and the elements were referred to as trace elements. Although mowadays very low concentrations determined accurately and precisely because of improvements to existing and development of new analytical techniques, the term “trace element” is stil in use. In general, one speaks of a trace element when the concentration is below 100 μg g^{-1}. At extremely low concentrations, below 10 ng g^{-1}, one also speaks of “ultra-trace” elements. In spite of their low concentration, these elements may play a very important role in many areas(biochemistry, medicine, enviromental, industrial) (Vandecasteele & Block,1993).

Based on current acceptance by the scientific community, a classification system of essentially for the trace elements considered in this study and identified in periodic table format as shown in Table 1.1. Among the elements in Table 1.1 some of them are essential but some of them are toxic.

Table 1.1 Classification of the trace element

Classification	Elements
Bulk structural elements	Carbon (C), hydrogen (H), oxygen (O), phosphorus (P), sulfur (S)
Macro elements	Calcium (Ca), chlorine (Cl), potassium (K), sodium (Na)
Trace elements	Copper (Cu), iron (Fe), zinc (Zn)
Ultra trace elements Non-metals	Arsenic (As), boron (B), fluorine (F), iodine (I), selenium (Se)
Ultra trace elements Metals	Cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), manganese (Mn), molybdenum (Mo), nicel (Ni), tin (Sn), vanadium (V)

1.2.2. Trace Metal Determination Techniques

The determination of these metals are of great importance and significance for environment science and life science to separate and determine trace amounts. It is possible to determine trace metal by chromatographic and spectroscopic techniques.

Sensitive analytical techniques such as FAAS, ICP-MS, ICP-AES and X-ray fluorescence are used for the determination of trace metals.

Chromatographic techniques such as HPLC, TLC, ion chromatography are performed for determination of trace metals.

1.3 Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a subdivision of liquid chromatography, in which the mobile phase is a liquid and the stationary phase is situated as a thin layer on the surface of a flat plate (Fried & Sherma, 1986). TLC is sometimes grouped under the term ‘‘planar chromatography’’ due to its flat geometry. Methods for the quantitative evaluation of thin-layer chromatograms can be divided into two

categories. In the first one, solutes are assayed directly on the layer either by visual comparison, area measurement or densitometry. In the second, solutes are eluted from the sorbent before being measured (Fried & Sherma, 1986) .

The basic procedure for classical TLC consists of the following steps;

1. The sample solution is applied to the plate origin as a spot or zone.
2. The sample solvent is allowed to evaporate from the plate.
3. The plate is placed in a closed chamber containing a shallow pool of mobile phase on the bottom.
4. The mobil phase rises by capillary action through the applied spot.
5. Development is continued until the solvent front is about 10 to 15 cm beyond the origin.
6. The plate is removed from the chamber and the solvent front is marked.
7. Mobil phase is removed from the plate by driying in air or applying heat.
8. If the compounds are not naturally colored or fluorescent, a detection reagent is applied to visualize the zones.
9. The positions of the zones are used for qualitative identification of compounds and the size and / or intensity of the zones for quantification.

1.3.1 High Performance Thin Layer Chromatography

The mid-1970s saw the introduction of high performance TLC plate, which vastly improved on the original thin-layer Chromatography separation methods. There are several differences between high performance TLC and conventional TLC, the most important being particle size (approx. 5 μm from 20 μm), particle uniformity, and the layer thickness on the plate (typcially 200 μm for HPTLC plates). These changes improve separation efficiency, decrease solvent consumption, lower zone diffusion, and provide better overall sensitivity and reproducibility.

1.3.2 Differences between HPTLC and TLC

Many of differences are available between TLC and HPTLC. Main difference is the particle and pore size of sorbents and the others are shown in Table 1.2 (Meyyanatha, 2003).

Table 1.2 Differences between HPTLC and TLC(Meyyanatha, 2003)

	HPTLC	**TLC**
Layer of Sorbent	100 μm	250 μm
Efficiency	High due to smaller particle size generated	Less
Separations	3 - 5 cm	10-15 cm
Analysis Time	Shorter migration distance and the analysis time is greatly reduced	Slower
Solid support	Wide choice of stationary phases like silica gel for normal phase and C8, C18 for reversed phase modes	Silica gel, Alumina, Kiesulguhr
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting
Scanning	Use of UV/ Visible/ Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible

1.3.3 HPTLC Theory

HPTLC separations are based on creating an interface between a stationary phase bound to a solid planar support and a mobil phase. The mobil phase migrates through the stationary phase by capillary action while the analytes are partitioned between the two phases. Analytes that partition themselves more in the mobile phase move further along the plate while analytes that partition more in the stationary phase migrate less.

The ratio of this partition of the analyte is described as capacity factor k^1 :

k^1= (retention time in the stationary phase) / (retention time in the mobile phase) **(1.1)**

The higher the retention for the stationary phase (larger capacity factor), the lower the migration of the compound on the TLC plate. The distance the analyte migrates on the plate is known as the R_F value:

R_F = (migration distance of analyte) / (migration distance of mobile phase) **(1.2)**

The R_F value is a fractional value of the mobility of the analyte in the mobile phase; therefore, values are always between zero and one, with zero being no migration of the analyte and one being an analyte that moves as far as the mobile phase migrates. Ideally, the solute will partially migrate with the mobile phase but will have attraction to the stationary phase so that migration values are between 0,1 and 0,9. The capacity factor and R_F value are related by equation 3:

$k^1 = (1- R_F) / R_F$ **(1.3)**

Occasionally, separation of samples in Chromatography is incomplete. Resolution (R_s) is used to determine the amount of overlap between chromatographic peaks:

$R_s=(Z_2-Z_1) / (0.5(W_1+W_2))$ **(1.4)**

Z_2 and Z_1 refer to the distance the middle of each peak has migrated from the initial spotting point, while W_2 and W_1 refer to peak widths. The larger the R_s value, the better the separation between the chromatographic peaks. Peaks with resolution values larger than 1 considered well resolved.

The efficiency of chromatographic separations can be modeled several ways. One of the most accepted models employs the use of theoretical plates (Martin & Synge, 1941; Martin, 1952). In this model, the mobility of compounds is based on the the theory of distillation. An equilibrium is established between the mobile and stationary phase at theoretical points(plates) over entire distance of the chromatographic surface, which provides the fundamental basis for the seperation. The height of a theoretical plate(N) can be calculated by the use of equation 5:

$$N= 16x ((LxZ) / W)^2 \quad \textbf{(1.5)}$$

L and Z are the migration distances of the mobile phase and the solute, respectively, and W is the width of peak in the direction of separation. The more theoretical plates across the chromatographic surface, the better the separation efficiency.

Another measure of separation efficiency is the height equivalent of a theoretical plate(H or HETP). H is a measure of chromatographic separation efficiency per unit lenght and can be calculated using equation 6:

$$H= L / N \quad \textbf{(1.6)}$$

Because of inverse reationship between H and N, it is obvious that shorter theoretical plates indicate better Chromatographic efficiency.(Sherma & Fried, 1996)

1.3.4 Advantages and Disadvantages of HPTLC

1.3.4.1 Advantages

High performance TLC has many distinct advantages (Table 1.3), which makes it an attractive analytical technique. The main advantage is the high throughput that the planar separation provides. This advantage is important for industries such as food and pharmaceuticals that need to analyze mass amounts of samples for quality and safety control purposes. The ability to analyze several samples in the same amount of time required to complete one separation from a serial technique (HPLC, capillary electrophoresis, gas chromatography, etc) saves a substantial amount of time and money.

A second advantage of an HPTLC system is the low amount of solvent consumed. HPLC and capillary electrophoretic separation instrumentation use a constant flow of mobil phase to elute the analytes off the stationary phase. Usual flow rates for these separations are between 1-5 ml per minute, causing an enormous amount of liquid waste. HPTLC uses a fraction of the mobil phase consumed by an HPLC or CE system to perform high throughput separation, because detection is performed off-line. Low solvent consumption is an advantage not only in and of itself but because there is far less waste requiring disposal than with other separation techniques, and therefore less enviromental impact. Decreased solvent consumption also saves the analyst / company time and money.

Another advantage of HPTLC system is that it produces total sample accountability. The entire HPTLC stationary phase can be imaged or scanned, thus revealing on components present. If an unknown sample is injected onto an HPLC column, it is possible for components of the sample to irreversibly absorb to the column without eluting. Substantial problems may result if the sample is a true unknown or if there are impurities that the analyst has not accounted for. The ability to account for all analytes is extremely important for pharmaceutical industry,

because impurities can cause mild to sever side effects or can inhibit the activity of the pharmaceutical itself.

HPTLC also allows for multiple detection modes without major modification to complex instrumentation. Several types of detectors can image or scan for fluorescence, absorbance, reflectance or fluorescence quenching without requiring modification of an elaborate flow system. There is also a wide variety of detectors available from which to choose, including scanning densitometers, video imaging devices, scientifically operated charge-coupled devices or infrared imaging detectors. Each type of detector provides advantages and disadvantages, but all have detection schemes separate from the chromatographic media, unlike HPLC and CE, which are directly linked to the detector through a flow system. The use of non-flow system like HPTLC also makes it possible to perform qualitative and quantitative analysis at a later time with another detection scheme, if it is necessary. Later detection by other methods is not an option for the online flow techniques, since the sample is discarded as waste.

A final advantage for HPTLC lies in its ability to performed less complex extractions. Since HPLC and CE are column techniques, they use fairly sophisticated and expensive equipment. Therefore, samples must be fairly clean before they can be injected into the system, which requires elaborate extraction schemes to remove harmful components of sample. Each HPTLC plate is used one time and is than either disposed of or filed after detection. Since no other separation occur on the chromatographic surface, components undesired in an HPLC or CE system can be present if they do not interfere with the detection of analytes of interest. This advantage is important for the food industry, since extraction from animal products (poultry, milk, meat, etc.) are typically complex, due to the presence of proteins and other large biomolecules.

1.3.4.2 Disadvantages

Although HPTLC has many advantages, it is not without faults. The separation efficiency of HPTLC is lower than that of its forced-flow competitors. Even with advances in HPTLC plate manufacturing, separation efficiency is no as high as with HPLC or CE. This decrease efficiency is linked to eluting analytes using a single plate volume of mobile phase while LC and CE techniques use multiple column volumes for their separations. The use of only one plate volume necessetitates lower capacity factors. The speed of certain separations can also be disadvantage. Normal phase separations using silica gel are quick, with development times of 20 minutes or less. Reverse phase separations by HPTLC, however, may take significantly longer; plate development times for reverse phase analyses can be in excess of one hour. Therefore, normal phase separations are the usual method of development. The time problem encountered in reversed phase development can be overcome by using a forced-flow system, but it is not common practice at this time.

Table 1.3 Advantages and disadvantages of HPTLC

Advantages	**Disadvantages**
High throughput	Poor separation efficiency (compared to column techniques)
Low solvent consumption	
Total sample accuntability	Speed of reverse phase separations
Multiple detection modes	
Less complex extractions	

1.3.5 Detector Technology

1.3.5.1 Scanning Densitometers

Scanning densitometry, the detection technique currently used for HPTLC analysis, involves rastering a detector (typically a photomultiplier tube), wavelenght selector (monochromator or filter system), and light source across a TLC plate,

detecting the compounds of each chromatogram individually. Quantitation can be performed by several detection modes. The main drawback to scanning densitometry is that sample throughput is decreased because the detector must scan each chromatographic lane of the TLC plate. Such scanning also contributes to problems associated with changes in the sample or instrument through time. Because each sample is scanned at different times, degradation of sample and fluctuations of the light source may drastically affect signal from individual scans. This problem can be corrected by imaging the entire surface of the plate at a single instance (Tamura, 1972).

1.3.5.2 Imaging Technology

The use of imaging technology has benefited the study and advancement of high-throughput techniques. Obtaining all the desired analytical information at one time can theoretically maximize throughput of a given spatial technique. Gathering information in this fashion also decreases the likelihood of changes in analytical signal due to degradation of sample, light intensity fluctuations, etc. In the past photodiode arrays, vidicon camera tubes, and vacuum tube multi-channel detectors have been used for imaging applications. However, their poor sensitivity and linear dynamic range limits their used today. Charge-couple device (CCD) detectors have many advantages over these other imaging techniques (Touchstone & Sherma, 1979). When treated scientifically (cooled with liquid nitrogen or thermoelectrically), CCDs are excellent imaging detectors with low read noise and dark current, excellent linearity through a large dynamic range, and superior sensitivity from x-ray to near infrared regions (Touchstone & Sherma, 1979).

1.3.6 TLC Detection Techniques

The detection technique for TLC and liquid Chromatography (LC) are quite different. LC is performed in a sequential manner so analytes are detected as they elute. In TLC, detection is a completely independent step and is carried out in the opaque non-uniform solid particles with large scattering interferences. Therefore,

some detection techniques that work very well for HPLC, such as refractive index detector, a polarimetry dedector (Yeung, Steenhoek, Woodruff & Kuo, 1980), and all differents kinds of electrochemical dedectors (Horvai & Pungor, 1989; Jandik, Haddad, Sturrock, 1988), do not work for TLC. TLC detection conventionally is limited to only a few optical methods such as adsorption and fluorescence, except use of radioactive technique. The detection limit is also hampered by the highly scattering background of the thin layer material. This probably is the main reason that, TLC lagged behing in the early years. In recent years, various advanced instruments and detection technique really bolstered the TLC method. Detection techniques for TLC will be briefly discussed in this overview.(Fried & Sherma, 1989)

1.3.6.1 Visual Detection

The success of separation of colored compounds is usually monitored visually. Such compounds absorb a particular portion of the polychromatic(white) light in the visible wavelenght range. The remaning radiation(complementary radiation) is reflected and detected by the eye; it determines the color of the substance zone. For colorless compounds, usually reagents are used to make them visible on the chromatgram. Spraying reagents can be divided into two classes: (1) general reagents, which detect a large number of different types of compounds, and (2) spesific reagents, which indicate type of compound or functional grup present. Up-to-date, hunders of spraying reagents have been found for the detection of different compounds (Fried & Sherma, 1986).

Visiual detection can only be used for qualitative and semiquantitative detection, and the search for a prope spraying reagent could be tedious job.

1.3.6.2 Fluorescence Detection

Fluorescence spectroscopy is a widely applied detection technique in both TLC and HPLC. It is especially useful for the quantitative determination of molecules at

trace levels, and it is one of the easiest techniques for achieving low limit of dedection (LOD), because of its high sensitivity and larger linear dynamic range basic prensible of fluorescence detection is that molecules that absorb radiation are raised to an excited state, when returning to the ground state, they emit the absorbed energy instanteneously as radiation energy in the form of fluorescence. The radiation emitted is usually longer is wavelenght than the incident light. Unfourtunately, fluorescence suits only fluorescent compunds or these compounds which can be derivatized to became fluorescent. Therefor it is not a universal detection method in spite of its high sensitivity and low LOD (Fried& Sherma, 1986).

1.3.6.3 Absorbance Detection

In situ densitometry is the most commonly used method to do quantitative determination on TLC (Grinberg, 1990; Poole & Schuette, 1984; Touchstone & Sherma, 1979). The TLC plate is scanned by a beam of light with predetermined wavelenght in the mode of reflectance, transmission, or both simultaneously. Light striking a spot on the plate will undergo absorption so that the light transmitted or reflected is diminished in intensity at those wavelenghts, forming the absorption profile of the spot. The measurement of the signal diminution due to this absorption by the spot provides the mechanism for in situ quantization. Since TLC plates are opaque and scatter light strongly, absorption measurements can not be expected to obey the Beer-Lambert law. The Kuybelka-Munk equation has to be used for correction. Its derivation can be found elsewhere (Grinberg, 1990; Hurtibise, 1981). The LOD this method is about 10 ng to 1 µg, varying with different samples.

1.3.7 Thin Layer Chromatography of Metal Chelates

In quantitative trace metal analysis, the analytical signal is affected from interferences because of matrix effects or contaminant elements in direct methods.

Both separation and enrichment can be possible in a single operation step simultaneously, by liquid_liquid extraction for trace metal analyses. After

complexation of metal ions in aqeous solution with organic reagents and separation from interfering inorganic matrix by extraction under enrichment into nonpolar organic solvents, they can be monitored by spectrophotometric or fluorometric measurements. All analytical operations lead to the chromatography of metal chelates as a powerful analytical method for trace metal determination (Figure1.1).

Generally, the following advantages can be utilized in the chromatography of the metal chelates for trace metal analysis;

1. Relatively, simple sample preparation (complex_formation at appropriate pH value),
2. Elements to be determined can be enriched before determination by extraction of their complexes,
3. Interfering inorganic matrix remain in aqeous phase during extraction,
4. Because of chromatographic separation, several elements can be identified and determined quantitatively in a single analytical step (multi_element analysis),
5. Sensitive detection of the metal chelates using UV-photometry (high extinction coefficient of the chelates) is possible,
6. Short analysis time, simple and rapid procedure.

Therefore, chromatography of metal chelates is a highly efficient analytical technique and it is able to replace other well known analytical methods of trace metal determination.

For separation metal chelates, generally the same chromatographic techniques like liquid chromatography (LC), gas chromatography (GC), arec used as for the analysis of organic substances. Adsorption chromatography is the most important one of the various liquid chromatographic separation techniques. Especially for chromatography of metal chelates, silica gel and alumina have been used more succesfully as stationary phases in TLC because of their good separation qualities.

Unfortunately not all elements can be used without problems in chromatography. Certain difficulties like decomposition of the chelates before or during elution, may appear as well as incomplete separation of the various complexes or strong tailing in chromatograms may appear. These difficulties often reduce the good qualification of chromatography of metal chelates for trace metal analysis. Therefore, only certain complexing agents which form chelates that can be separated chromatographically and determined analytically, can be used successfully (Merdivan, 1994).

1.3.7.1 Relation Between Complex Chemical Properties and Chromatography of Metal Chelates

The well known complexing agents in analytical chemistry for photometric, gravimetric and volumetric determinations, in spite of their wide range of application, do not form metal chelates which can be chromatographed successfully. Several reasons are responsible for that;

1-Lack of solubility of the formed chelates in nonpolar solvents.

-thus it is not possible to enrich the metal ions from aqueous solution by extraction.

-thus only limited fractions of the chelates carried out in adsorption chromatography on silica gel phases.

2- Lack of stability of the chelates during contact with stationary phase.

3- Decomposition of the chelates during elution.

4- Strong tailing, because of reaching equilibrium between adsorption and desorption of the chelates on the stationary phase slowly.

5- In spite of successful chromatographic behaviour for individual analytes, not enough differences in chromatographic retention for the mixture.

A close relationship exists between the chromatographic behaviour and the complex chemical properties of the metal chelates because these are responsible for type and intensity of interaction with stationary and mobile phases. The active

site of silica gel is the end positioned with silanol groups (-SiOH). They form a weak linkage with each neighbouring molecule with one of the following interactions:

1- dipole- dipole
2- dipole-induced dipole
3- Π-complex- linkage to double bondings of the metal chelate.
4- Hydrogen bonding between the silanol group (as proton donor) and the functional groups of the ligand with a free electron pair (as a proton acceptor)
5- Polar electron donor bonding of the silanol anion ($-SiO^-$) with the central atom of the complex.

The interaction between adsorption of the chelate onto the surface of the stationary phase depends on the properties of the chelates. These are dipole moment, basicity of the coordination sites, metal-spesific properties of the central atom like its affinity to oxygen or sulphur, coordination number, steric structure of the chelate including additional ligands like water, solvent mlecules or oxygen.

The power of the interaction between the mobile phase and the stationary phase is very important for retention of complexes, because mobile phase and analyte compete for the active sites of the stationary phase.

Elements coordinated to ligands with oxygen as coordination site (oxygen affinic elements) can be chromatographed less successfully than those with sulphur on polar stationary phases (Merdivan, 1994).

Moreover, most of these complexes are less soluble in aqueous as well as in nonpolar organic solvents.

Tailing can be reduced by addition of strongly polar substances (H_2O,acids) or complexing agents to the mobile phase. This can be solved by ligand-derivatisation, but substitution is restricted, because too big substituents would level out chromatographic retention, or decrease the solubility in organic solvents by gravimetric effects.

Therefore, low polarity of the metal-ligand bonding is a prerequisite for successful application of a chelate to adsorption chromatography. If the linkage is too polar, tailing or decomposition of the complex appear, or no elution of the substance takes place. The chromatographic behaviour can be improved by substitution of oxygen with sulphur as coordination site. The metal-sulphur bonding is more covalent, thus the enthalpy of adsorption of the complexes decreases on the stationary phases (Merdivan, 1994).

Metal chelates must have the following conditions and prerequisites;

1- Formation of stable neutral metal chelates,

2- Control of complex formation by pH-variation or by masking reagents,

3- Formation of well defined chelates with clear stoichiometry,

4- Five-membered chelate-rings between metal and ligand possess the highest stability. But four- and six-membered chelate-rings reduce stability,

5- Chelating agents with N-, O-, S or Se- atoms as coordination sites has good chemical properties for complex formation. In addition the ligands are stable under normal circumstances for analytical applications.

6-The metal chelates should have high solubility in the mobil phase,

7- The ligand must be too large,

8-The chelating conjugated Π-system should cover the complete ligand for sensitivce photometric detection (high extinction coefficients).

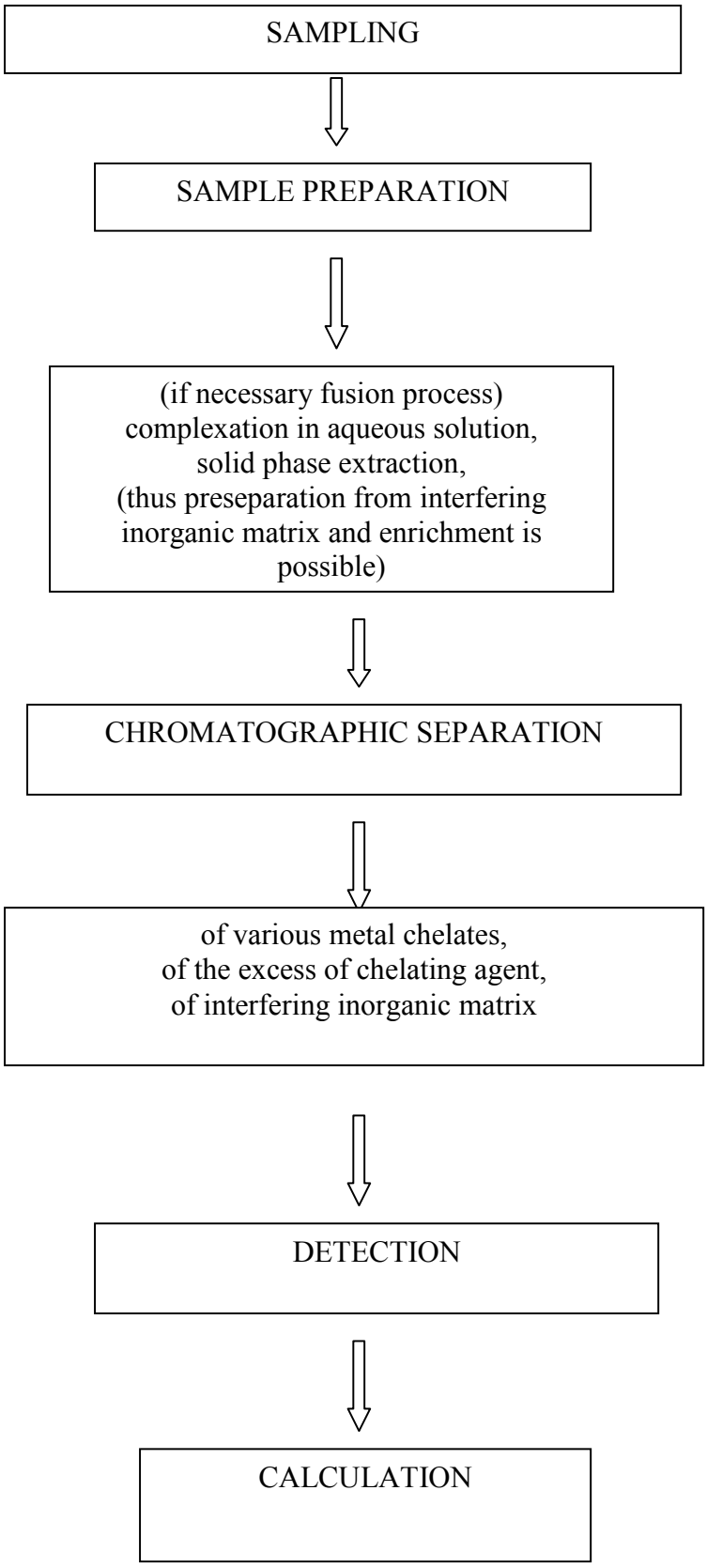


Figure 1.1 Trace metal analysis by the chromatography of metal chelates

1.4 Porphyrin

The porphyrins are a class of naturally occurring macrocyclic compounds, which play a very important role in the metabolism of living organisms. The porphyrin molecule contains four pyrrole rings linked via methine bridges (Figure 1.2). The porphyrin nucleus is a tetradentate ligand in which the space available for a coordinated metal has a maximum diameter of approximately 3.7 Å (Falk, 1975).

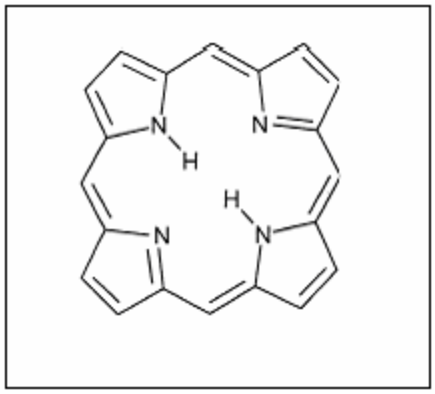


Figure1.2 Porphyrin macrocyclic system

When coordination occurs, two protons are removed from the pyrrole nitrogen atoms, leaving two negative charges. The porphyrin ring system is very stable and exhibits aromatic character. The porphyrin complexes with transition metal ions are very stable (Figure 1.3).

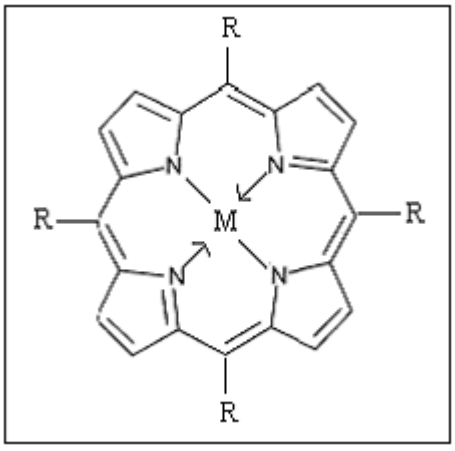


Figure 1.3 Porphyrin-metal complex

Almost all metals form complexes 1:1, although Na, K, Li complexes are 2:1 in which the metal atoms are incorporated slightly below and above the porphyrin macrocycle plane. When divalent metal ions (e.g. Co(II), Ni(II), Cu(II)) are chelated, the resulting tetracoordinate chelate has no residual charge. While Cu(II) and Ni(II) in their porphyrin complexes have generally low affinity for additional ligands, the chelates with Mg(II), Cd(II) and Zn(II) readily combine with one more ligand to form pentacoordinated complexes with square-pyramidal structure. Some metalloporphyrins (Fe(II), Co(II), Mn(II)) are able to form distorted octahedral with two extra ligand molecules.

Porphyrin metal complexes play an important role in biological activities as for instance iron complex in the haemoproteins, magnesium complexes in the chlorophylls, and a cobalt complex in Vitamin B12. Complexes of many metals with various porphyrins have been extensively studied in order to understand the biosynthetic formation and biological activity of natural compounds. Porphyrin derivatives play a key role in essential biological processes such as photosynthesis, dioxygen transport and storage. From the perspective of coordination chemistry, the porphyrin ligand has turned out to be very versatile, and almost all metals have been combined with porphyrins. Such complexes have been used in a variety of applications as models for biological electron transport, oxygen transport and metalloenzymes.

It is well known that porphyrin is also a high sensitive chromogenic reagent. Porphyrins and their metal chelates generally exhibit characteristic sharp and intensive absorption bands in the visible region. The region from 400 to 500 nm, which is called the Soret band, shows the most intensive absorption and molar absorptivities of the order of 10^5 are often found. The Soret band is widely used for spectrophotometric determination of metalloporphyrins.

Owing to its well known photochemical andn redox activity, the porphyrin macrocycle is an attractive building block on which to append additional recognition sites for anion binding. The combination with Lewis acid, such as zinc, complexed in

the porphyrin macrocycle cavity, may produce new selective redox active reagents for anions. Indeed various metalloporphyrins have shown a potentiometric response to anions with selectivity sequences solely dependent on the centrally bonded metal (Beer, Drew & Jagessar, 1997; Falk, 1975). The metalloporphyrins have a rich redox chemistry since they have the advantage of including coordination of additional ligands above and below the porphyrin plane.

Due to strong complexing properties and catalytic behaviour of metalloporphyrins, these compounds have found numerous applications in chemical analysis. This review presents applications of porphyrin compounds in spectroscopy, electroanalytical chemistry, flow injection analysis, and chromatography.

1.5 Porphyrins for Metal Determination

Separation of many porphyrins and metalloporphyrins by TLC(Lai, Lam & Chan, 1994; Podgorna & Kus, 2000; Podgorna & Dziegielewski, 2006; Saitoh, Kashiwa, Tada, Kiyohara & Suzuki, 1992; Stefaniak; 2007) and HPLC (Hu, Yang, Yin & Yao, 2002; Igarashi, Ide & Takagai, 2000; Itoh, Liu & Komata, 2006) has been described in literature. Up to now, mobility of porphyrins such as crown ether, tetrahydroxyl and tetraalkyloxy derivatives of tetraphenylporphyrin, tetra-(p-tolyl)porphyrin have been investigated in TLC (Kobayashi, Saitoh & Suzuki, 1984; Kus, 1996; Kus, 2002; Podgorna & Kus, 2000; Podgorna, 2002). Similarly, a few metalloporphyrins have been studied (Kobayashi, Saitoh & Suzuki, 1984; Podgorna, 2002; Podgorna & Dziegielewski, 2006; Stefaniak, 2007).

In 2002, Podgorna determined that porphyrins and their complexes with Cu(II) and Ni(II) have been separated by adsorption thin-layer chromatography. The effects of Cu(II) and Ni(II) cations on the separation of porphyrins and metalloporphyrins have been determined. In this study, carbon tetrachloride:chloroform (1:1) as mobile phase was used (Podgorna, 2002).

In 1992, the R_F values of twelve synthetic porphyrins including porphine and phenyl- and alkyl-substituted porphines, and also of their copper complexes, were obtained on thin-layer chromatographic plates coated with silica gel, cellulose, NH_2-bonded silica gel and octadecyl-bonded silica gel (ODS) with different developing solvents by Saitoh et al. (Saitoh, Kashiwa, Tada, Kiyohara & Suzuki, 1992).

In 1984, Kobayashi et al studied the migration behavior of metal complexes of meso-tetrakis (p-tolyl) porphyrin (TTP) in HPTLC systems with cellulose and silica gel thin layers and various organic solvents of low polarity. The mobility of the metal complex tends to increase in the following orders of its central-metal elements: on cellulose, Mg(II)<Zn(II)<V(IV)<Pd(II)<Ni(II)~Cu(II); on silica gel, Mg(II)<V(IV)<Zn(II)<Ni(II)~Pd(II)<Cu(II) (Kobayaski, Saitoh & Suzuki, 1984).

In 2004, Yang, Guangyu and Lin determined heavy metal ions in Chinese herbal medicine by microwave digestion and reversed-phase high-performance liquid chromatography. Lead, cadmium, mercury, nickel, copper, zinc, and tin ions in the digested samples were pre-column derivatized with tetra-(4-chlorophenyl)-porphyrin to form the colored chelates which were then enriched by solid phase extraction with C18 cartridge and eluted from the cartridge with tetrahydrofuran (THF). In the original samples the detection limits of lead, cadmium, mercury, nickel, copper, zinc and tin are 4 ng L^{-1}, 3 ng L^{-1}, 6 ng L^{-1}, 5 ng L^{-1}, 2 ng L^{-1}, 6ngL^{-1}, and 4 ng L^{-1}, respectively (Yang, Guangyu & Lin, 2004).

In 2000, a HPLC procedure has been optimized and applied to porphyrins of environmental samples such as marine sediment without purification using detectors as diode array and/or mass spectrometry, such as etio and octaethylporphyrins and their Vo and Ni compounds by Magi et al. (Magi, Ianni,Rivaro & Frache ; 2000).

In 2002, tetra-(4-bromophenyl)-porphyrin (T_4BPP) as a chelating reagent has been used for the on-line column enrichment and the separation of trace lead, cadmium and mercury ions by reversed-phase high-performance liquid chromatography (RP-HPLC) with photodiode array detector by Hu et al. When the Hg–T_4BPP, Pb–T_4BPP

and Cd–T_4BPP chelates were injected into the injector and sent to the enrichment column with 0.05 mol l^{-1} of pH 10.0 pyrrolidine–phosphoric acid buffer solution as mobile phase. This method can be applied to the determination (g l^{-1}) level of lead, cadmium and mercury in drinking water (Hu, Yang, Yin & Yao, 2002).

By Yang et al., heavy-metal ions in tobacco and tobacco additive were determined by microwave digestion and reversed-phase high-performance liquid chromatography (RP-HPLC). The lead, cadmium, mercury, nickel, copper, and tin ions in the digested samples were precolumn derivated with tetra-(4- aminophenyl)-porphyrin (T_4-APP) to form color chelates; the Hg-T_4-APP, Cd-T_4-APP, Pb-T_4-APP, Ni-T_4-APP, Cu-T_4-APP and Sn-T_4-APP chelates were then enriched by solid-phase extraction with C_{18} disks and the retained chelates were eluted from the disks using tetrahydrofuran. The method was applied to the determination of lead, cadmium, mercury, nickel, copper, and tin in tobacco and tobacco additive (Yang, Li, Shi & Wang, 2005).

Porphyrins have been used for metal determination by the techniques of AAS, spectrophotometry and spectrofluorometry.

By Pyrzyn'ska and Wierzbicki, Amberlite IRA-904 resin modified with tetrakis (*p*-carboxyphenyl) porphyrin (TCPP) was used to pre-concentrate vanadium species. Several parameters, such as sorption capacity of the chelating resin, pH for retention of V(IV) and V(V), volume of sample and eluent, were evaluated. Both vanadium species sorbed on TCPP-modified resin were eluted by use of 2 M nitric acid and determined by atomic absorption spectrometry.The recovery values were >94% and pre-concentration factor of 110 was obtained.The proposed method was examined for reference standard material and river water sample (Pyrzyn'ska & Wierzbicki, 2004).

In 2003, the reaction of 5,10,15,20-tetrakis(4-carboxylphenyl)porphyrin with Cd(II), Pb(II), Hg(II) and Zn(II) was studied spectrophotometrically and kinetics, equilibrium constants as well as photodecomposition of complexes were determined

by Kilian and Pyrzyn'ska.. The detection limit for the recommended procedure was $1.4x10^{-9}$ M (0.9 ng ml^{-1}) and precision in range 20-100 ng ml^{-1} not exceeds 2.7% RSD (Kilian & Pyrzyn'ska, 2003).

In 2005, an indirect substitution spectrophotometric methodology using porphyrin has been developed for the determination of some metals that do not react with porphyrin directly by Itoh et al. The method is concerned with a multistep reaction system, which consists of 3 complexation reactions that occur in a sequence of EDTA with metal, EDTA with Cu(II) and a cationic porphyrin, *meso*-tetrakis (4-*N*-trimethylaminophenyl)-porphine (ttmapp),with Cu(II) (Itoh, Liu & Komata, 2005).

By Dargiewicz-Nowicka et al., methyl-pyridyl porphyrin and its europium complex were prepared in the monolith gels by sol-gel method. The samples doped with the porphyrins were prepared by tetraethoxysilane hydrolysis and condensation. Their absorption and emission spectroscopic properties in comparison with the spectra of the same compounds in various solvents were investigated. It was seen that fluorescent properties of the europium in sol-gel matrices maked this complex useful for some special applications as sensing of molecular oxygen or biomolecules (Dargiewicz-Nowicka, Makarska, Villegas, Legendziewicz & Radzki, 2004).

By Guo et al., a new optical sensor membrane was developed for sensing Hg^{2+} in aqueous solution by using 5,10,15,20-tetraphenylporphyrin as a lipophilized indicator, which was dissolved in an organically modified sol-gel (Guo, Zhang, Xie, Lin & Chen, 2006).

By Delmarre et al., the influence of different solvents and of the pH on the spectroscopic properties of Sn(IV)tetrapyridyl and tetramethylpyridinium porphyrins were studied. Then these porphyrins were immobilized in sol-gel matrices. The spectroscopic properties of the immobilized porphyrins are dependent on the composition of the matrix and on its polarity (Delmarre, Lemarinier & Bied-Charreton, 1999).

1.6 Objective of This Work

In trace metal analysis spectrometric techniques such as flame AAS, graphite furnace AAS, ICP-OES and ICP-MS have been commonly used. Besides spectrometric techniques, metal analysis has been carried out using chromatographic techniques. Separation and determination steps are performed together with this technique. Thin layer chromatography is commonly used technique and has high resolution and has been used in many different industrial areas. High performance thin layer chromatography (HPTLC) is generally not time consuming and not required expensive sample pre-treatment. In contrary, it is simple, economic and fast technique. Depending on these reasons, in this study quantitative analysis of metals was achieved by densitometric thin layer chromatography. In proposed work, quantitative separation of metalloporphyrin was achieved by densitometric TLC process. To achieve this goal we aimed at :

1. Synthesis of chelating agent 5,10,15,20-tetrakis(bromohydroxyphenyl) porphyrin (TBHPP) and verification of its formation using UV, FTIR, ^{1}H NMR and fluoresence spectrophotometric analysis,
2. Complexation of Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(II), Pd(II) ions with TBHPP and verification of their formation using uv-vis and fluoresence spectrophotometric analysis,
3. Passing of metalloporphyrins through C18 cartridge and preconcentrate Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(II), Pd(II).
4. Optimization for HPTLC.
5. Determination the linear range and plotting calibration graphs for metalloporphyrins.
6. Controlling of precision with between-day and within-day analysis applying the proposed method to determine the amounts of metals in various samples.

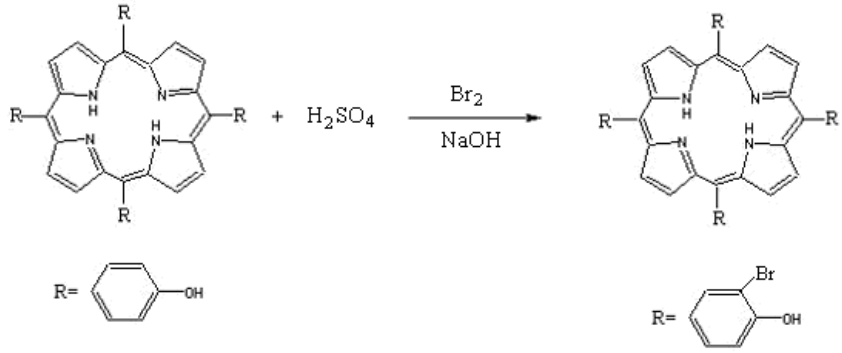
CHAPTER TWO

MATERIALS AND METHODS

2.1 Synthesis of 5,10,15,20-tetrakis(bromohydroxyphenyl) porphyrin (TBHPP) and Metal Chelates

2.1.1 Synthesis of 5,10,15,20-tetrakis(bromohydroxyphenyl) porphyrin (TBHPP)

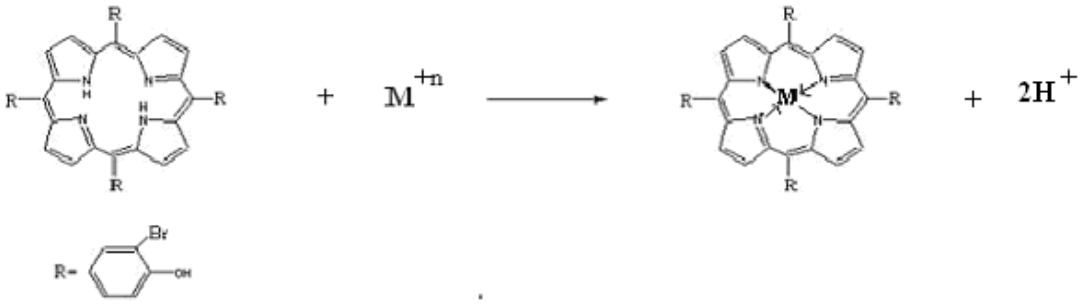
0.170 g tetrahydroxyphenyl porphyrin was mixed with 1.2 mL concentrated H_2SO_4 and 150 mL of tetrahydrofuran (THF) in a round-bottomed flask and refluxed for 3 h at 70^0 C. The reaction mixture was left to cool, and 5 mL of 0.6 mol L^{-1} NaOH solution and 250 µL of bromine were added drop by drop. The mixture was refluxed for 1 h at 70^0 C and after left to cool, 1.0 mol L^{-1} 5 mL H_2SO_4 was also added and mixed for 30 min. The reaction mixture was extracted by diethyl ether and THF in upper phase was evaporated by rotary evaporator. After 10 mL of distilled water was added, the formed residue was filtered off and finally dried under vacuum. Synthesis of TBHPP is shown with chemical equition below (Scheme 2.1).



Scheme 2.1 Preparation of TBHPP

2.1.2 Preparation of Metal Chelates

Metal ion solutions were prepared in ultra-pure water using nitrate salts of Zn((II), Ni(II), Cu(II), Co(II), Hg(II), Pb(II), Pd(II), Mn(II), Pt(IV). $1x10^{-3}$ mol L^{-1} TBHPP was prepared in ethyl alcohol.



Scheme 2.2 Preparation of metal chelates

2.2 Characterization of TBHPP and Metal Chelates

To determine the chemical properties of the TBHPP and metal complexes, FTIR, ^{1}H NMR, UV and fluoresence spectrophotometer have been used.

2.2.1 FTIR Measurements

FTIR spectra were recorded with Perkin Elmer Spectrum (Minnesota, MN, USA) BX Fourier Transform IR spectrometer using KBr discs in the range 4000-700 cm^{-1}. 1 mg of sample was intimately mixed with about 100 mg of dried potassium bromide powder (Merck) using an agate mortar. The mixture was then pressed into pellets under pressure. The pellet was investigated by FTIR.

2.2.2 ^{1}H NMR Measurements

^{1}H NMR spectra were recorded on Bruker instruments (Billerica, MA, USA) operating at 400 MHz using tetramethylsilane as internal standard.~ 0.1 g of sample was dissolved in chloroform with deuterium and this solution was taken in NMR tube and then was located in NMR device. ^{1}H NMR spectra obtained was evaluated.

2.2.3 UV Measurements

Absorbance measurements were recorded on a Shimadzu uv-vis spectrophotometer, equipped with deuterium and tungsten lamp sources and 1.0 cm quartz cell.

2.2.4 Fluoresence Measurements

Fluorescence measurements were recorded on a Varian Cary Eclipse Fluoresence Spectrophotometer, equipped with a xenon lamp source and 1.0 cm quartz cell. Slit widths of both monochromators were set at 10 nm.

2.3 Optimization of HPTLC Technique for Separation

2.3.1 Instrumentation

Samples were spotted on the plates using Linomat V sample applicator. 10 x 10 cm horizontal Camag TLC tank and 10 x 10 cm or 20 x 10 cm The CAMAG twin trough chamber were used for development. The migration of the compounds was measured with a Camag TLC Scanner 3 (Figure 2.1, 2.2, 2.3).

Figure 2.1 Linomat V sample applicator

Figure 2.2 Horizontal developing chamber and twin trough chamber

Figure 2.3 Camag TLC Scanner 3

2.3.2 Procedure of HPTLC on R_F Determination

HPTLC was performed on commercially available precoated silica gel $60F_{254}$ and $RP18F_{254}$ glass plates from Merck (Darmstadt, Germany). Before use silica gel plates were activated at 110^0C for 30 min and then cooled in a desiccator. RP18 plates were used without preliminary treatment. On R_F determination of ligand, standard solutions of TBHPP in ethanol were prepared as 1 mg mL^{-1}. For qualitative analysis, 1 µL of samples were applied by Linomat V semi automatic sample applicator. Chromatograms were developed to a distance of 5 cm with single, binary or ternary mobile phases (Table 2.1) at room temperature (22-25^0 C) in a 10 x 10 cm horizontal Camag TLC tank. After development the plates were dried at room temperature. Each experiment was repeated three times. The migration of the test

compounds was measured with a Camag TLC Scanner III at 420 nm on silica gel plate and 454 nm on RP18 plate for detection.

2.4 Analysis of Samples

3 mL of $1x10^{-3}$ mol L^{-1} (100 μg mL^{-1}) standard TBHPP solution was transferred to 25 mL of volumetric flask. Then, 5 ml of pH 10 buffer solution (pyrrolidine-acetic acid) and 15 ml of 1 μg mL^{-1} standart metal solution were added in this volumetric flask. The solution was diluted to 25 mL with ethyl alcohol. The mixture was heated in a boiling water bath for 20-30 minutes. The prepared metal chelate solutions were passed through 200 mg C18 cartridge. Finally, the retained metal complex was eluted with 2.5 mL of THF. The ratio of metal ion solution to ligand solution, in volume, was 20:1; this results in a solution where metal ion concentration is 0.6 μg mL^{-1} and ligand concentration is 12 μg mL^{-1}, this molar ratio is in excess for the ligand.

1 μl volumes of all samples were spotted on the plates and were dried. Then, prepared metal porphyrin chelates standard solutions at 6.0 ng μL^{-1} were added to all samples as 0.8 μL.

Table 2.1 Studied mobile phases for R_F determination of TBHPP and metal-TBHPP

	TBHPP	**Metal-TBHPP**
Stat. phase: silica gel $60F_{254}$ plate		
single mobile phases	chloroform, benzene, acetone , n-hexane, dichlorometane, methanol, acetonitrile, carbontetrachloride	
binary mobile phases	carbontetrachloride:chloroform chloroform:n-hexane acetone:chloroform methanol:chloroform methanol:tetrahydrofuran ethylacetate:n-Hexane acetonitrile- chloroform	acetone:chloroform methanol-chloroform ethylacetate:n-Hexane carbontetrachloride: chloroform
ternary mobile phase	dichlorometane:chloroform: n-hexane acetone:acetonitrile: benzene	
Stat. phase: RP18 plate		
single mobile phases	Chloroformacetone, methanol, tetrahydrofuran, water	acetone,
binary mobile phases	methanol:tetrahydrofuran methanol:acetone methanol:chloroform ethanol:water	Methanol:tetrahydrofuran methanol:acetone methanol:chloroform

2.5 Preparation of Samples

2.5.1 Tomato Samples

Dried and homogenized tomatoes sample was purchased from the local market. Tomatoes sample was stored in darkness at +4 $^{\circ}$C.

0.25 g of the sample was treated a PTFE beaker with 2 mL of concentrated nitric acid and the mixture was heated until dryness. This procedure is made until using 10 mL of concentrated nitric acid to achieve complete solubility (Gundersen, McCall & Bechmann, 2001). The volume was made up to 50 mL with distilled water. This solution was used by applying procedure given 2. 4.

2.5.2 Water Samples

Tap water from our research laboratory, geothermal waters (Balçova) and river waters (Arda and Tuna river) were used for this study. All water samples were filtered through from 0.45 μm filter paper (Millipore Millex-HV, Hydrophilic PVDF) and were used by applying procedure given 2. 4.

2.6 Method Validation

The analytical methods were validated according to the quideliness of IUPAC (IUPAC, 2002). Linearity, precision, and limit of detection and quantitiation were determined using solutions of each metal complex in ethanol. Concentration ranges for calibration solutions of each metal at working wavelengths were selected with respect to linear range of signal versus concentration of each elements. For calibration, stock solutions containing 6 μg mL^{-1} for each metal were prepared by procedure given 2.4 and increasing volume of standard metal complex solution from 0.1 to 10 μL was applied on plate.

The method precision was assessed by consecutively analyzing three replicates of metal complex solutions containing 6 ng μL^{-1} and was expressed in terms of percent relative Standard deviation(RSD%). The within and between day variation fort he determination of metals was carried at three different concentration levels of 6 and 12 ng μL^{-1}

The limit of detection (LOD) (3 s) was calculated based on the Standard deviation of the response and the slope of the calibration curve at low calibration levels. The LOD was calculated as follows:

$$LOD = k \times (\overline{s_{bl}} / m)$$

Where:

$\overline{s_{bl}}$: The mean standard deviation of the response based on the standard deviation of y-intercept of regression line,

k: The constant which is 3,

m: The slope of the calibration curve.

The limit of quantitation (LOQ) was calculated using the calibration curves with the Formula;

$$LOQ = = t \times (\overline{s_{bl}} / m)$$

Where:

$\overline{s_{bl}}$: The mean standard deviation of the response based on the standard deviation of y-intercept of regression line,

t: The constant which is 10,

m: The slope of the calibration curve.

CHAPTER THREE

RESULTS AND DISCUSSIONS

3.1 Characterization of TBHPP and Metal Chelates

3.1.1 FTIR Measurements

FTIR spectrum of TBHPP was compared with FTIR spectrum of tetrahydroxyphenyl porphyrin. In the FTIR spectrum of TBHPP, C-Br stretching vibration at 897 cm^{-1} in the TBHPP was absent in the THPP. The C-OH stretching vibration at 3125 cm^{-1} was observed in the TBHPP. Thus, FTIR spectrum of TBHPP is different from FTIR spectrum of tetrahydroxyphenyl porphyrin. In the metal complexes, N-H band was disappeared. Also, the metal complexes exhibit small shifts to the red region. (Figure 3.1)

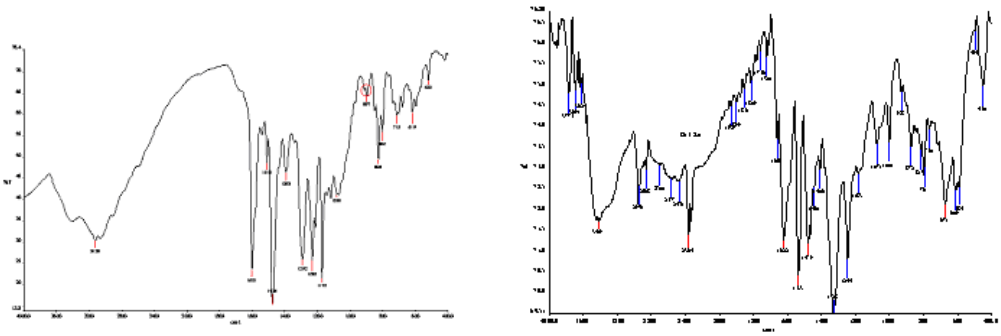


Figure 3.1 FTIR spectrum of TBHPP and Zn-TBHPP

3.1.2 ^{1}H NMR Measurements

For the ^{1}H NMR spectrum of TBHPP ($CDCl_3$, 400 MHz); δ (ppm): 9.88 ppm (s, 4H), 7.55 ppm (s, 2H), 7.05-6.70 ppm (m, 20H). The ^{1}H NMR spectrum of TBHPP was given in Figure 3.2.

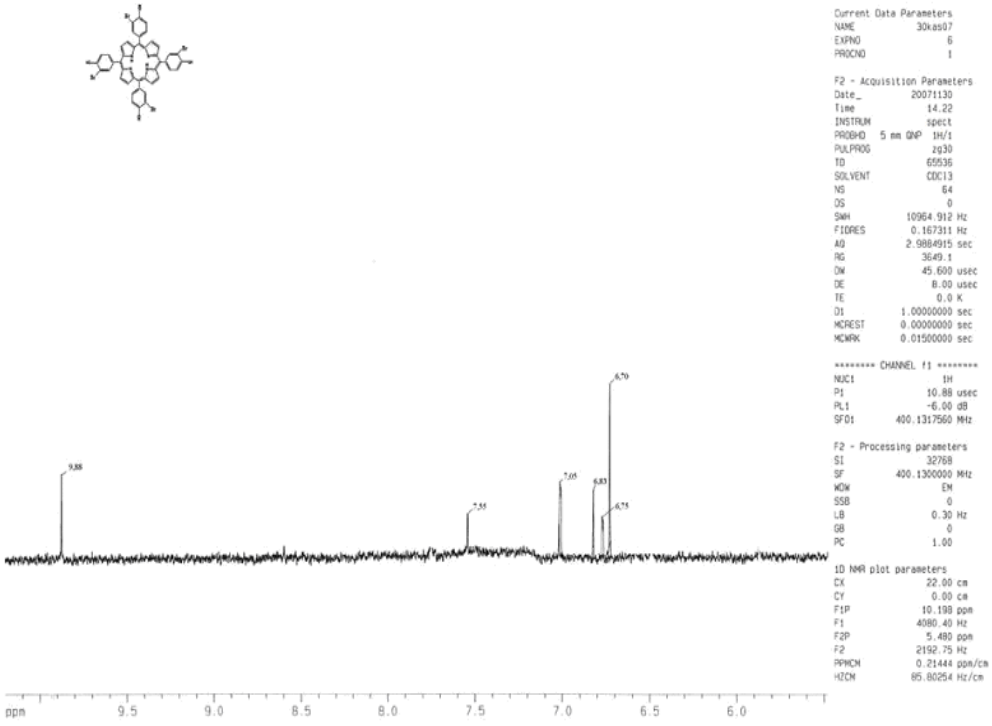


Figure 3.2 ^{1}H NMR spectrum of TBHPP

3.1.3 UV Measurements

A characteristic UV-spectrum of TBHPP is given Figure 3.3. TBHPP has a strong absorption band at 419 nm.

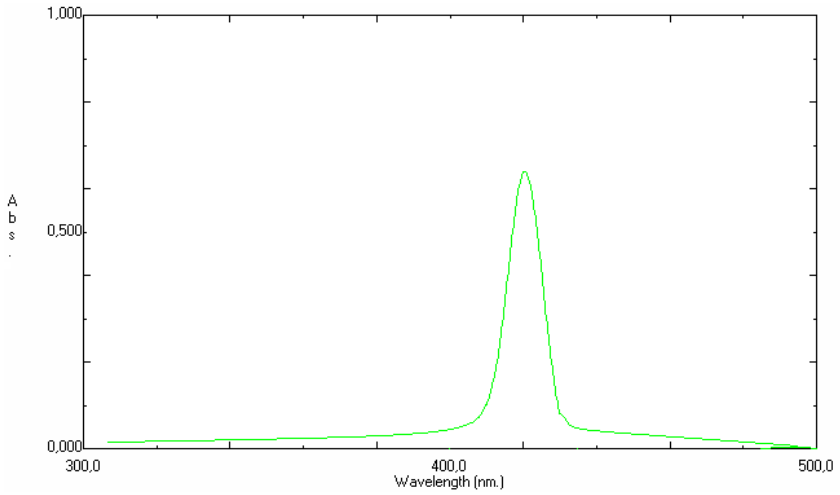


Figure 3.3 Absorbance spectrum of TBHPP in ethanol. λ_{max} =419 nm

The absorption bands of metal chelates have been observed around 415- 425 nm in ethanol.

3.1.4 Fluoresence Measurements

In order to determine the optimum wavelength, the emission spectrum of TBHPP in ethanol was taken and was shown in Figure 3.4. Owing to the conjugated double bond system and the high mobility of its π-electrons, The TBHPP exhibits fluorescence emission at 659 nm when excited by the radiation of 559 nm.

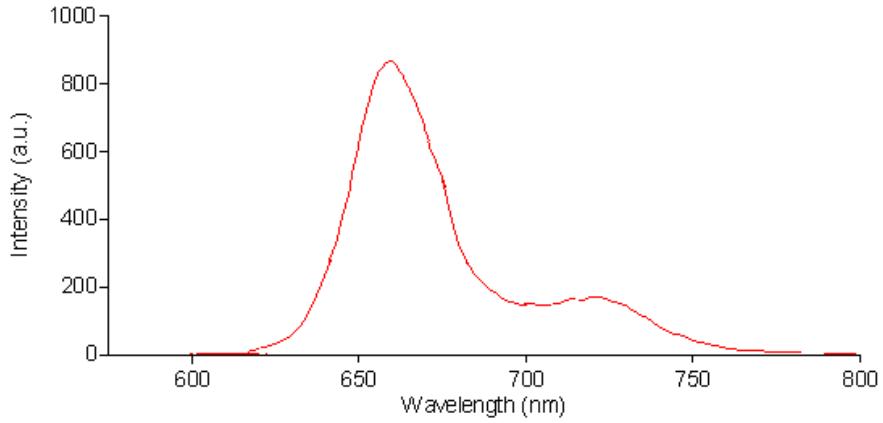


Figure 3.4 Emission spectrum of TBHPP in ethanol $\lambda_{ex:}$ 559nm, $\lambda_{em:}$ 659 nm

The emission spectra of metal chelates were taken and were shown in Figure 3.5. According to Figure 3.5, when the atom radii of the metal ions decrease, the nitrogen atoms in porphyrin ring are bound more strongly with the metal ions. The formation of complex of TBHPP with metal ions caused static fluorescence quenching in the pH 10. It could be explained that, metal ions had effect of invertible charge transfer or electron spin-orbit coupling, which accelerated the singlet-triplet transition and internal conversion deactivation of excited TBHPP. The fluorescence intensity of TBHPP was changed

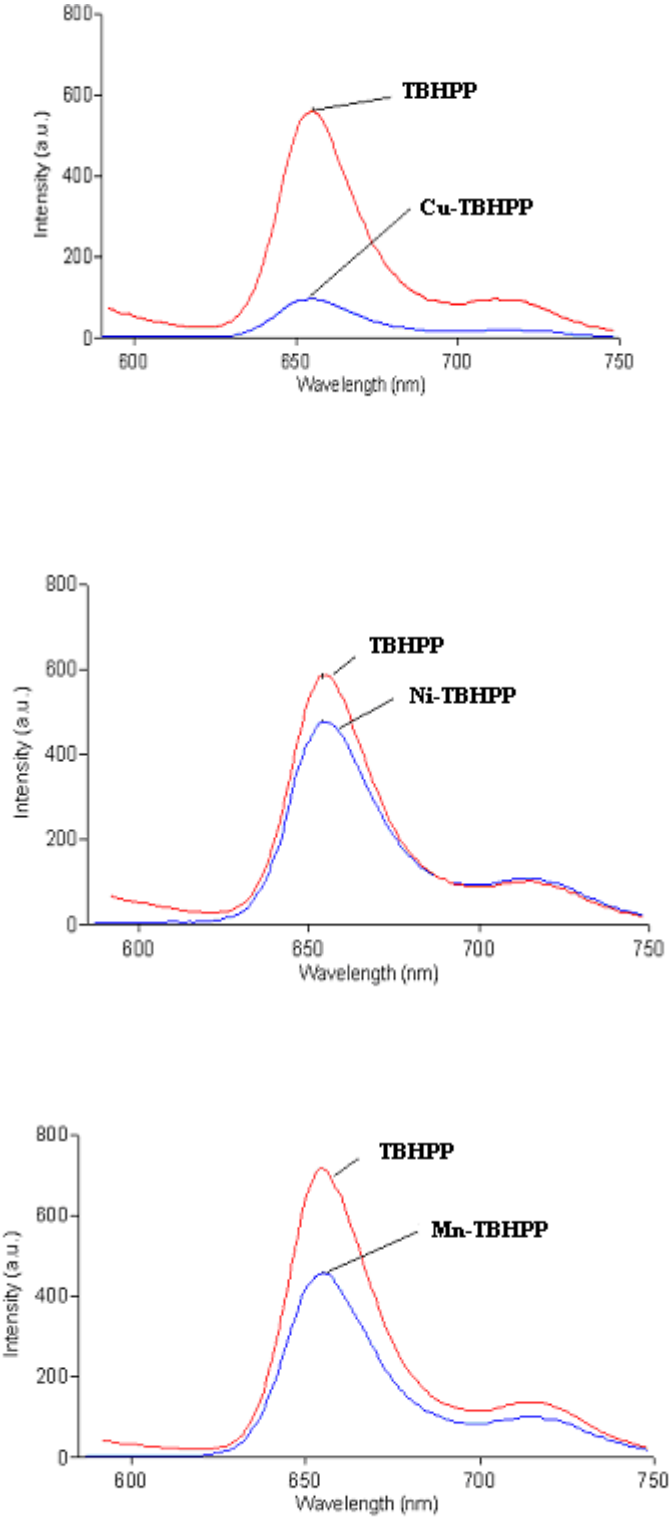


Figure 3.5 Emission spectra of metal-TBrHPP; a) Cu(II), b) Ni(II), c) Mn(II), d) Co(II), e) Zn(II), f) Cd(II), g) Hg(II), h) Pb(II), ı) Pt(IV), j) Pd(II) c_{metal} = 5 µg mL^{-1}, c_{TBHPP}= $2x10^{-6}$ mol L^{-1}

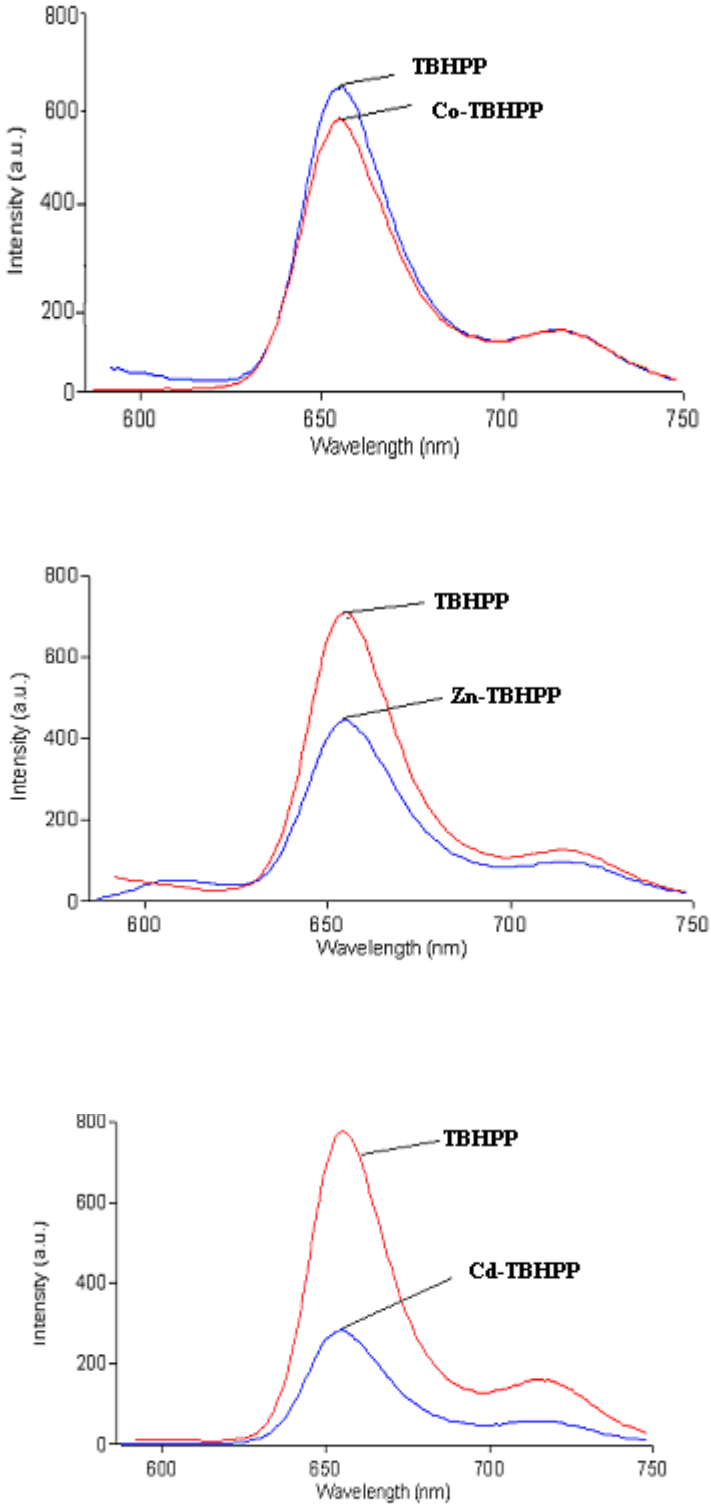
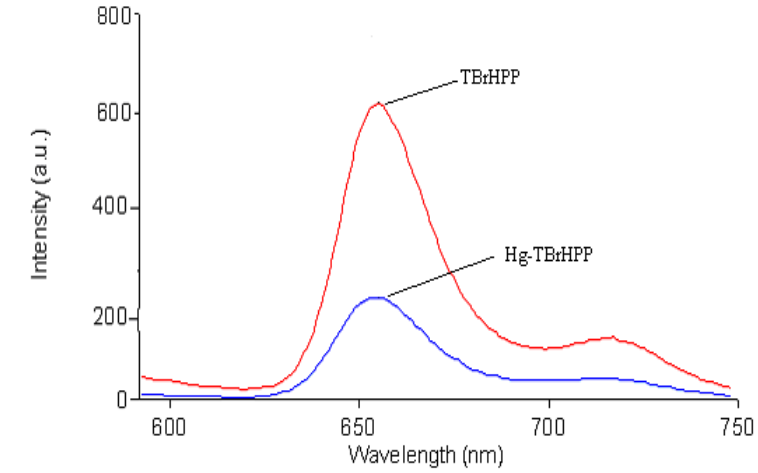
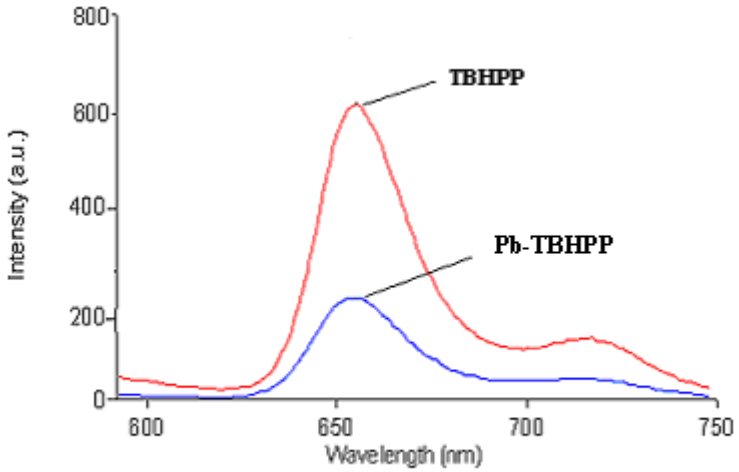


Figure 3.5 Emission spectra of metal-TBrHPP; a) Cu(II), b) Ni(II), c) Mn(II), d) Co(II), e) Zn(II), f) Cd(II),g) Hg(II), h) Pb(II), ı) Pt(IV), j) Pd(II)c_{metal} = 5 µg mL^{-1}, c_{TBHPP}= $2x10^{-6}$ mol L^{-1} (cont'd)





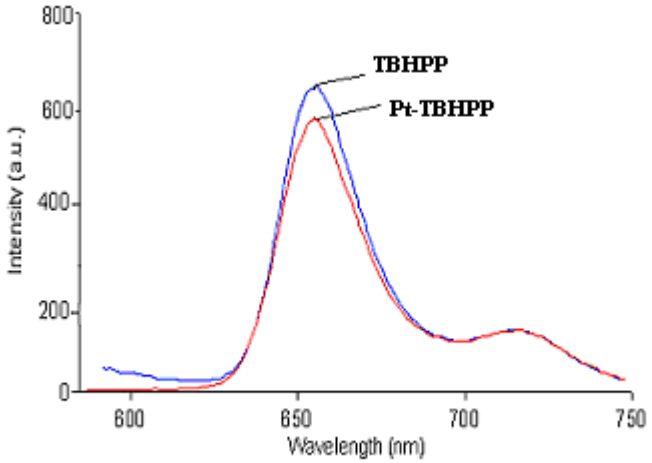


Figure 3.5 Emission spectra of metal-TBrHPP; a) Cu(II), b) Ni(II), c) Mn(II), d) Co(II), e) Zn(II), f) Cd(II), g) Hg(II), h) Pb(II), ı) Pt(IV), j) Pd(II) c_{metal} = 5 µg mL^{-1}, c_{TBHPP}= 2x10^{-6} mol L^{-1} (cont'd)

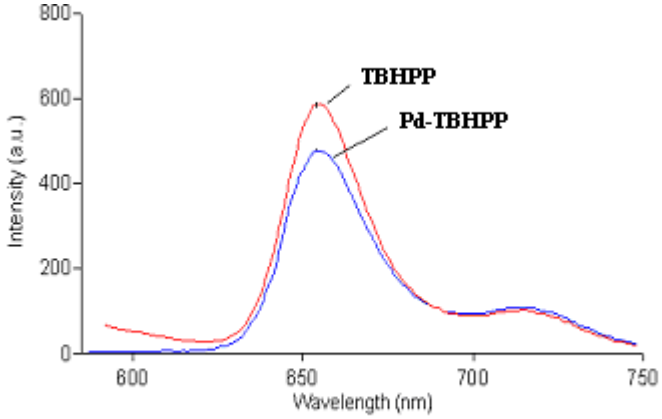


Figure 3.5 Emission spectra of metal-TBrHPP; a) Cu(II), b) Ni(II), c) Mn(II), d) Co(II), e) Zn(II), f) Cd(II), g) Hg(II), h) Pb(II), ı) Pt(IV), j) Pd(II) c_{metal} = 5 µg mL^{-1}, c_{TBHPP}= $2x10^{-6}$ mol L^{-1} (cont'd)

3.2 Optimizated Parameters for TBHPP and its Metal Complexes in HPTLC

3.2.1 Solvent Dependence of the Mobility of TBHPP

TBHPP was chromatographed with silica gel and RP18 stationary phases and several single, binary or ternary mobile phases (Table 3.1-3.6). On a high performance thin layer plates porphyrin gave a compact spot without tailing on the chromatogram.

The halogen in substituent group form H-bonds with the surface of the stationary phase. Polar interactions like hydrogen-bonding take place between hydrogen of the silica and the halogen in the substituent group, and also between the oxygen atoms of the sorbent and the 'inner' hydrogen atoms of the porphyrin ring. However, the second interaction should be weakened by 'projecting' phenyl groups which separate the porphyrin plane from the sorbent surface (Kus, 1996).

Table 3.1 The hR_F values of TBHPP on silica gel on variou single solvents.

Mobile phase	Polarity index	hR_F*
n-hexane	0	0
carbontetrachloride	1.7	0
benzene	3.0	0
dichlorometane	3.4	0
chloroform	3.8	0
acetone	5,4	91±3
acetonitrile	6.2	94±2
methanol	6.6	98±3

* $hR_F = R_F \times 100$

Table 3.2 The hR_F values of TBHPP on silica gel on various binary solvents

Mobile phase	Polarity index	hR_F
n-hekzan:chloroform (1:1)	1.9	0
carbontetrachloride:chloroform (4:6)	2.96	0
ethylacetate: n-hexane (1:1)	2.15	22±3
acetonitrile:chloroform (2:8)	4.28	28±2
acetonitrile:chloroform (3:7)	4.52	46±2
acetone:chloroform (1:1)	4.6	62±3
acetonitrile:chloroform (4:6)	4.76	78±1
methanol: tetrahydrofuran (1.1)	5.4	88±4
methanol:chloroform (1:9)	4.08	42±2
methanol:chloroform (2:8)	4.36	68±2
methanol:chloroform (3:7)	4.64	90±3
acetonitrile:benzene (3:7)	3.96	54±3

Table 3.3 The hR_F values of TBHPP on silica gel on various ternary solvents

Mobile phase	Polarity index	hR_F
dichlorometane: chloroform: n-hexane (1:1:3)	1.38	89±2
dichlorometane: chloroform: n-Hexane (2:1:2)	2.12	91±1
acetone: acetonitrile: benzene (1:1:8)	3.56	28±3

Table 3.4 The hR_F values of TBHPP on silica gel as a function of the composition of the acetone:chloroform binary mobile phases

Mobile Phases	Polarity index	hR_F
acetone:chloroform (0:10)	3.8	9±1
acetone:chloroform (1:9)	3.96	18±2
acetone-chloroform (2:8)	4.12	35±3
acetone:chloroform (3:7)	4.28	52±3
acetone:chloroform (4:6)	4.44	53±1
acetone:chloroform (1:1)	4.6	62±3
acetone:chloroform (6:4)	4.76	75±3
acetone:chloroform (7:3)	4.92	85±4
acetone:chloroform (8:2)	5.08	85±2
acetone:chloroform (9:1)	5.2	94±2
acetone:chloroform (10:0)	5.4	98±1

The migration rate of TBHPP has moderate mobilities in polar mobile phases. This compound is strongly adsorbed on silica gel for non-polar mobile phases since it has halogen atom in its substituent groups. But, this interaction is weakened by using polar mobile phases. The mobilities of TBHPP is higher because of their higher solubilities in polar mobile phases. The mobilities of TBHPP on RP18 stationary phase is high because of its higher solubilities in polar mobile phases. TBHPP was traveled up to nearly the solvent front at all the mobile phases.

Table 3.5 The hRF values of TBHPP on RP 18 plate on single solvent

Mobile phase	Polarity index	hR_F
acetone	5.4	66±2
chloroform	3.8	94±3
tetrahydrofuran	4.2	95±2
methanol	6.6	96±2
water	9.0	85±1

Table 3.6 The hRF values of TBHPP on RP 18 plate on binary solvent

Mobile phase	Polarity index	hR_F
methanol: tetrahydrofuran (5:95)	4.32	92±2
methanol: acetone (2:8)	5.64	84±3
methanol: chloroform (4:6)	4.92	96±3
methanol: chloroform (2:8)	4.36	98±2
ethanol: water (9:1)	5.58	99±1

3.2.2 Solvent Dependence of the Mobility of Metal-TBHPP

The chromatographic behaviours of metal complexes of the TBHPP were also investigated and the hR_F values of these metal complexes were summarized in Table 3.7 and 3.8 for the best binary mobile phases on silica gel and RP18 plate. In adsorption chromatography on silica gel, the optimum separations of TBHPP and its Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(IV), Pd(II) complexes were obtained with acetone:chloroform (2:8, v:v). The metal complexes showed generally higher hR_F values. This means that these metal complexes are less polar than their ligand. The reason for higher mobilities of metalloporphyrins could be decrease in dipole moment and also disappearance of polar NH bond. Cu(II)-TBHPP in acetone:chloroform (2:8) mobile phase has the highest hR_F value. However, the complexes of Zn(II) had lower mobility than the porphyrin. This can be explained by its lower electrostatic field strength than the other studied ions as mentioned by Kobayashi and coworkers before. When the positive charge of metal cation is more dispersed over the π-electron system of the porphyrin derivatives and then the metal-ligand bond is more delocalized, the dipole-dipole interaction between the metal-porphyrin complex and polar stationary phase is reduced, and so larger mobility may be observed (Kobayashi, Saitoh & Suzuki, 1984).

Unfortunately, on RP18 plates, the separation of porphyrin and its metal complexes was not accomplished. The all studied metal complexes with the different composition of mobile phases traveled up to nearly the solvent front.

The migration distance of Zn(II), Co(II), Cu(II) and Hg(II)-TBHPP complexes in aceton:chloroform (8:2) on HPTLC siilca gel plate are different at acceptable degree as shown the chromatograms of the mixture of these metal complexes in Figure 3.6. So, the quantitative analysis of these metal ions in certain analytical matrices has been studied. In quantitative analysis of metal ions, the optimum parameters used for TLC of metal-porphyrin chelates were summarized in Table 3.9. Well defined spots were obtained when the chamber was saturated with mobile phase for 30 min at room temperature. The absorption spectra of metal-TBHPP chelates were obtained from the three-dimensional chromatograms as illustrated in Figure 3.7 and 3.8. The maximum absorption wavelengths of Zn-TBHPP, Cu-TBHPP, Hg-TBHPP, Co-TBHPP are found as 427 nm, 421 nm, 418nm and 419 nm, respectively. During analysis, each metal-TBHPP chelate was detected at its maximum absorption wavelength.

Table 3.9 Optimized parameters in HPTLC

Stationary phase	20 x 10 cm HPTLC silica gel $60F_{254}$
Injector	100 µL conic pointed syringe
Sampler	Linomat V semi automatic sampler
Temperature	22-25 °C
Distance from the plate side edge	7 mm
Distance from the bottom of the plate	10 mm
Distance between bands	9.2 mm
Volume of sampling	1 µL
Developing tank	20 x 10 cm twin through chamber
Mobile phase	acetone-chloroform (2:8)
Detection	TLC Scanner III, absorbance-reflection mode
Wavelength	420-430 nm
Length of band x width	6.0 x 0.3 mm
Light source	D_2
Slit dimension	4.0 x 0.3 mm
Scanning rate	20 mm s^{-1}

Table 3.7 The hRF values of Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(IV), Pd(II)-TBHPP on silica gel with binary solvents

Mobile phase	TBHPP	Zn(II)	Ni(II)	Cu(II)	Co(II)	Cd(II)	Hg(II)	Pb(II)	Mn(II)	Pt(IV)	Pd(II)
acetone: chloroform (2:8)	25±3	16±3	25±3	83±3	45±2	25±3	68±4	65±4	28±2	58±1	25±4
methanol: chloroform (1:9)	42±3	42±2	42±2	86±1	41±1	40±2	92±1	39±2	20±2	41±2	42±1
ethyl acetate: n-hexane (1:1)	22±3	16±1	21±3	78±2	15±2	17±1	20±2	19±2	21±1	21±2	21±3
carbontetrachloride: chloroform (1:9)	0	0	0	18±3	0	0	0	0	0	0	0
acetone: chloroform (1:1)	75±2	75±3	75±1	75±3	75±3	75±2	75±3	75±2	75±1	75±2	75±3
methanol: chloroform (3:7)	91±1	89±2	88±1	87±1	88±3	88±3	89±2	90±2	88±2	91±1	90±3

Table 3.8 The hR_F values of Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(IV), Pd(II)-TBHPP on RPl8 plate with solvent

Mobile phase	TBHPP	Zn(II)	Ni(II)	Cu(II)	Co(II)	Cd(II)	Hg(II)	Pb(II)	Mn(II)	Pt(IV)	Pd(II)
methanol: tetrahydrofuran (5:95)	92±1	92±1	93±2	90±2	90±2	90±2	91±1	91±2	89±1	89±2	91±2
acetone	86±2	88±2	87±2	86±2	87±2	86±2	88±2	87±2	91±2	90±2	88±2
methanol: acetone (2:8)	84±1	87±2	85±2	79±3	86±1	83±2	83±3	83±2	85±3	81±2	82±2
methanol: chloroform (4:6)	96±2	93±1	94±3	97±1	97±2	95±1	96±2	97±1	97±3	98±2	95±1

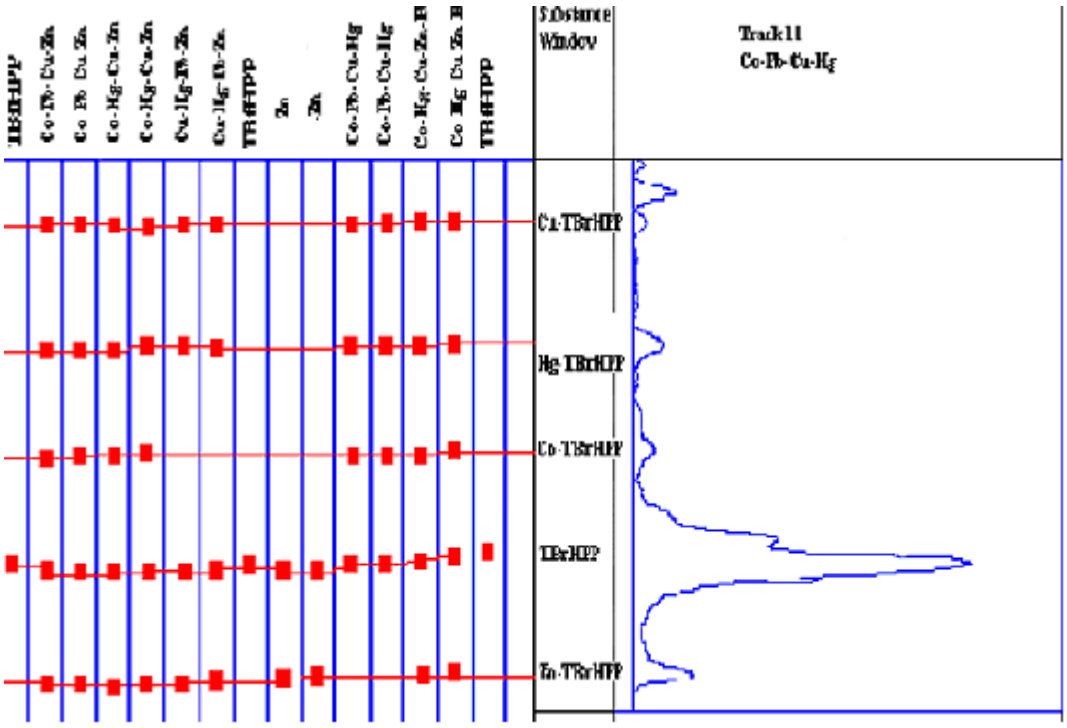


Figure 3.6 The chromatograms of the mixture of TBHPP complexes with Hg (II), Zn (II), Cu (II) and Co (II) in acetone:chloroform (2:8) on HPTLC-silikajel 60F_{254} plate.

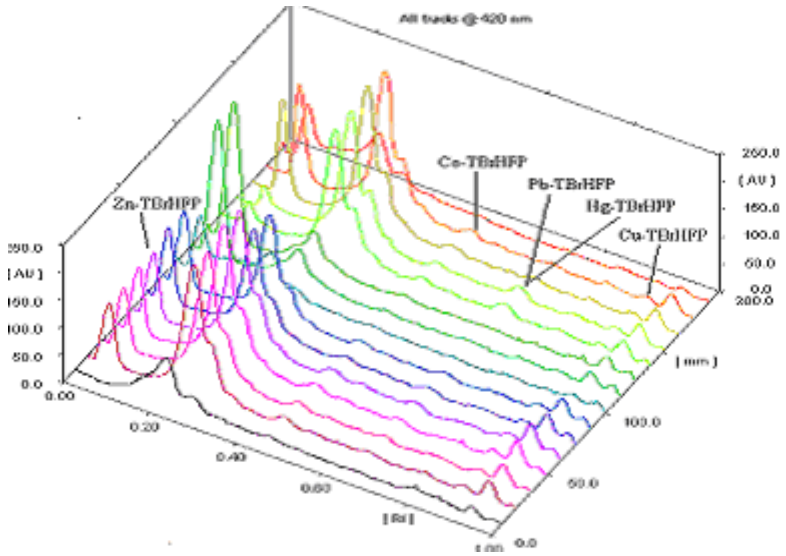


Figure 3.7 The three-dimensional image of TBHPP and metalloporphyrins in acetone:chloroform (2:8) on HPTLC-silikajel 60F_{254} plate

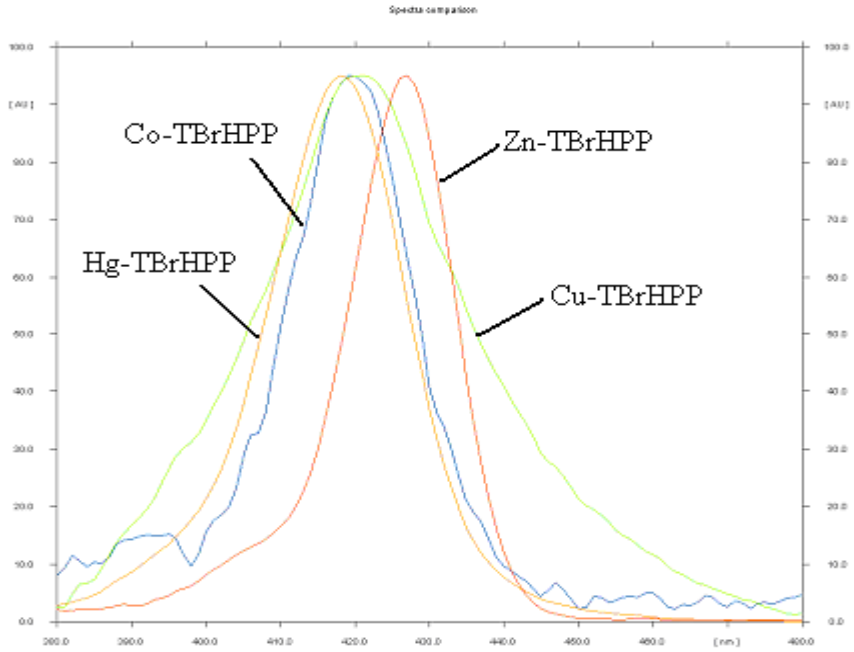


Figure 3.8Absorption spectra of TBHPP complexes in acetone:chloroform(2:8) on HPTLC-silikajel 60F_{254} plate

3.3. Quality of Parameters

The performance of the proposed HPTLC method was evaluted by establishing the analytical parameters. These parameters were determined using standard and sample solutions. The results obtained are tabulated in Table 3.10. Under optimum conditions, the regression equations of the metal-TBHPP complexes were established on the basis of the standard samples injected at their peak areas. As can be seen from Table 3.10, Zn and Hg-TBHPP exhibited same calibration range and are narrower than Co and Cu-TBHPP. All studied metal-TBHPP complexes showed linear relationship over the determined concentration range with correlation coefficients beter than 0.99. The results pointed out a good precision with relative standard deviation below RSD% for the within-day (intermediate) and between-day (repeatability) precision. The LODs and LOQs were estimated from the regression lines. For Co-TBHPP, the lowest value for each one was obtained. For Zn and Hg-TBHPP, these values are found higher.

Table 3.10 Summary of analytical statistical data

	Hg-TBHPP	**Zn-TBHPP**	**Cu-TBHPP**	**Co-TBHPP**
Linear range (ng μL^{-1})	3.6-60	3.6-30	1.2-30	0.6-30
LOD (ng μL^{-1})	0.90	0.92	0.36	0.19
LOQ (ng μL^{-1})	3.01	3.06	1.11	0.54
Repeatibility (RSD%, n=4) (12 ng μL^{-1})	4.51	5.24	3.51	4.10
Intermediate precision (RSD%,n =4) (6 ng μL^{-1})	3.09	3.67	2.66	1.87
Linear regression equation	y=16.114x +43.37	y=20.406x - 25.705	y=42.235x +7.386	y=50.934x +16.52
Regression coefficient (R^2)	0.9908	0.9904	0.9944	0.9961
Retention factor (hR_F)	63	16	83	42

3. 4 Analysis of Samples

As mentioned above, the validated proposed method was used for the analysis of water samples such as tap water, geothermal water and river water and food sample such as dried tomato. The values obtained for the real samples are presented in the Table 3.11. The concentrations of studied metal ions were not determined in most samples due to their low concentrations. The recoveries for the addition of 4.8 ng of Cu(II), Co(II), Zn(II) and Hg(II) were from 99-105%. Analyses were validated by spiking 4.8 ng. There was a good agreement between the added and recovered amount of the sample.

Table 3.11 a) Determination results of Zn(II) and Co(II) in the samples with this method.

Sample	**Zn amount ($ng\ \mu L^{-1}$)**		**Recovery (%)**	**Co amount ($ng\ \mu L^{-1}$)**		**Recovery (%)**
	Added	**Found**		**Added**	**Found**	
Tap water	0	ND*	102	0	ND*	102
	4.8	4.88		4.8	4.91	
Geothermal	0	0.26	101	0	ND*	101
water-1	4.8	5.1		4.8	4.88	
Geothermal	0	ND*	102	0	ND*	100
water-2	4.8	4.1		4.8	4.82	
Arda river water	0	ND*	104	0	0.66	99
	4.8	4.99		4.8	5.43	
Tuna river water	0	ND*	103	0	0.10	100
	4.8	4.7		4.8	4.89	
Tomatoes	0	ND*	104	0	0.65	100
	4.8	4.99		4.8	5.47	

*ND:not detected

Table 3.11 b) Determination results of Hg(II) and Cu(II) in the samples with this method

Sample	**Hg amount ($ng\ \mu L^{-1}$)**		**Recovery (%)**	**Cu amount ($ng\ \mu L^{-1}$)**		**Recovery (%)**
	Added	**Found**		**Added**	**Found**	
Tap water	0	ND*	102	0	0.24	100
	4.8	4.89		4.8	5.04	
Geothermal	0	ND*	101	0	ND*	102
water-1	4.8	4.86		4.8	4.92	
Geothermal	0	ND*	101	0	ND*	100
water-2	4.8	4.86		4.8	4.8	
Arda river water	0	ND*	105	0	0.30	100
	4.8	5.03		4.8	5.1	
Tuna river water	0	ND*	101	0	ND*	104
	4.8	4.85		4.8	4.99	
Tomatoes	0	ND*	103	0	0.33	100
	4.8	4.94		4.8	5.14	

*ND:not detected

CHAPTER FOUR

CONCLUSION

High performance thin layer chromatography with silica gel plates and densitometric detection has been shown to constitute a good approach for the analysis of metal ions in certain analytical matrices.

For this; 5, 10, 15, 20-tetrakis(bromohydroxyphenyl) porphyrin was synhesized and metal chelates wre prepared at optimum pH. By FTIR, ^{1}H NMR, UV and fluoresence spectrophotometric techniques, TBHPP and Zn(II), Ni(II), Cu(II), Co(II), Cd(II), Hg(II), Pb(II), Mn(II), Pt(IV) and Pd(II)-TBHPP chelates were chracterized. Chromatographic behaviours of TBHPP and its metal chelates were investigated in different solvents on silica gel and RP 18 plates. Thus, optimized conditions were determined. According to this, metal chelates and TBHPP had good chromatographic behaviour on silica gel plate with acetone:chloroform (2:8, v:v). TBHPP complexes with Zn(II), Cu(II), Co(II) and Hg(II) had different migration distance from each other. So, these metal chelates were analyzed quantitatively in certain analytical matrices.

In this study, hR_F values of TBHPP complexes with Zn(II), Cu(II), Co(II) and Hg(II) are 16, 83, 42 and 63, respectively. Quality parameters have shown that a good precision and low detection limits can be obtained, providing an easy and rapid procedure for the determination of metal ions.

The proposed HPTLC method is simple, fast and reliable and applicable to the determination of some transition metal ions in water and food samples. By this technique, the number of samples can be analysed on a single HPTLC plate. The proposed method represents a good alternative to save money and time with respect to atomic spectrometric and liquid chromatographic techniques

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