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Design of stable Di and tetraradical nitroxides as magnetic resonance imaging agents

Radha V. Iyer

New Jersey Institute of Technology

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ABSTRACT

Design of Stable Di and Tetraradical Nitroxides as Magnetic Resonance Imaging Agents

by

Radha V. Iyer

Several nitroxides have been prepared in the Biotechnology Laboratory at NJIT to study their image enhancement capability. These nitroxides contain different moieties within one molecule and their paramagnetism is found to be initially increasing proportionally to the number of their radical centers. It is found that the addition of nitroxides at very close distances causes interference and a reduction of intensity due to the interaction of the two paramagnetic centers with each other. Thus MRI contrast agents are not improved by simple addition of nitroxide centers to a given molecule and drug designers must pay special attention to intramolecular spacing. To demonstrate techniques, rigorous controls and more detailed studies are being undertaken.

In this research, methods for the synthesis of multiradical nitroxides by structural modification as well as the synthesis of nitroxides with a functional group in close proximity to the radical center have been developed. Specifically, two methods for synthesis of the multiradical nitroxide [bis-(2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) phthalate] have been tested and their overall effect on the yield evaluated.

The compounds intended for synthesis were bis (2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) phthalate (by two different routes) and tetrakis (2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) pyromellitate focusing on molecular structure and multi paramagnetic centers.

The investigations described here establish the versatility of the disubstitution of phthaloyl chloride under varying ratios of 4-hydroxy-2,2,6,6 tetramethyl 1-oxyl piperidine (TEMPO) and the associated effect on the yield of bis-(2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) phthalate. Optimal reaction requirements for each method have been established. The overall yield of ester formation depends on the amount of 4-hydroxy-2,2,6,6 tetramethyl 1-oxyl piperidine taken. The yield increases two fold with an increase in the amount of 4-hydroxy-2,2,6,6 tetramethyl 1-oxyl piperidine (TEMPO) available. It is found that only the di-substituted product is formed.

In the preparation of tetrakis-(2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) pyromellitate, the pyromellitoyl chloride was further reacted in-situ with 4-hydroxy-2,2,6,6 tetramethyl-1-oxyl-piperidine without isolation.

The identification of these multiradical nitroxides was done using thin layer chromatography, Nuclear Magnetic Resonance spectroscopy, and mass spectrometry. ESR spectroscopy was used to verify the formation of nitroxide free radicals.

DESIGN OF STABLE DI AND TETRARADICAL NITROXIDES AS MAGNETIC RESONANCE IMAGING AGENTS

by
Radha V. Iyer

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APPROVAL PAGE

Design of Stable Di and Tetraradical Nitroxides as Magnetic Resonance Imaging Agents

by

Radha V. Iyer

Dr. Sam S. Sofer, Thesis Adviser
Professor of Chemical Engineering, Chemistry, and Environmental Science, NJIT

Dr. Howard Perlmutter, Committee Member
Professor of Chemical Engineering, Chemistry, and Environmental Science, NJIT

Dr. George Lei, Committee Member
Professor of Chemical Engineering, Chemistry, and Environmental Science, NJIT

BIOGRAPHICAL SKETCH

Author: Radha V. Iyer

Degree: Master of Science in Applied Chemistry

Date: May, 1992

Undergraduate and Graduate Education:

- Master of Science in Applied Chemistry, New Jersey Institute of Technology, Newark, NJ, 1992
- Master of Science in Organic Chemistry, M.S. University, Baroda, Gujarat, India, 1988
- Bachelor of Science in Chemistry, M.S. University, Baroda, Gujarat, India, 1986

Major: Applied Chemistry

Professional Position Held:

January 1991 - May 1992: Research Assistant at the Biotechnology Laboratory of the New Jersey Institute of Technology, Newark, New Jersey

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CHAPTER I
INTRODUCTION

1.1 Background

A great deal of study has been done on stable free radical nitroxides. Nitroxides without alpha hydrogens are probably the most stable known free radicals. Polynitroxides, also known as multiradical nitroxides, are well known diagnostic drugs for nuclear magnetic imaging. These multiradical nitroxides have potential applications as spin labels, paramagnetic substances and contrast agents. The toxicity appears to be less with polynitroxides as compared to that of mononitroxides.

Brik, Pechine *et al* [1] studied the influence of contrast media on the NMR signal. They found that the NMR signal depends on the concentration of the injected paramagnetic nitroxides. A magnetic resonance imaging was done on some of the polynitroxide compounds synthesized. They found that their relaxant effects on water were much higher than those of mononitroxides. On injection of the reduced doses of polynitroxides; interesting contrast for lungs and liver was obtained with diminished toxicological risks.

Polynitroxides are assemblies of molecules or polymeric molecules which incorporate multiple nitroxyl groups. These nitroxides do not serve as delivery systems for other contrast agents but act as contrast agents themselves.

The compounds prepared are stable bi- and tetra radical nitroxides developed as contrast agents for magnetic resonance imaging and as paramagnetic agents for imaging studies by electron spin resonance spectroscopy.

1.2 Magnetic Resonance Imaging

Magnetic Resonance Imaging is a powerful non-invasive medical diagnostic tool, that is currently undergoing rapid development. MRI can reveal the distribution of the internal structure of the body by generating images without the use of X-rays. This can be used to discriminate between the healthy and the diseased tissues. MRI techniques were used to monitor metabolic reactions in animals and human beings. This is now used as a diagnostic tool by presenting information in pictorial form.

Keana *et al* [2] have reported that polynitroxides are agents that selectively enhance the contrast among various tissues, organs and fluids or of lesions within the body. Active investigation on the polynitroxides as contrast enhancing agents is done due to their paramagnetic nature. In order to achieve significant enhancement in a given target area, it is necessary to introduce many paramagnetic centers at the site. Thus if the nitroxides could be concentrated in certain areas of the body, we can get a good contrast.

The inherent MRI contrast between tissues generally depends on physical characteristics such as viscosity & temperature and chemical characteristics such as hydrogen concentration and local environment. The NMR contrast is obtained due to the difference in the intensity of nuclear resonance signals from various tissues of the body.

1.3 Contrast Enhancers

MRI contrast agents are analogous to iodinated contrast media used in radiography. For example, MRI contrast agents excreted in the urine provide useful information about renal function. A radiological review by the University of California, School of Medicine [3], reports that the use of stable nitroxides as

contrast enhancers help in enhancing the differences in signals observed between magnetically similar tissues. These may produce the best contrast that give the most diagnostic information. These nitroxides alter the local magnetic environments. Such MRI contrast agents are not observed directly on the images; rather, their magnetic effects on neighboring hydrogen nucleus are the means of contrast enhancement. That is, these paramagnetic molecules enhance the proton relaxation of a nearby hydrogen nucleus. The proton relaxation is very weak in nuclear paramagnetic substances and no substantial contrast is observed. Therefore, paramagnetic substances having unpaired electrons should be used for the proton relaxation enhancement (PRE).

Some tissues have similar MRI characteristics (just as some tissues absorb X-rays equally) and hence free radical polynitroxides can be used as MRI contrast enhancers for tumors that cannot be identified without contrast enhancement.

Bydder, Steiner, Young *et al.* [4] have reported a potential clinical application of free radical polynitroxides as MRI contrast enhancers in the identification of an abnormal tissue surrounded by normal tissues of similar MRI characteristics. An ideal contrast enhancer is the one which is reproducible, chemically versatile, stable, easily stored and available for immediate administration.

1.4 Spin Labels

Free radicals are paramagnetic in nature and have an unpaired electron in the outermost molecular orbital. These unpaired electrons are readily available for electron pairing with reducing agents (electron donors). Benson , Maienthal *et al* [5,6] have reported a class of free radicals that are protected from pairing called

the stable free radicals. The free radicals that persist in biological systems for several hours or longer without electron pairing are not 'Spin Labels'. The term 'Spin Label' is used for those stable free radicals that have been used as probes in the study of biochemical systems. Spin labels are chemically versatile and can be covalently attached to specific groups of biomolecules. The magnetic effects of these 'probes' have been studied *in-vitro* by electron spin resonance spectroscopy. Also, the proton relaxation effect of spin labels on the relaxation values of neighboring hydrogen nuclei can be observed *in-vitro* by NMR spectroscopy.

1.5 Nitroxide Stable Free Radicals (NSFR)

The polynitroxides constitute the above "Stable free radicals" (SFR) class of compounds. These are called nitroxide stable free radicals (NSFR). Most NSFR compounds are derivatives of piperidine and pyrrolidine.

The NSFR compounds studied for this thesis are bi- and tetra radical nitroxide derivatives of piperidine. The stability of these compounds can be attributed to the steric hindrance provided by the bulky methyl groups on the alpha carbons and also to the delocalization of the unpaired electron between the nitrogen and oxygen atoms. These are stable when heated up to $123^{o}C$ or above over a wide pH range of 1.7 to 10.7 [7].

These NSFR compounds have relatively strong proton relaxation enhancement because of electron paramagnetism. The strong magnetic moment of the unpaired electron promotes both the spin lattice and spin-spin relaxation of the surrounding protons. Low concentrations of the NSFR can result in a marked increase in the intensity of the image. These NSFR are well suited for labeling of target specific biomolecules. By complexing NSFR compounds to

substances of known specificity, it seems possible to make them selective for different tissues and receptors [5,6].

1.6 Biooxidations

Over and above the chemical synthesis of nitroxide free radicals; biosynthesis of nitroxide free radicals (NFR) is a very important field. This would be very useful in the *in-vivo* studies due to the possible reversible oxidation of the reduced forms of nitroxides used as contrast agents in the body. *In-vitro* biosynthesis of nitroxides is thus an important field for investigation.

1.7 Literature Survey

Valvis *et al* [7] of this laboratory have reported an *in-vitro* synthesis of nitroxide free radicals by hog liver microsomes. The *in-vitro* biooxidation of 4-hydroxy-2,2,6,6 tetramethyl piperidine (TEMP), 4-hydroxy-2,2,4,4 tetramethyl-1,3 oxazolidine (TEMO) and diphenyl amine (DPA) by hog liver microsomes to their respective nitroxide free radicals; 4-hydroxy-2,2,6,6 tetramethyl-piperidine-1- oxyl (TEMPO), 2,2,4,4, tetramethyl-1,3 oxazolidine 1-oxyl (TEMO) and diphenyl nitroxide (DPNO) was investigated. A calcium alginate immobilization procedure was used. The biooxidation rates of the above amines to their respective nitroxide metabolites were measured by means of oxygen uptake at $37^{O}C$ and pH 7.4. N-octylamine was found to be an activator in the biooxidation of the amines.

The proton relaxation enhancement characteristics of seven potential MRI contrast agents containing two nitroxyl spin labels per molecule (diradicals) were compared with eight similar agents with only one spin label per molecule (monoradical) by Sosnovsky *et al* [9]. The hypothesis, that multiple paramagnetic

centers in one molecule will result in stronger proton relaxation enhancement characteristics, was tested. Diradical nitroxyls were evaluated at low molar concentrations for effective contrast enhancement. The acute toxicity of these agents is believed to be largely related to the osmotic load. The lower molar concentrations reduces the osmotic load. Five of the seven diradical nitroxyls tested had spin-lattice relaxivities that were substantially greater than all the eight monoradicals tested. The spin-spin relaxation properties of these agents are favorable for contrast enhancement. The results indicate that diradical nitroxyl spin labels may be used advantageously for the design of safer and more effective MRI contrast agents.

A study of acute mutagenesis on nitroxyl spin label contrast enhancers for MRI was done by Sheldon Wolff *et al*[10]. Two nitroxyl spin label (NSL) compounds that are used experimentally as *in-vivo* contrast enhancers in magnetic resonance imaging were tested for acute toxicity in rats and for genotoxic effects in cell cultures. These compounds, 2,2,6,6 tetramethyl 1-pyrolidinyl-1-oxyl-3-carboxylic acid (PCA) and 2,2,6,6 tetramethyl -oxido-4-piperidinyl-1-succinic acid (TES) and their hydroxylamine and amine derivatives did not induce sister chromatid exchanges or mutations in Chinese hamster ovary cells at the HGPRT or Na^+/K^+ AT*pase* loci. The acute LD_{50} doses in rats for PCA and TES was found to be 15.1 mmol/kg or greater, suggesting relatively high tolerance[10].

Some applications of nitroxyl radical to organic reactions based on its redox function are described in a review by Yamaguchi *et al* [11]. It demonstrates hydroxylamine as a hydration reagent of nitriles to produce amides and also shows the reduction of oxygen to superoxide ion by an anion of hydroxylamine.

Magnetic properties of bi- and tetraradicals are presented in a review by Andre Rassat [12]. He reports that the individual properties of monoradicals are g values and hyperfine coupling constants. In multiradicals, (except the polyradicals which are polymers obtained from stable monomer radicals) dipolar D and exchange J interactions must be added. He also explains that the organic stable free-radicals are paramagnetic at high temperature and follow the curie law [$X(T-0) = C_t 0$] with negative Weiss constants. It also reports that few purely organic nitroxides have positive Weiss constants indicating the ferromagnetism of these compounds.

A series of compounds demonstrating the effects of Mn-nitroxyl interaction with the nitroxyl EPR spectra is reported by J.K More *et al* [13]. It highlights the long-range nature of electron-electron exchange interaction. When a nitroxyl oxygen binds to the Mn(III) complex of a capped tetraphenylporphyrin there is a strong antiferromagnetic coupling of the Mn $S=2$ and the nitroxyl $S=1/2$ to give an $S=3/2$ ground state with a characteristic EPR signal at $g\sim4$. When a spin labeled pyridine coordinates to the Mn(III) porphyrin via the pyridine nitrogen, the electron-electron spin-spin interaction causes broadening and splitting of the nitroxyl EPR signal.

Harold M. Swartz [14] has determined some of the principles of interactions between nitroxides and cells. He has demonstrated the feasibility of using metabolic interactions of nitroxides with cells to measure hypoxia *in-vivo*. He reports that the principal metabolism of nitroxides by cells is the reversible reduction to hydroxyl amines. The rate of reduction depends on the physical characteristics of the nitroxides. Reduction occurs primarily in the intracellular compartment and therefore only nitroxides that can cross the cell membrane readily (e.g small molecules that are lipid soluble) can be reduced readily by

cells. For some nitroxides the rate of reduction is up to thirty times faster in severely hypoxic cells which helps in the detection of the hypoxic areas *in-vivo* by using the nitroxides as contrast agents. These reduce themselves into non paramagnetic hydroxyl amines and do not affect the n.m.r images. Thus it may be feasible to use the nitroxides to detect and follow processes *in-vivo* that are associated with hypoxia. These include cancer, ischemia (i.e drastically reduced blood flow) and inflammation.

Ingold *et al* [15] have reviewed the reversible dimerization of stable nitroxides in solution. X-ray analysis was done and the two nitroxyls were found to be in a four centered bond proving the dimerization of some stable nitroxides. In continuation of the earlier work Ingold *et al* [16] have measured the bimolecular rate constants and activation parameters for the self-reactions of several dialkyl nitroxides in a number of solvents including the equilibrium constant for the dimer formation. The results were found to be consistent with a mechanism involving the reversible formation of a diamagnetic nitroxide dimer and its slow decomposition to a hydroxylamine and a nitrone.

A predictive design was explored with nitroxyl labeled derivatives, and other congeners of alkylating drugs streptozotocin/chlorozotocin and many others by Sosnovsky [17]. The design was based on the correlations of lipophilicities of these drugs with their antineoplastic activites in-vivo against the murine lymphocytic leukemia P388. Several of these compounds were found to be possessing considerably higher therapeutic indices than those of the parent drugs which are in clinical use presently.

A computerized literature search for the topic relevant to this work is presented in Appendix.

CHAPTER II
OBJECTIVES

The primary objective of this study is to develop a feasible method for the synthesis of nitroxides with various free radical centers in one moiety. The compounds to be synthesised are the bi and tetra radical nitroxides; bis-(2,2,6,6 tetra methyl-1-oxyl-4-piperidyl) phthalate and tetrakis-(2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) pyromellitate.

The accomplishment of this objective to establish a sucessful and economic process in high yield involves the following steps:

i) designing the drug to determine at what spacing between the nitroxide centers the relative intensity begins to diminish due to interference,

ii) synthesizing the biradical nitroxide, bis-(2,2,6,6 tetra methyl-1-oxyl-4-piperidyl) phthalate, by different possible routes,

iii) optimizing the reaction conditions for each method,

iv) following the most feasible method (giving high yield) for the synthesis of the tetra radical, tetrakis-(2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) pyromellitate,

v) optimizing the reaction conditions for the preperation of tetrakis-(2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) pyromellitate in high yield,

vi) investigation and understanding of the mechanisms involved in the oxidation of these nitroxides,

vii) Obtain the yield data and thus compare the different methods,

viii) Purification of the compounds by column chromatography, and

ix) Analysis of these purified nitroxides by various analytical techniques such as thin layer chromatography, nuclear magnetic resonance, mass spectrophotometry and electron spin resonance for the confirmation of these products obtained.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials and Apparatus/Instrumentation

3.1.1 Chemicals

Ethyl acetate, hexane, thionyl chloride, benzene and hydrogen peroxide obtained were of a suitable reagent grade. All other materials, 4-hydroxy-2,2,6,6 tetramethyl piperidine , phthaloyl chloride, pyromellitic acid, sodium bicarbonate, sodium tungstate dihydrate and pyridine were obtained from FLUKA Chemical Corp., Ronkonkoma,NY.

Anhydrous calcium sulfate was obtained from DRIERITE.

3.1.2 Melting Point Apparatus

Thomas hoover capillary melting point apparatus (Unimelt).

3.1.3 Ultraviolet Lamp

Model UVSL-25 mineral lamp of perkins elmer.

3.1.4 IR Spectrophotometer

Fourier-transform infrared spectrometer - Perkin Elmer 1760X.

3.1.5 TLC

Kiesel gel 60 F_{254} ; 25 Folien 20x20 cm Schichtdicke 0.2 mm from E.M. Science.

3.1.6 Column Chromatography

Silica gel (40-140 mesh) by Baker and

Silic AR CC-7 (special) by Mallinckrodt Inc.

3.1.7 NMR Spectrophotometer

The solvent used for running the NMR was $CDCl_3$. The spectra were taken with respect to TMS. EM360 60 MHz NMR Spectrophotometer.

3.1.8 Mass Spectrometer

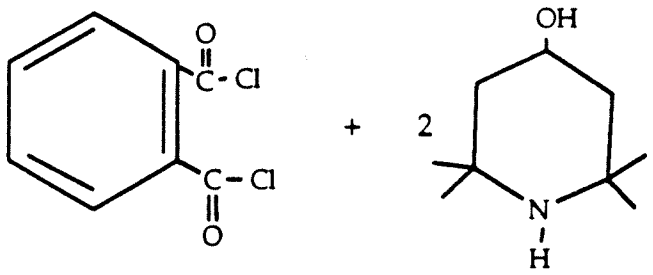
JEOL 303 double focussing magnetic sector.

3.1.9 ESR Spectrophotometer

Varian E-12 ESR Spectrophotometer.

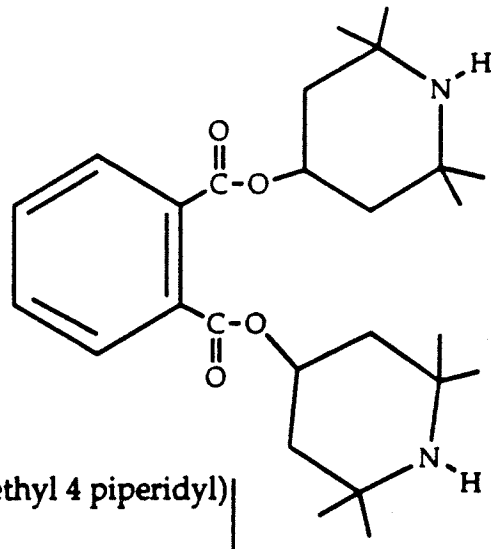
3.2 SYNTHESIS OF BIS(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) PHTHALATE

3.2.1 SCHEME I :

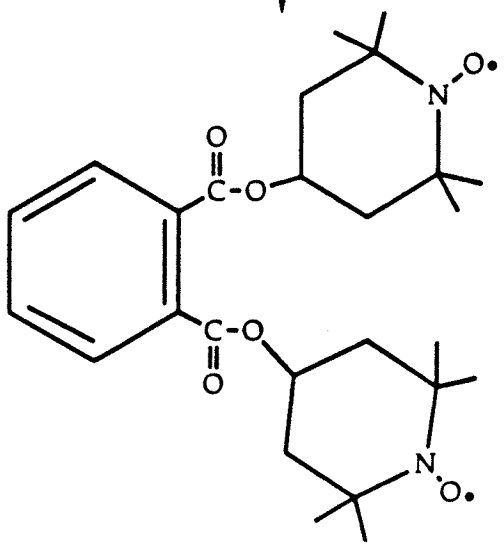


Phthaloyl chloride

4 Hydroxy 2,2,6,6 tetramethyl piperidine



Bis (2,2 ,6, 6 tetramethyl 4 piperidyl) phthalate



Bis (2,2 ,6, 6 tetramethyl 1-oxyl 4-piperidyl) phthalate

Synthesis of the Ester Before Oxidation

a) Reaction Between Phthaloyl Chloride and TEMP

A one liter flask was attached with a magnetic stirrer, a reflux condensor and a dropping funnel. 8 G (0.039 moles) of 4-hydroxy- 2,2,6,6 tetramethyl piperidine with 50 mL of benzene was placed in the flask. The stirrer was set in motion and the mixture heated to a gentle refluxing. 3 G (0.015 moles) of phthaloyl chloride was added at such a rate that moderate refluxing continued even after the source of heat was removed. When about two-thirds of the phthaloyl chloride had been introduced, the mixture refluxed very vigorously.

The reaction flask was cooled immediately in an icebath, and the remainder of the phthaloyl chloride added. The mixture was then heated and refluxed for 2 hours. The reaction mixture was washed with 250 ml of cold 2 % sodium hydrogen carbonate. The powder, white in colour was dried and purified using column chromatography. 1.8 gm of the purified ester was obtained. Thus the yield obtained was 60 %.

Purification of the Ester product

The ester was dissolved in a 25:75 ratio of MeOH:$CHCl_3$ solution, and then separated by column chromatography. Silica gel was used as the packing material.

b) Oxidation of bis-(2,2,6,6 tetramethyl-4-piperidyl) phthalate

1 gm (0.0023 moles) of the ester obtained was dissolved in benzene and kept for stirring for about 1 to 2 hours. The solution was cooled to 10 °C and perbenzoic acid was added to the reaction flask and stirred for two hours at 10 to 15 °C.

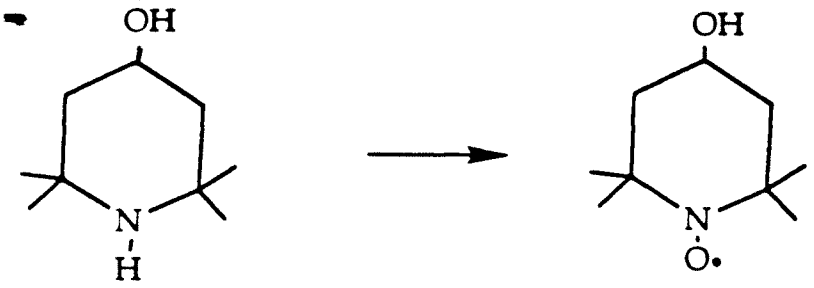
This was then kept at room temperature, stirring overnight. The benzene was evaporated with vaccum and the product was purified using column chromatography. The purified product obtained was 0.53 gm (53 % yield).

Purification of bis-(2,2,6,6 tetramethyl-1-oxyl-4 piperidyl) phthalate

The oxidized product was dissolved in hexane:ethylacetate (3:1) and purified by column chromatography, packed with silica gel. The melting point was found to be 155.5°C.

3.2 .2) SCHEME II:

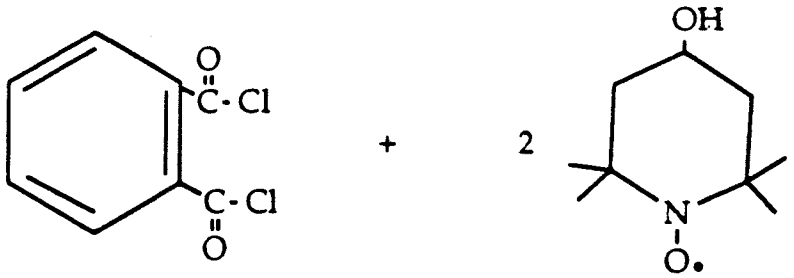
A)



4 Hydroxy 2,2,6,6 tetramethyl piperidine

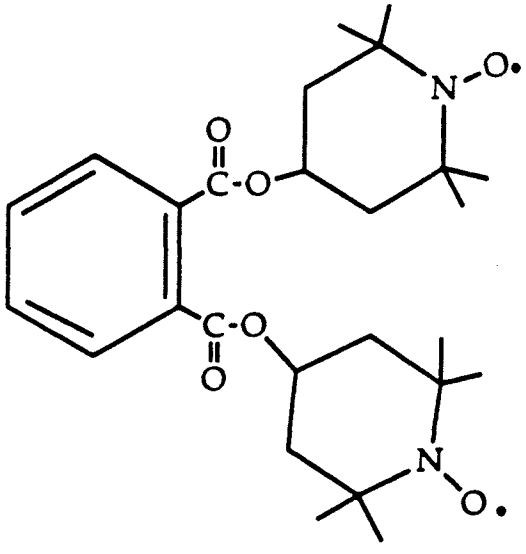
4 Hydroxy 2,2,6,6 tetramethyl 1-oxyl piperidine (TEMPO)

B)



Phthaloyl chloride

TEMPO



Bis (2,2 ,6, 6 tetramethyl 1-oxyl 4-piperidyl) phthalate

Synthesis of the Ester After Oxidation

a) Oxidation of 4-hydroxy-2,2,6,6 Tetramethyl Piperidine

5.0 gms of TEMP was placed in a 250 ml flask with a thermometer. A solution containing 0.07 g sodium tungstate (Na_2WO_4) in 40 ml of water was prepared. The mixure was dissolved and cooled to about 0-5 °C.

5 g of hydrogen-peroxide (30%) was added slowly (drop-wise) at 0-5 °C. The reaction was kept for stirring for another hour at 0-5 °C. This was then kept at room temperature, stirring overnight. After 15 hours of stirring, the solution was saturated with sodium chloride. The 4-hydroxy-2,2,6,6 tetramethyl 1-oxyl piperidine formed was extracted using ethyl acetate as solvent and separated. The solvent was dried using few graciers of drierite and then evaporated using the Rota Vapor BUCHI 461 - Brinkmann. The solution obtained was cooled in a refrigerator and the solid product was weighed and used directly for the next reaction without purification. The yield obtained was 89 %.

b) Reaction of TEMPO With Phthaloyl Chloride

A three necked flask was equipped with a thermometer, condensor and stop corks. The required amount of TEMPO (Table I) was placed with appropriate amount of absolute pyridine into the flask. This was then cooled to 0-4 °C. An appropriate amount of phthaloyl chloride was added (Table I) slowly at 0-4 °C. The reaction was kept for stirring for one more hour at 0-4 °C and then at room temperature (overnight). Next day, the resulting mixture was poured to 150 ml ice-water and 10 % hydrochloric acid was added to adjust to the appropriate pH (Table I). The precipitate was collected and washed with water and then dried.

This was then purified using column chromatography. The melting point of the product was found to be 155.5 C.

Table - I

Wt of TEMPO	Vol. in ml of Pyridine	Wt of Phtaloyl Chloride	pH adjusted	Yield g(%)
1.9 g (0.01 mol)	25	2.10g (0.01 mol)	2.0	2.33g (51)
1.9 g (0.01 mol)	25	1.05g (0.005 mol)	4.5	2.20g (90)

The above was carried out to find the possibility of occurence of mono substitution on phthaloyl chloride at equimolar concentrations of the reactants.

Purification of bis-(2,2,6,6 tetramethyl-1-oxyl-4 piperidyl) Phthalate

The obtained product was purified by column chromatography using the eluent hexane:ethyl acetate in a 3:1 ratio and the packing material being silica gel.

Table II - Column chromatographic results of bis (2,2,6,6 tetramethyl 1-oxyl-4 piperidyl) phthalate

Fraction	Colour	Volume (ml)	TLC R_f		
			0.067	0.8-0.75	0.097
1-3	Clear	30	-	-	-
4-5	Yellow	20	-	-	+
6-8	Orange	30	-	-	+
9-10	Light Yellow	20	-	+	+
11-13	Orange	20	+	-	+
14-15	Orange	20	+	-	-

The R_f value of the 4- hydroxy- 2,2,6,6 tetramethyl- 1-oxyl-piperidine was found to be 0.06 to 0.07 and that of bis- (2,2,6,6 tetramethyl 1-oxyl-4 piperidyl) phthalate was found to be 0.097.

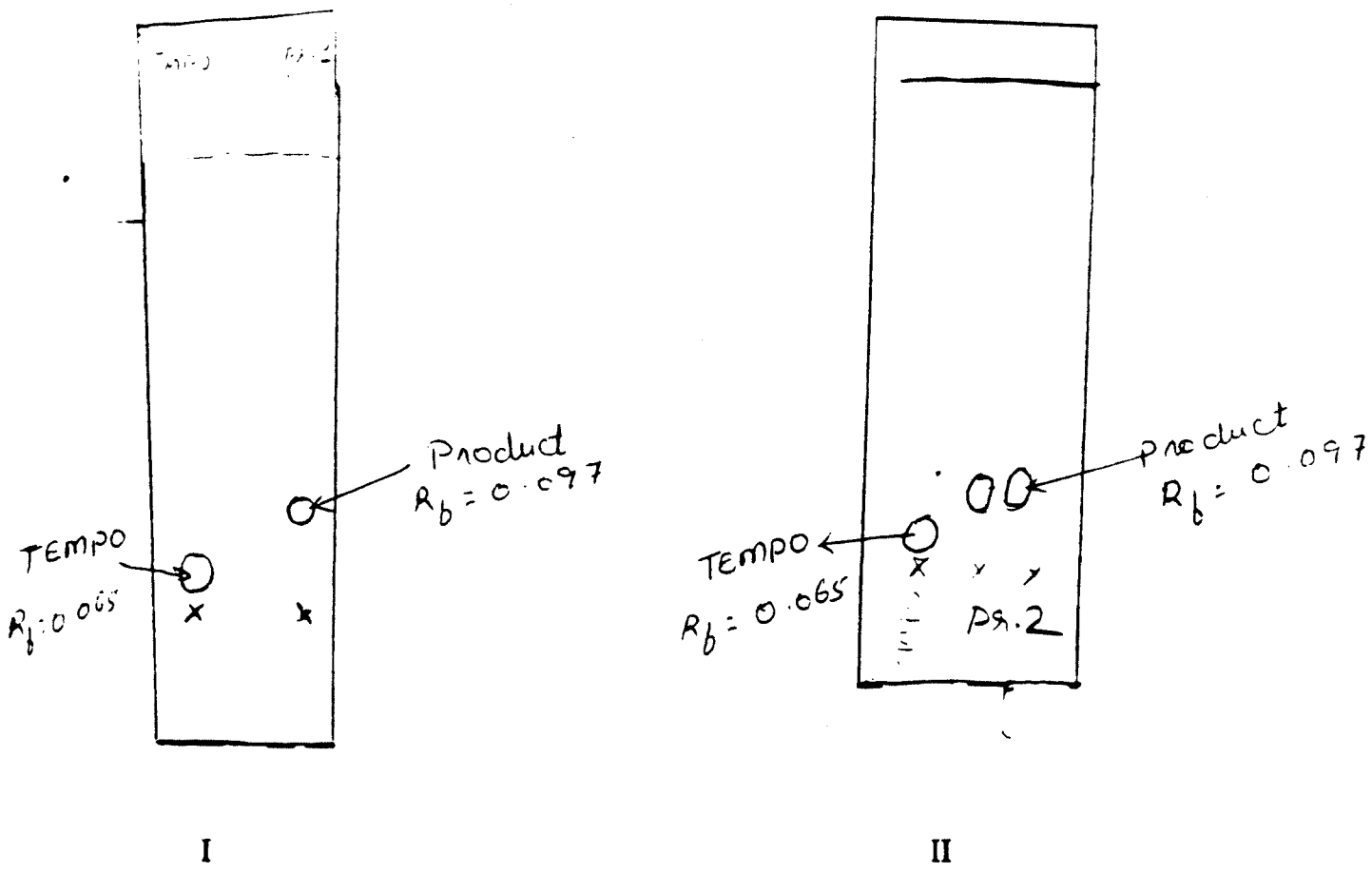


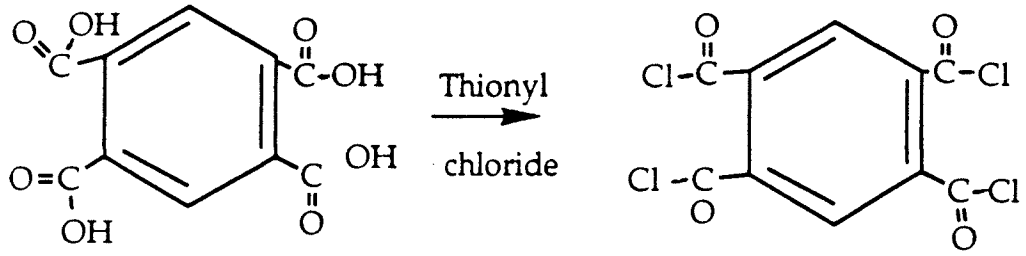
Figure 3.1 Identification of Bis(2,2,6,6 teramethyl-
1- oxyl 4-piperidyl)phthalate,using Hexane:Ethylacetate

I: Product by scheme I
II:Product by scheme II

3.3) SYNTHESIS OF TETRAKIS (2,2,6,6 tetramethyl-1-oxyl-4-piperidyl) PYROMELLITATE:

SCHEME III:

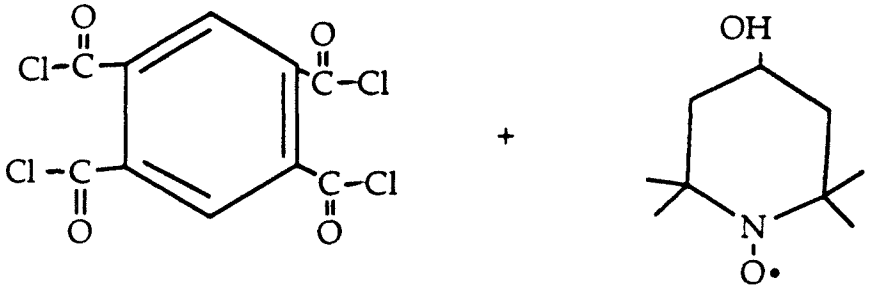
A)



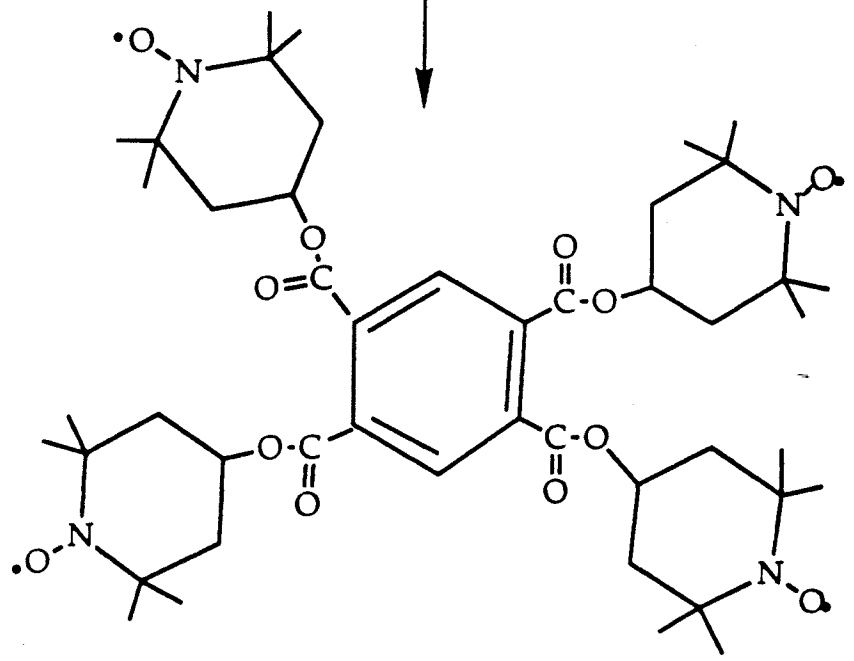
Pyromellitic acid

Pyromellitoyl chloride

B)



Pyromellitoyl chloride + TEMPO



Tetrakis (2,2,6,6 tetramethyl-1 oxyl-4-piperidyl) Pyromellitate

3.3 Synthesis of Tetrakis-(2,2,6,6 tetramethyl 1-oxyl-4 piperidyl) Pyromellitate

a) Formation of Pyromellitoyl Chloride

One gram (0.004 moles) of solid pyromellitic acid was mixed with 50 gms of thionyl chloride and 0.08 gm of $FeCl_3.6H_2O$. The reaction mixture was rapidly agitated to initiate gas evolution. The gases hydrochloric acid and sulphur dioxide were vented through a caustic guard tube connected to a vessel containing water. The temperature maintained was 74-76 °C in the reaction flask for about 2½ hours. When the acid seemed to have dissolved in thionyl chloride , it was refluxed for another half hour until no gas came out of the vent. The excess thionyl chloride was removed by distillation. The pyromellitoyl chloride in the reaction flask was dissolved in methylene chloride (CH_2Cl_2 : product) in a 4:1 ratio. The product was stored for extended period without deterioration. This was taken directly for further reaction without isolation. Pyromellitoyl chloride, on isolation in powder form, decomposed easily. Hence, it was taken directly for further reaction.

b) Reaction of Pyromellitoyl Chloride With 4-hydroxy 2,2,6,6 Tetramethyl 1-oxyl-piperidine

A two necked flask fitted with a stirrer and calcium chloride tube was charged with 3.4 gm (0.02M) of TEMPO and 20 mL of absolute pyridine. With stirring and ice cooling, the pyromellitoyl chloride from (a) was added slowly at 0-4°C. The reaction mixture was stirred with ice cooling for one hour and then at room temperature for 14-15 hrs. The reaction product was poured into 200 mL of ice-water and acidified with dilute hydrochloric acid to pH 5. The top aqueous layer was reextracted with methylene chloride. The organic layers were pooled together and washed thoroughly with water to pH~7. The methylene chloride

solvent was removed by rotary vaccum (Rota-vapor, BUCHI 461- Brinkmann). The product was dried and purified by column chromatography.

Purification:

The tetraradical obtained was purified by column chromatography using hexane:ethylacetate (3:1) as eluent. The weight of the purified product was 0.8 gms (30.6% Yield).

Table III

Column chromatographic results of tetrakis (2,2,6,6 tetramethyl-1-oxyl-4 piperidyl) pyromellitate

Fraction	Colour	Volume (ml)	TLC R_f	
			0.067	0.16-0.17
1-3	Clear	30	-	-
4-6	Yellow	30	-	+
7-8	Orange	20	-	+
9-10	Clear	20	-	-
11-12	Orange	20	+	+

The R_f value of TEMPO was found to be 0.06-0.07 and that of tetrakis-(2,2,6,6 tetramethyl 1-oxyl-4-piperidyl) promellitate was found to be 0.16-0.17.

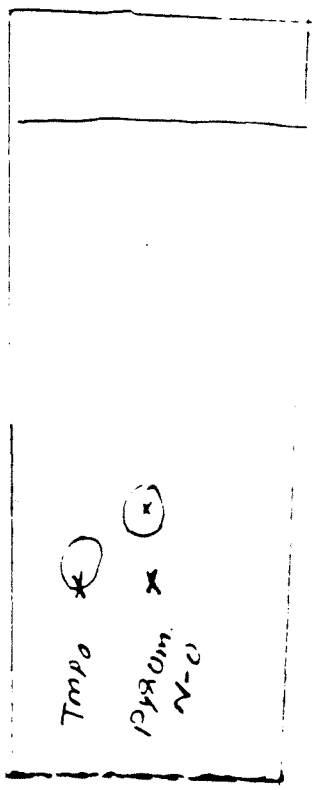


Figure 3.2 Identification of tetrakis-(2,2,6,6, tetramethyl-1-oxyl-4-piperidyl) pyromellitate (Scheme III)

3.4 TLC Analysis of the Nitroxide Products

The TLC of all the nitroxide compounds was done on silica gel and eluted using hexane:ethylacetate (3:1). The TLC plate was dried in air and kept in iodine chambers. The plates were then observed using U.V. lamp and the dark spots marked.

3.5 Column Chromatography

20-30 G of silica gel was used for each gram of sample. The column was clamped securely using a ring stand in a vertical position. A small piece of glass wool plug was gently pressed through the column and 1 inch autoclaved sand put as mechanical support. The silica gel was put in the beaker containing the solvent and poured into the column with continous tapping of the side of the column. A 1cm sand layer was added at the top of the column. The sample was drawn into the column in such a fashion that the column didn't dry at anytime. The eluent was then eluted continously and different fractions collected. The compounds separated according to their polarity with the less polar component eluting first. Refer to Figure 3.3.

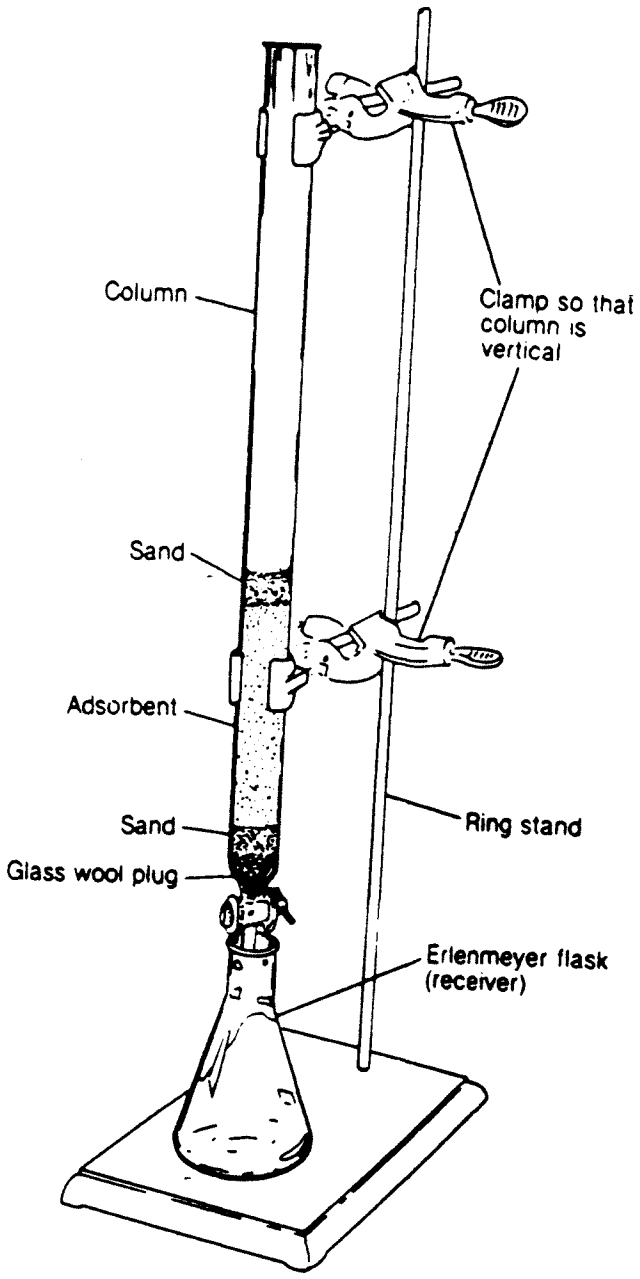


Figure 3.3 Column Chromatography

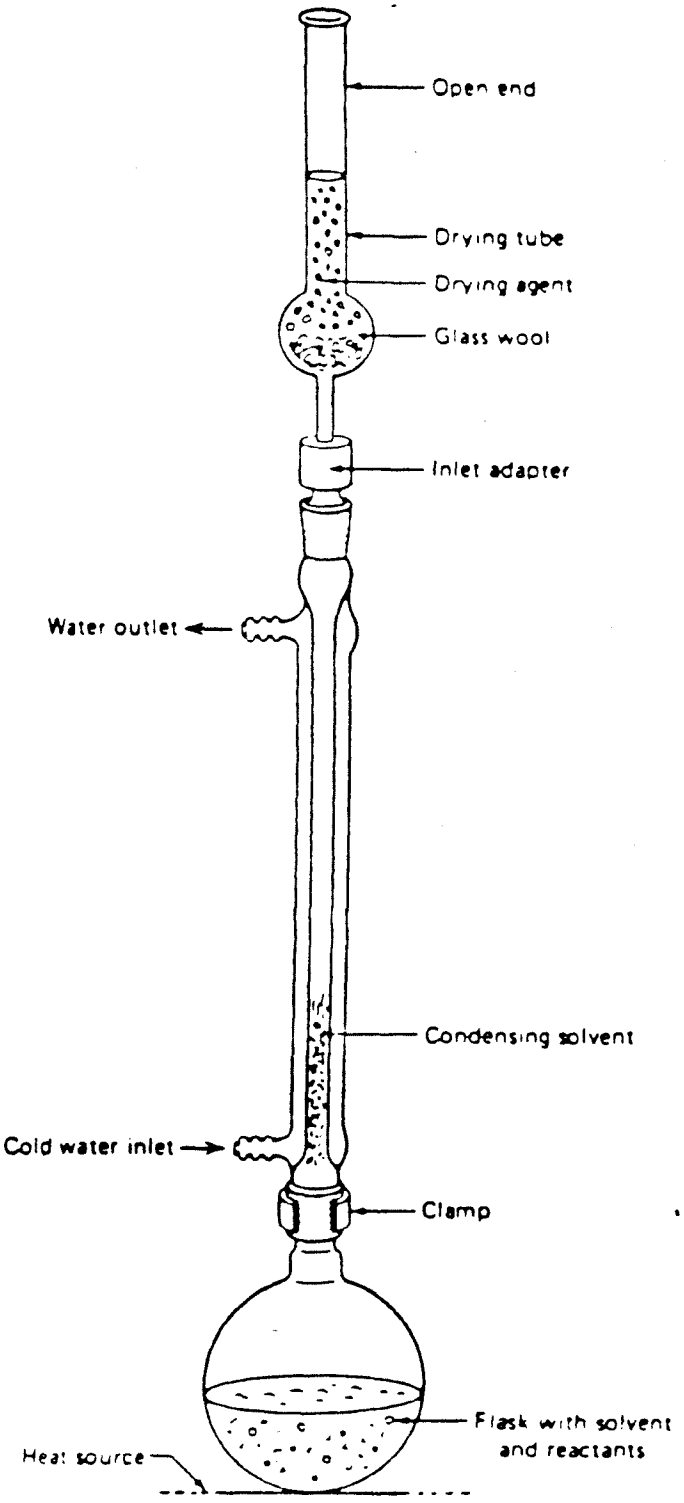


Figure 3.4 Reflux Apparatus

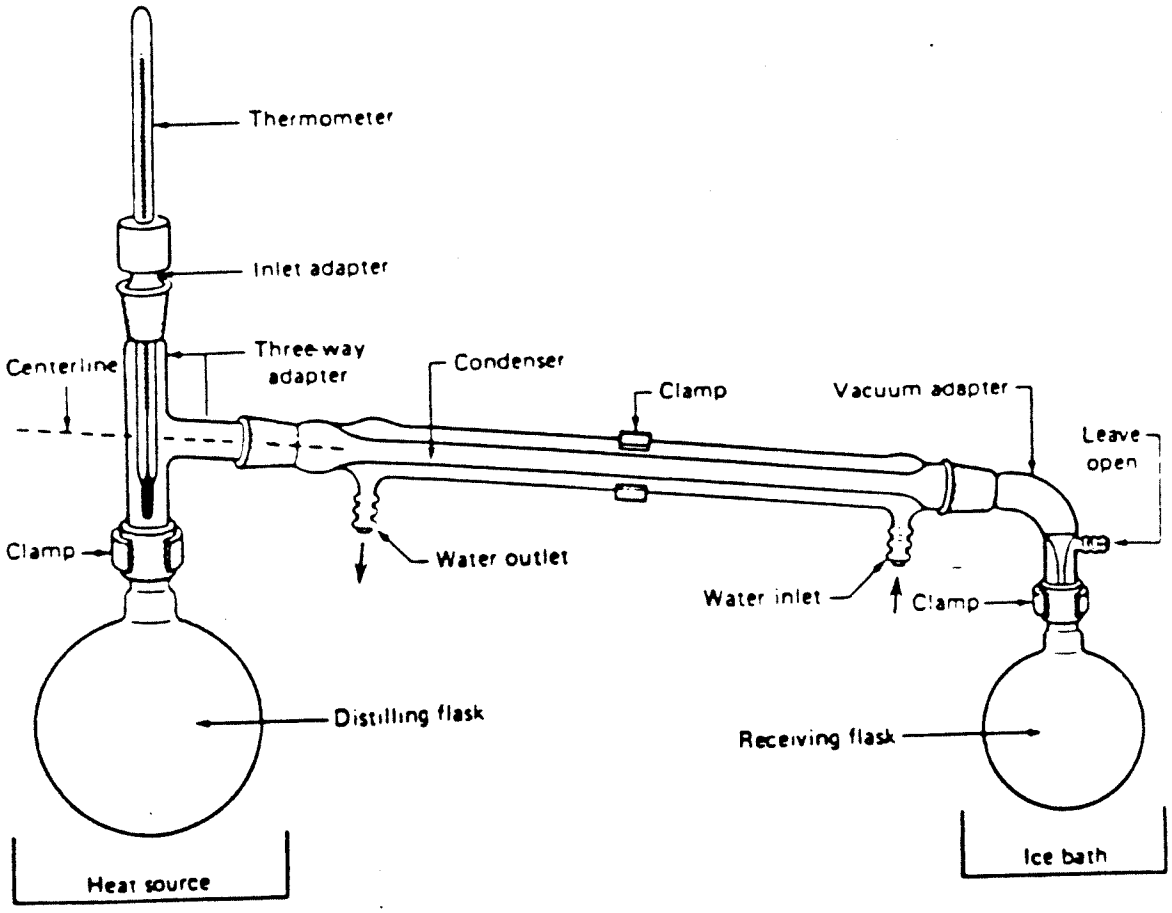


Figure 3.5 Distillation Apparatus

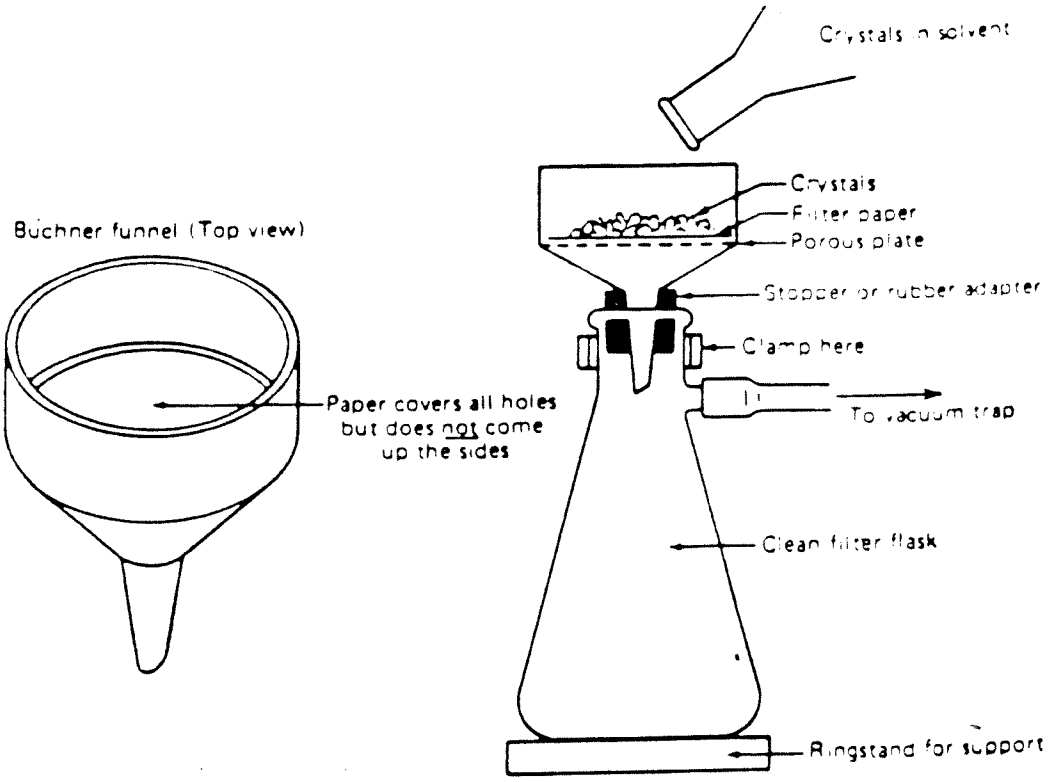


Figure 3.6 Filteration (suction) Apparatus

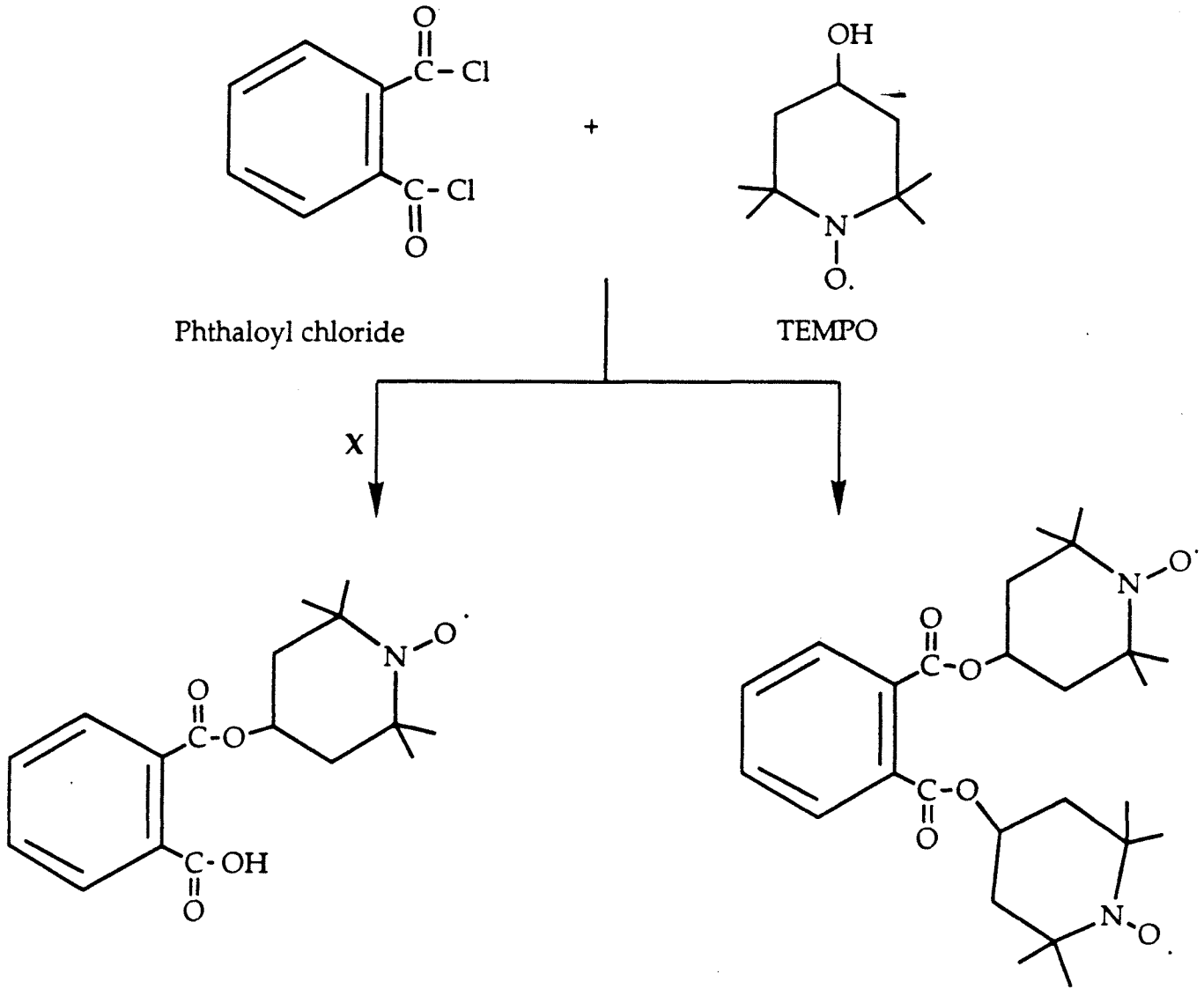
CHAPTER IV
RESULTS AND DISCUSSION

In this study, we investigated the effect on the yield by different methods and by varying the amount of TEMPO.

4.1 Effect of the Varying Amount of TEMPO

The reaction of TEMPO with phthaloyl chloride was studied to observe the possibility of any mono substitution at equimolar ratio of the raw materials; phthaloyl chloride and TEMPO. It is found that when equimolar ratios of TEMPO and phthaloyl chloride are taken, there is no evidence of the formation of any mono substituted product of phthaloyl chloride. The free radical nitroxides were attached ortho to each other. There was an observable lowering of yield of the final product.

The disubstituted product formed was in low yield. When the molar ratio of 4-hydroxy-2,2,6,6 tetramethyl 1-oxyl piperidine was increased two fold, the yield dramatically increased two fold. This study proves the formation of only the disubstituted product.



Bis (2,2 ,6, 6 tetramethyl-1-oxyl-4-piperidyl) phthalate

4.2 Comparison of the Two Methods Under Study

The method which uses the already oxidized TEMPO product for the formation of the bis-(2,2,6,6, tetramethyl 1-oxyl-4-piperidyl) phthalate is found to be the most versatile reaction with respect to the yield obtained (90% yield). The other method is less versatile due to the possible formation of amide along with the ester formation and a low final yield (53% yield) of the expected product.

4.3 Mechanism of the Nitroxide Formation

These amines are oxidized to the corresponding nitroxides, generally in good yield, by treatment with hydrogen peroxide in the presence of pyridine and a salt of either vanadium, molybdenum, or tungsten. Alternatively, hydrogen peroxide may be used. This method was introduced by Lebedev *et al* (18).

The oxidation of secondary amines to nitroxides by hydrogen peroxides or peroxyl radicals is considered to take place as follows:

$$RO_2OH \longrightarrow RO_2\cdot + \cdot OH$$

$$R_2NH + RO_2\cdot \longrightarrow R_2N\cdot + RO_2H$$

Or

$$R_2NH + RO\cdot \longrightarrow R_2N\cdot + ROH$$

$$R_2N\cdot + RO_2\cdot \longrightarrow R_2NOOR \longrightarrow R_2NO\cdot + \cdot OR$$

Or

$$R_2N\cdot + \cdot OH \longrightarrow R_2NOH$$

$$R_2NOH + RO\cdot \longrightarrow R_2NO\cdot + ROH$$

4.4 Infrared Spectra

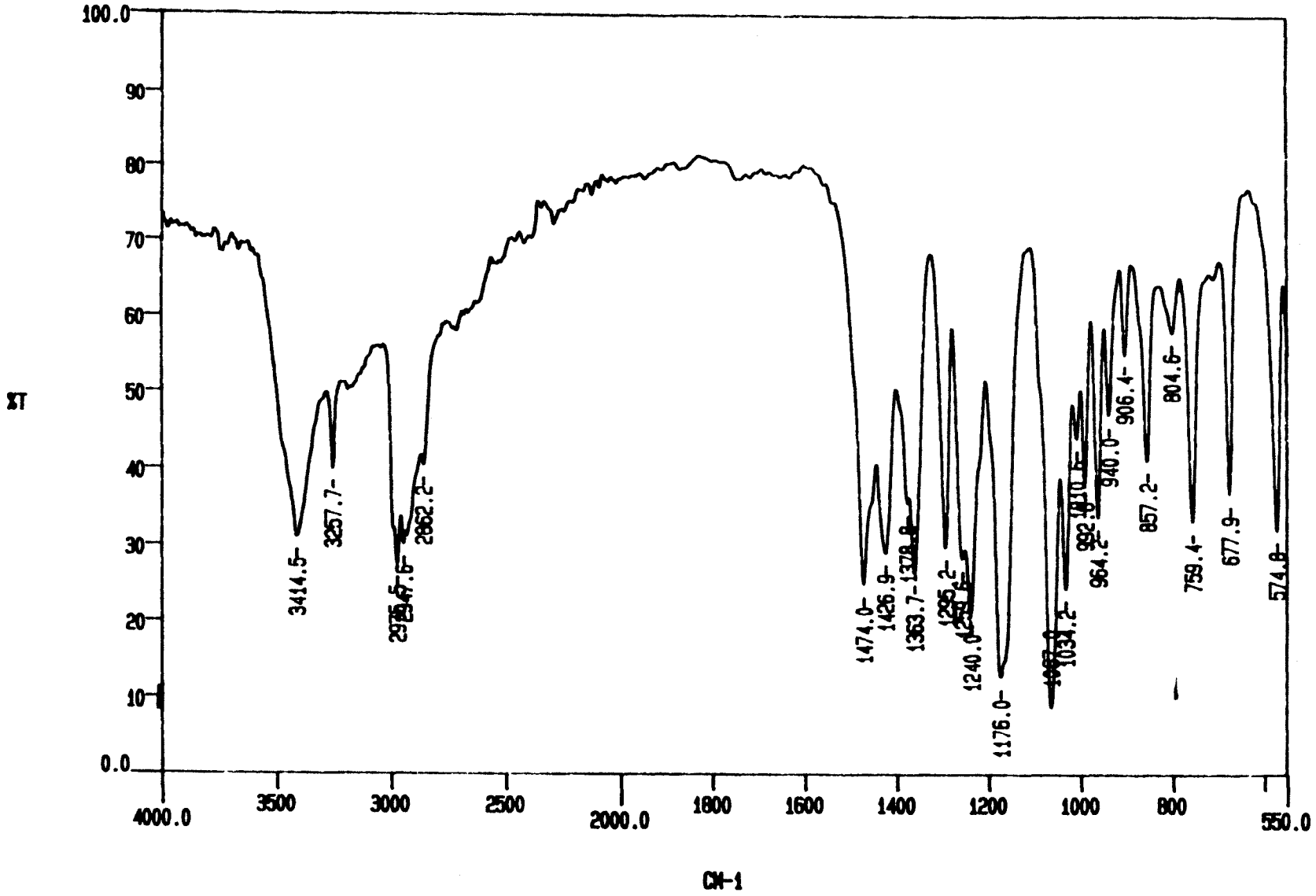
The IR spectra show the characteristic N-O frequency of nitroxide.

Table IV

COMPOUND	FREQUENCY OF N-O.
TEMPO	1363.7 cm^{-1}
Bis-(2,2,6,6, tetramethyl -1-oxyl-4-piperidyl) phthalate (Route I)	1365.2 cm^{-1}
-do- (Route II)	1368.5 cm^{-1}
Tetrakis-(2,2,6,6 tetramethyl 1-oxyl-4-piperidyl)pyromellitate	1365.1 cm^{-1}, 1377 cm^{-1}

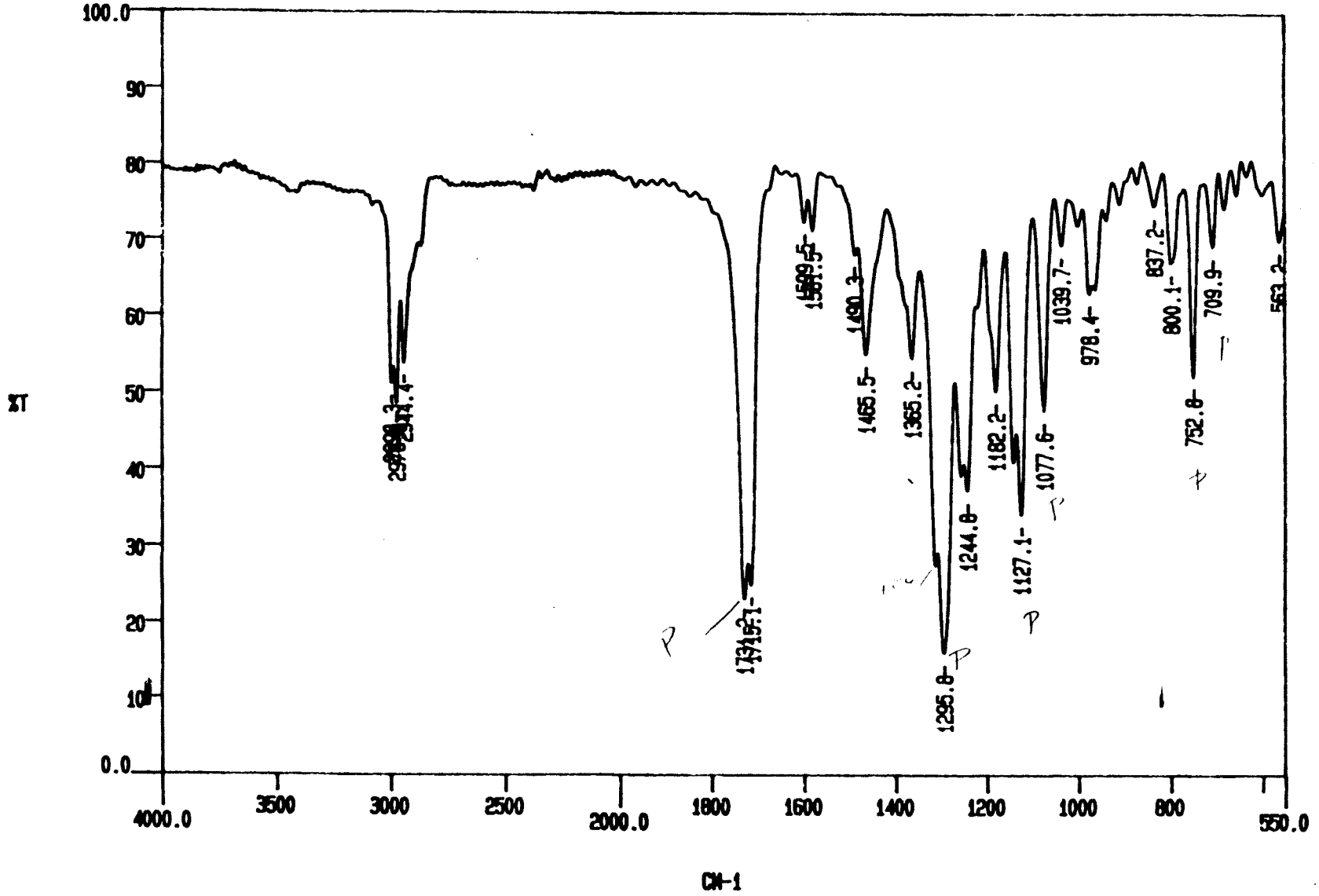
The ester frequencies observed in bis-(2,2,6,6, tetramethyl 1-oxyl-4-piperidyl) phthalate by the first route was 1731.2 and 1715.1 cm^{-1} and by the second route was 1738.7 and 1717.9 cm^{-1}. The ester frequencies observed in tetrakis-(2,2,6,6, tetramethyl-1-oxyl-4 piperidyl) pyromellitate were 1735.6 and 1718.9 cm^{-1}. The IR spectra for all the four compounds follow.

See Figures 4.1, 4.2, 4.3, 4.4.



P-E 1720 Filename: 1-HHADA.SP Date: 92/04/01 Time: 13:27:02.00
Scans: 16 Resolution: 4.00 Operator: ATE
Sample: 4-OH-TMPO, orange powder, KBr pellet

Figure 4.1 IR Spectra of TEMPO



P-E 1720 Filename: 2-RADHA.SP Date: 92/04/01 Time: 13:58:49.00
Scans: 16 Resolution: 4.00 Operator: ATE
Sample: D1 TMPO phthalate, light reddish powder, KBr pellet

Figure 4.2 IR Spectra of Bis(2,2,6,6 tetramethyl -1-oxyl-4-piperidyl)phthalate (Method I)

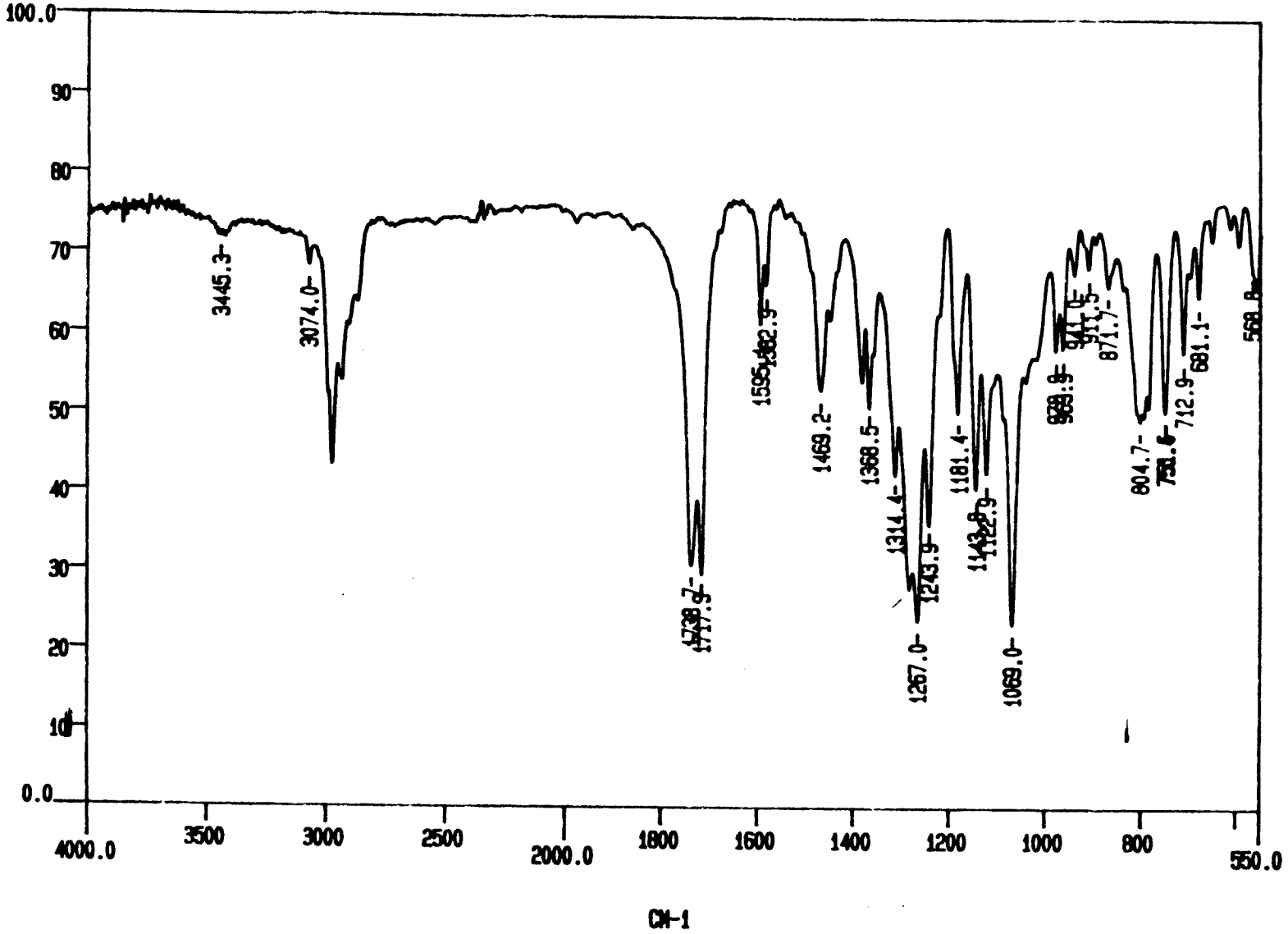
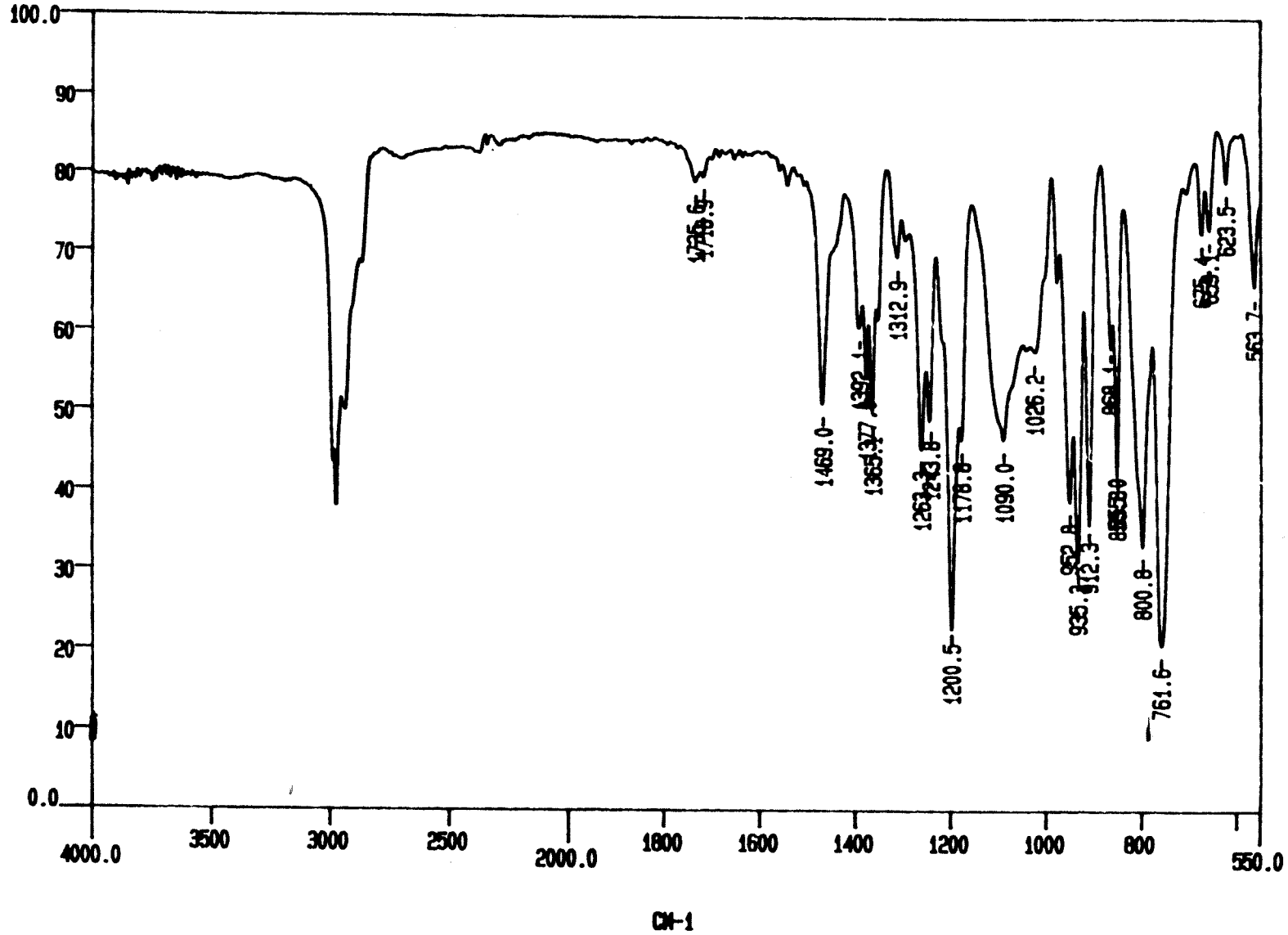


Figure 4.3 IR spectra of Bis(2,2,6,6 tetramethyl 1-oxyl-4-piperidyl)Phthalate (Method II)



P-E 1720 Filename: 4-RADHA.SP Date: 92/04/01 Time: 14:57:33.00
Scans: 16 Resolution: 4.00 Operator:
Sample:

Figure 4.4 IR Spectra of Tetrakis(2,2,6,6 tetramethyl 1-oxyl-4 piperidyl)Pyromellitate

4.5 NMR Spectra and Mass spectra

The NMR Spectra and the mass spectra of all the three products are attached.

The mass spectral parameters are:

Probe temperature programme	: 35 °C 8 ° C/min 250° C
Ionization Voltage	: 70 ev
Ion Current	: 100 ua
Mode	: Electron Impact, Positive Ion
Ion Multiplier	: 7.2 KV
Data System	: JMA - DA 500

The following mass spectra of the three compounds show the molecular ion peaks M^+. The TEMPO M^+ ion peak is found to be 172 m/e, bis-(2,2,6,6 tetramethyl 1-oxyl-4 piperidyl) phthalate (Method 1 & 2) show M^+ ion peak at 474 m/e. The tetrakis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) pyromellitate shows M^+ ion as impurity and the major peak is at 390 m/e. This compound is probably very unstable to detect the M^+ ion peak.

The NMR spectra shows the respective intensities of signals and the multiplicity of signals indicating the number of protons to which there is coupling.

Figures 4.5 to 4.18 show the NMR and the mass spectra of all the three products prepared including that of TEMPO.

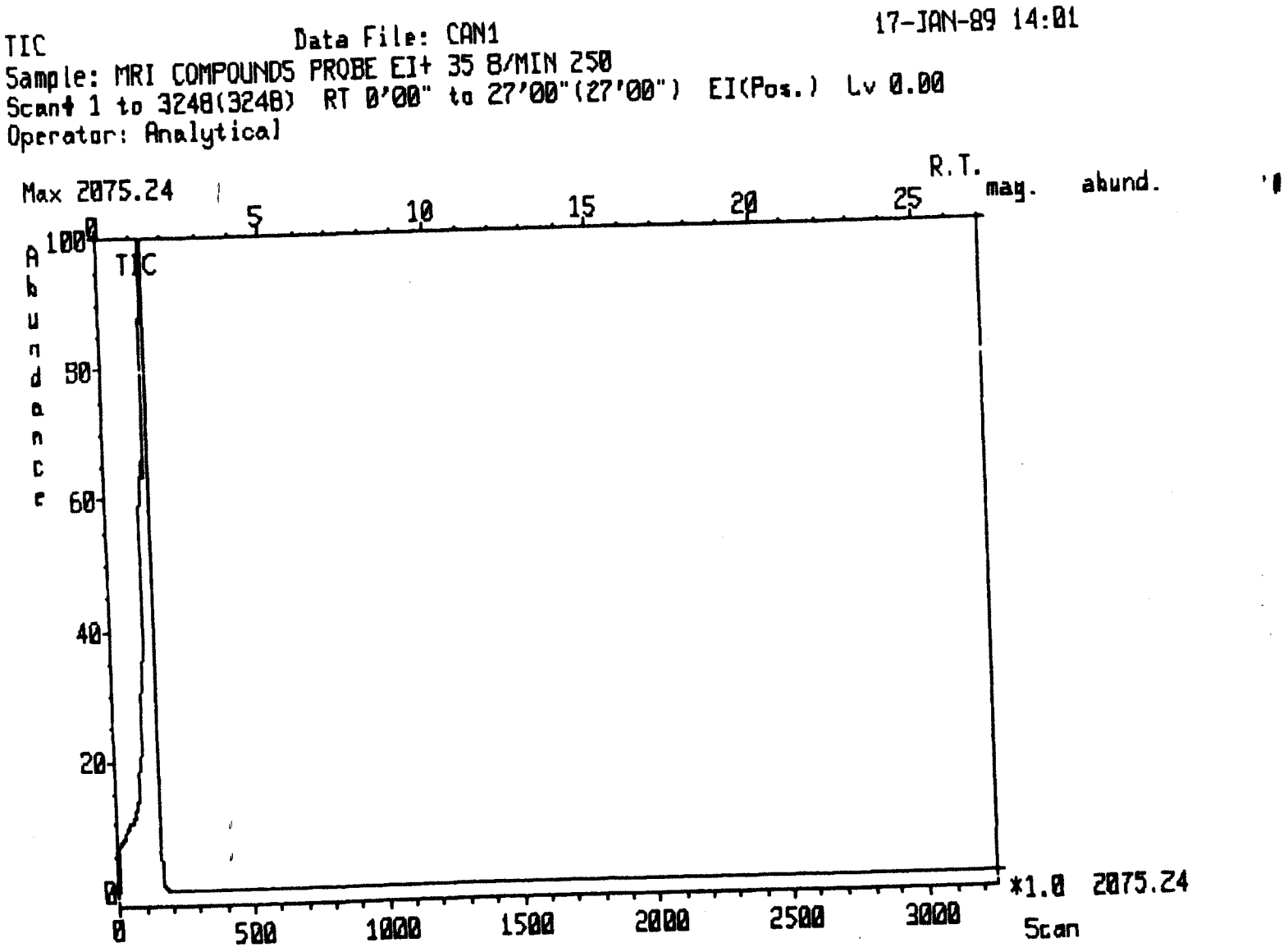


Figure 4.5 TEMPO

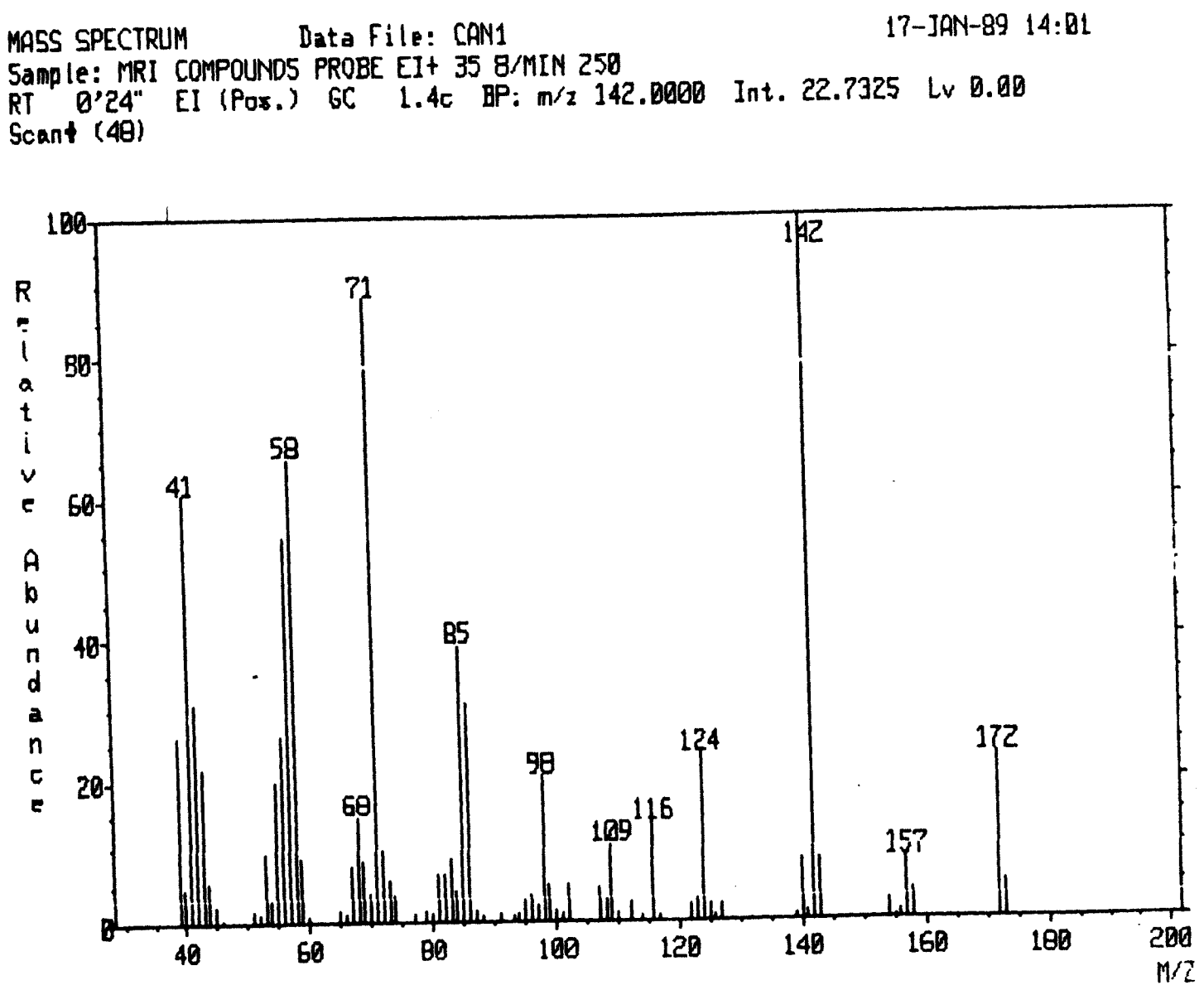


Figure 4.6 TEMPO

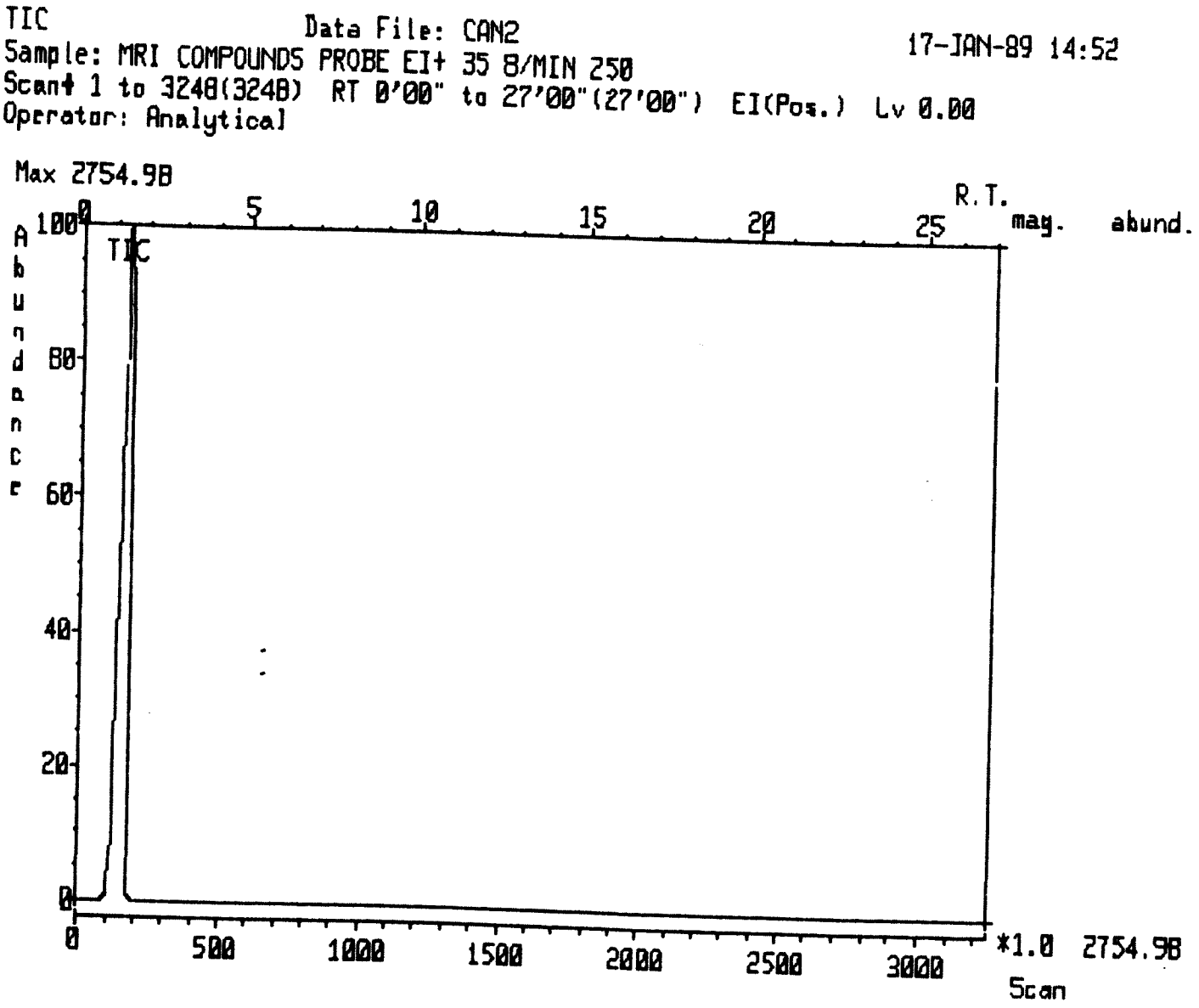


Figure 4.7 Bis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate(Method 1)

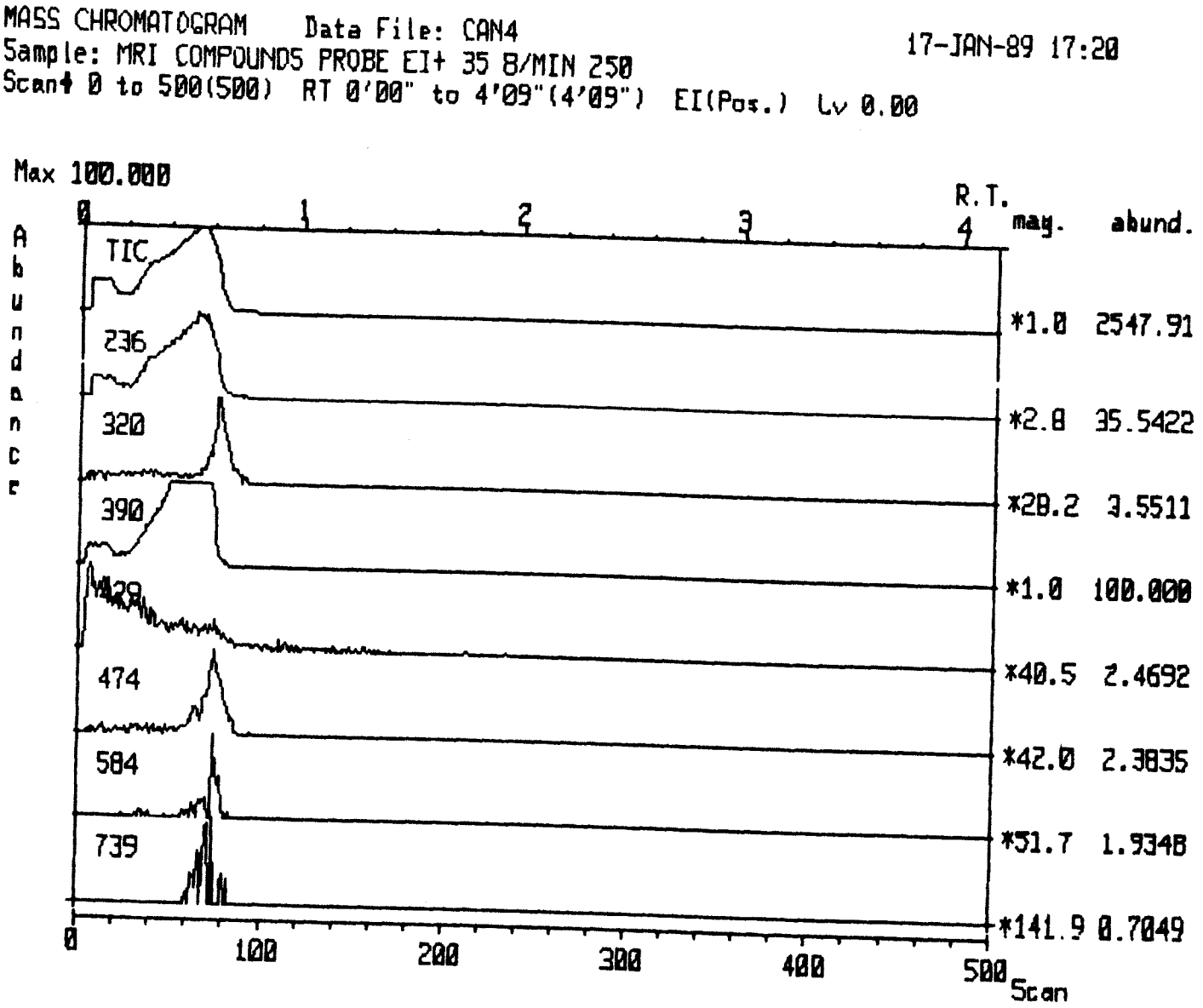


Figure 4.11 Wide range scan of Tetrakis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl)Pyromellitate

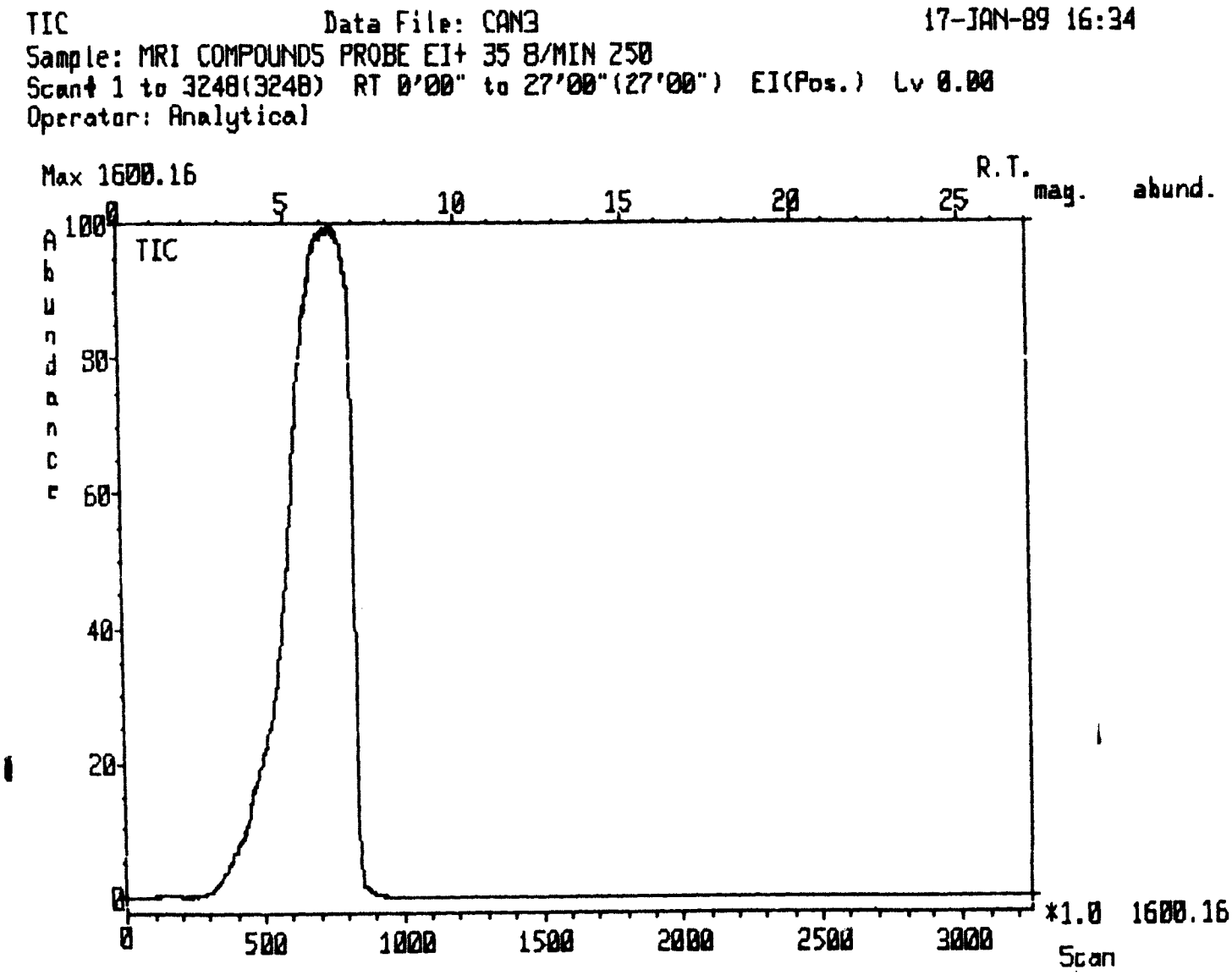


Figure 4.9 Bis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate (Method 2)

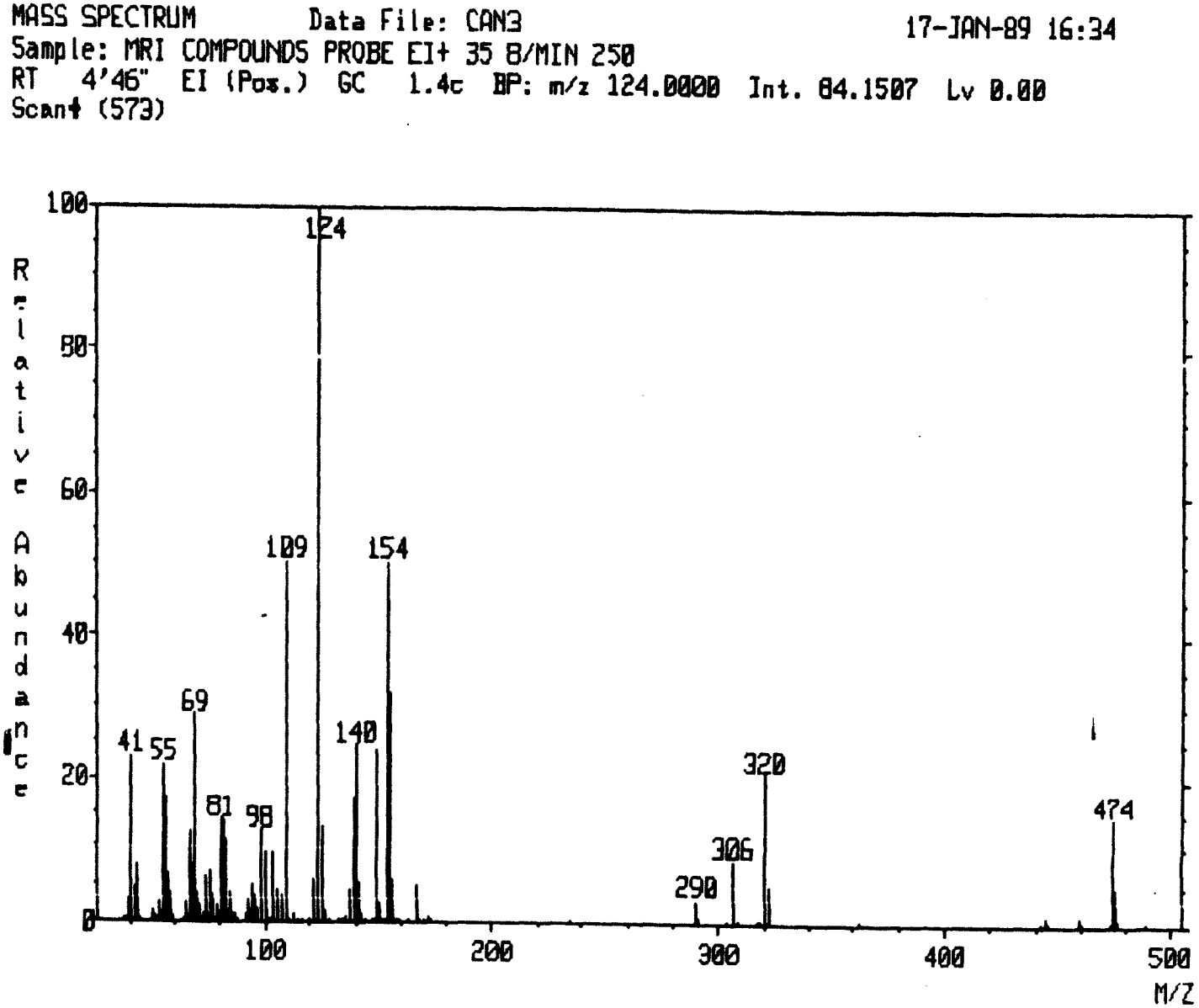


Figure 4.10 Bis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate (Method 2)

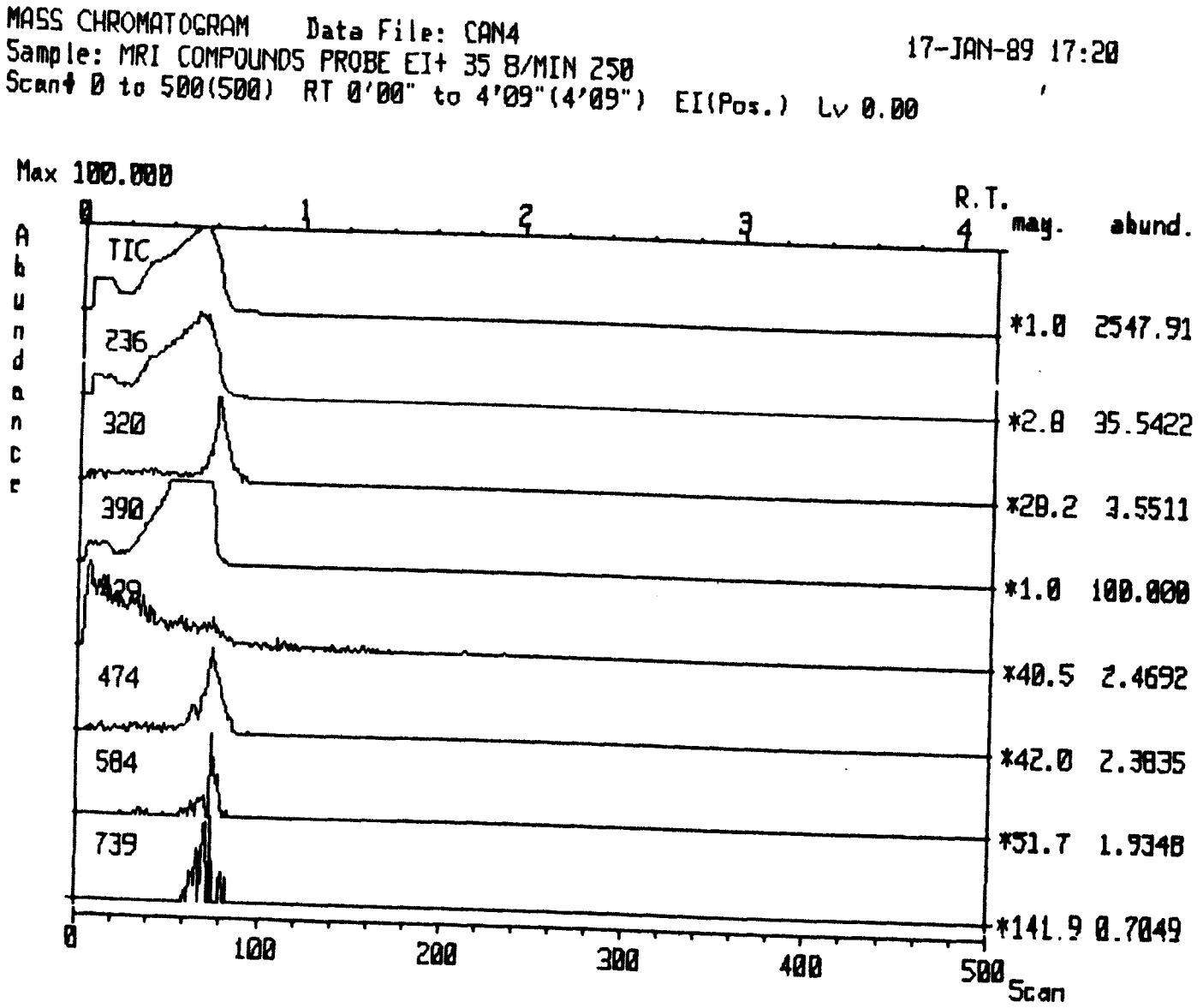


Figure 4.11 Wide range scan of Tetrakis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl)Pyromellitate

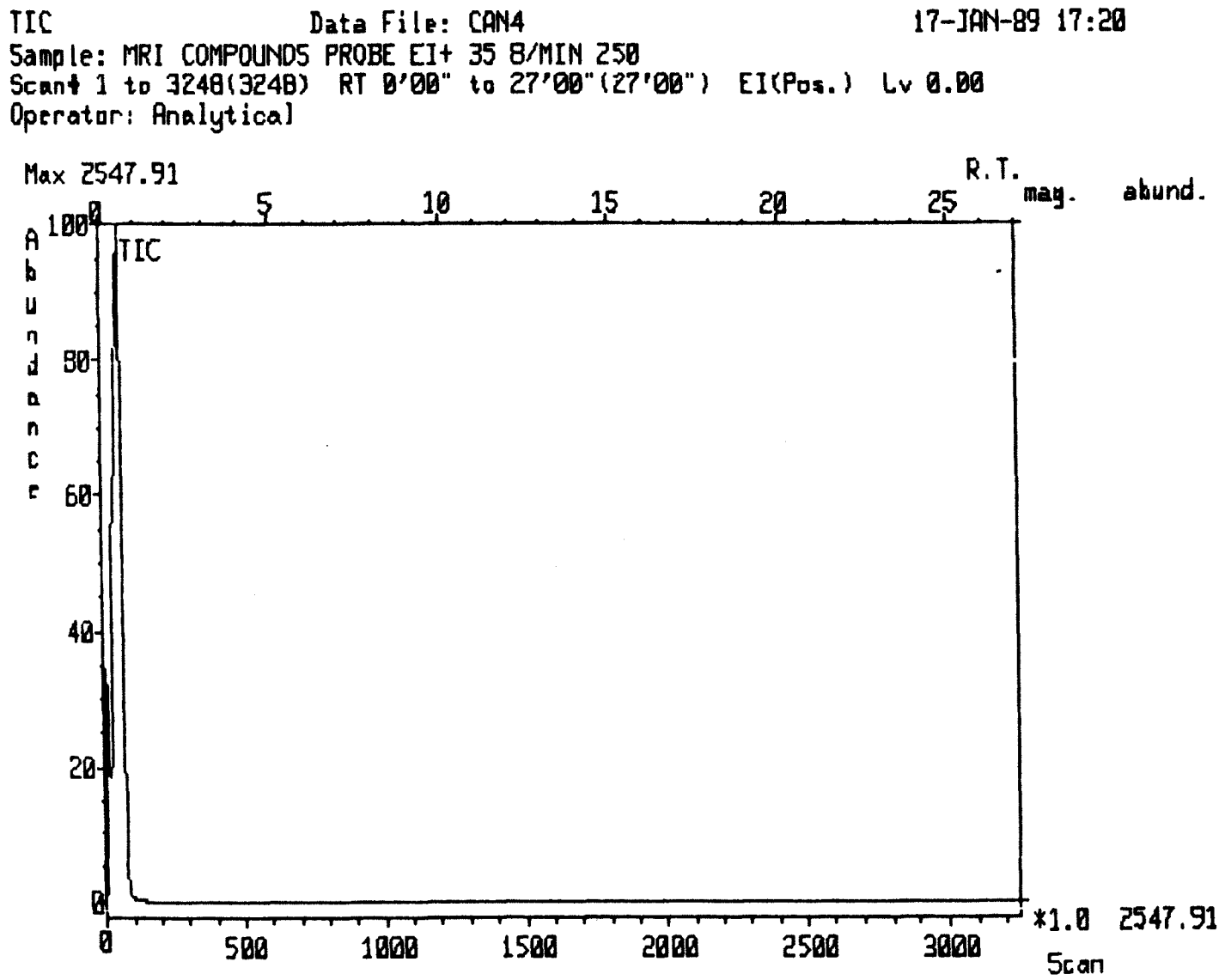


Figure 4.12 Tetrakis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl)pyromellitate

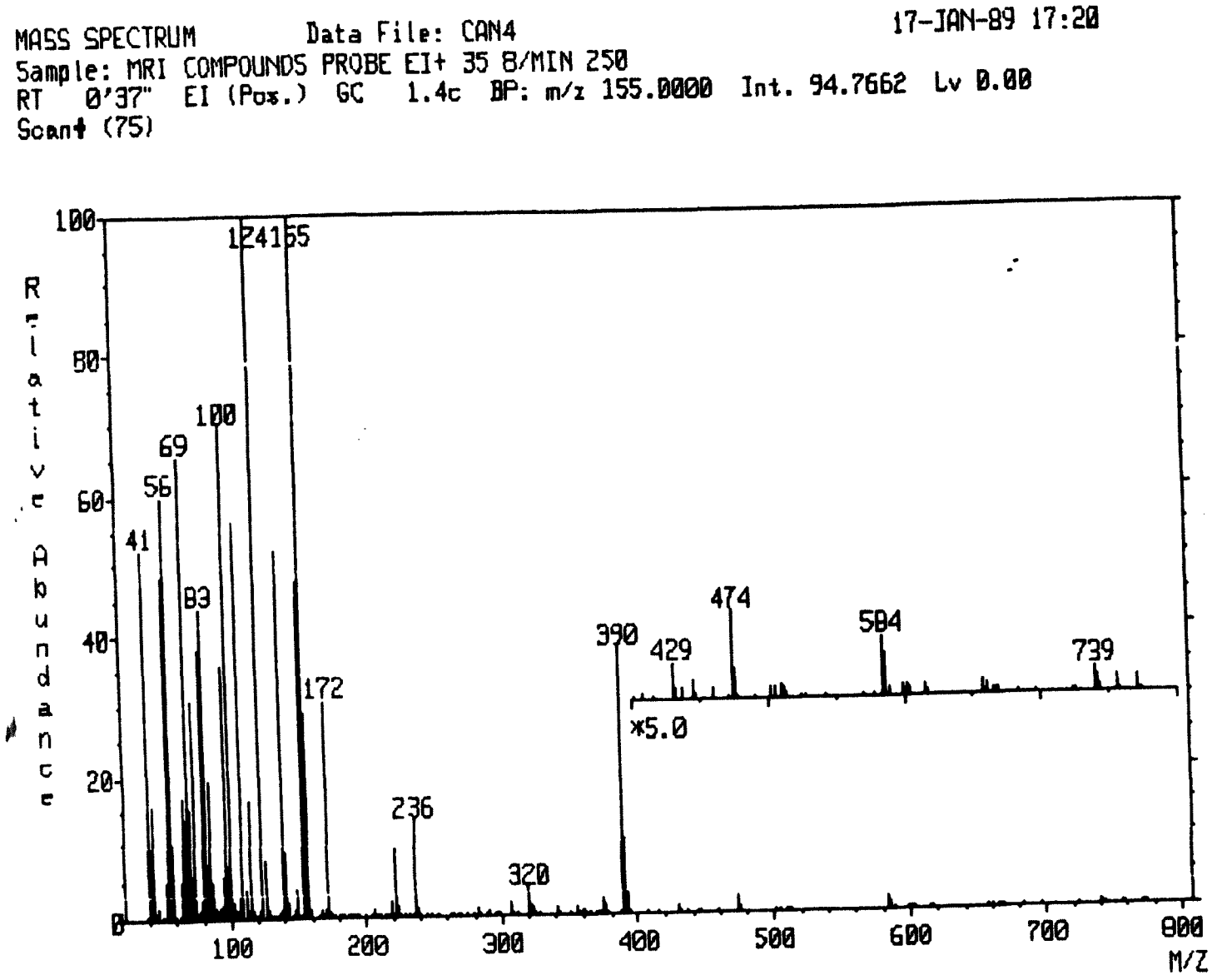


Figure 4.13 Tetrakis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) pyromellitate

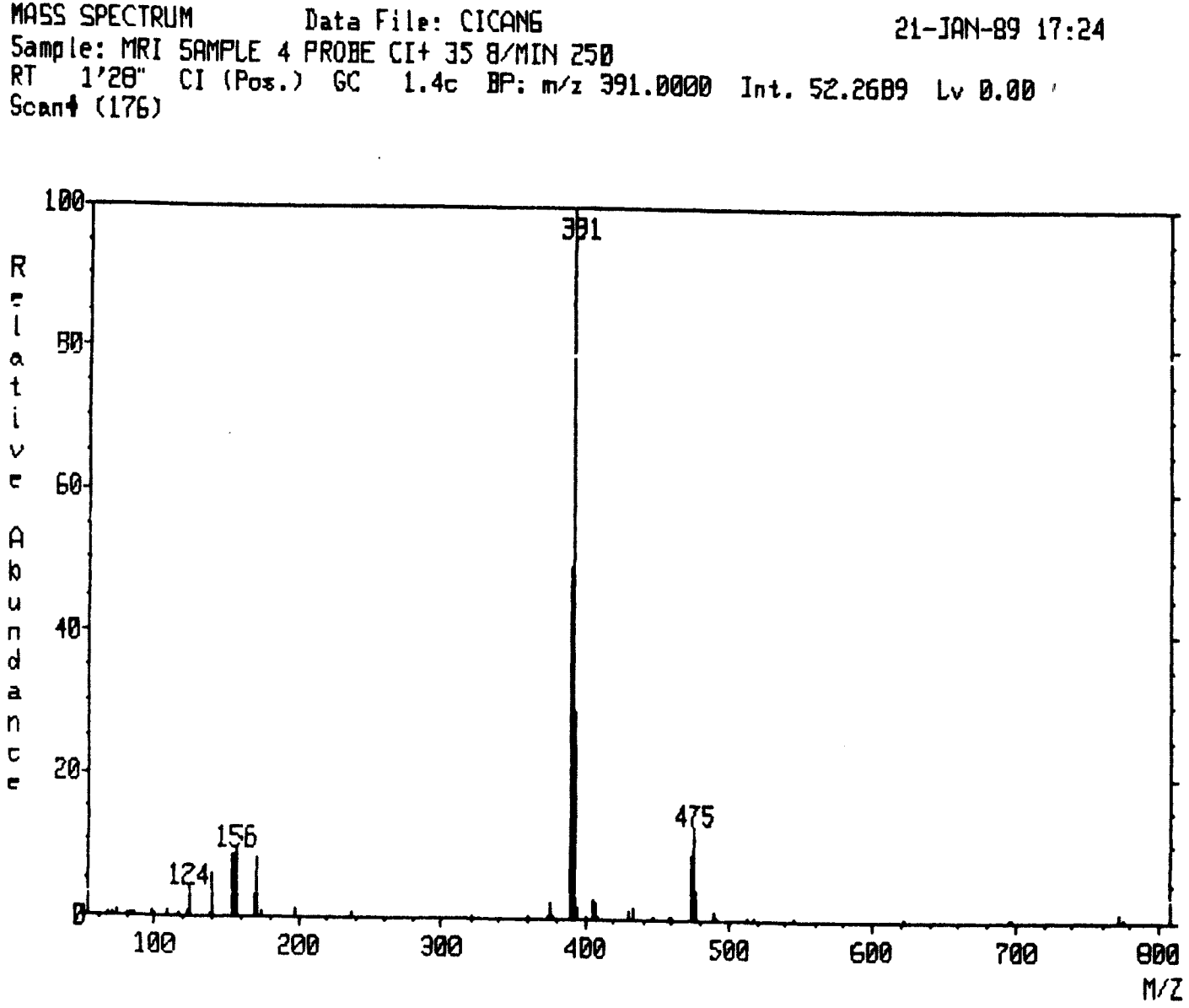


Figure 4.14 Chemical Ionisation spectra of Tetrakis(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) pyromellitate

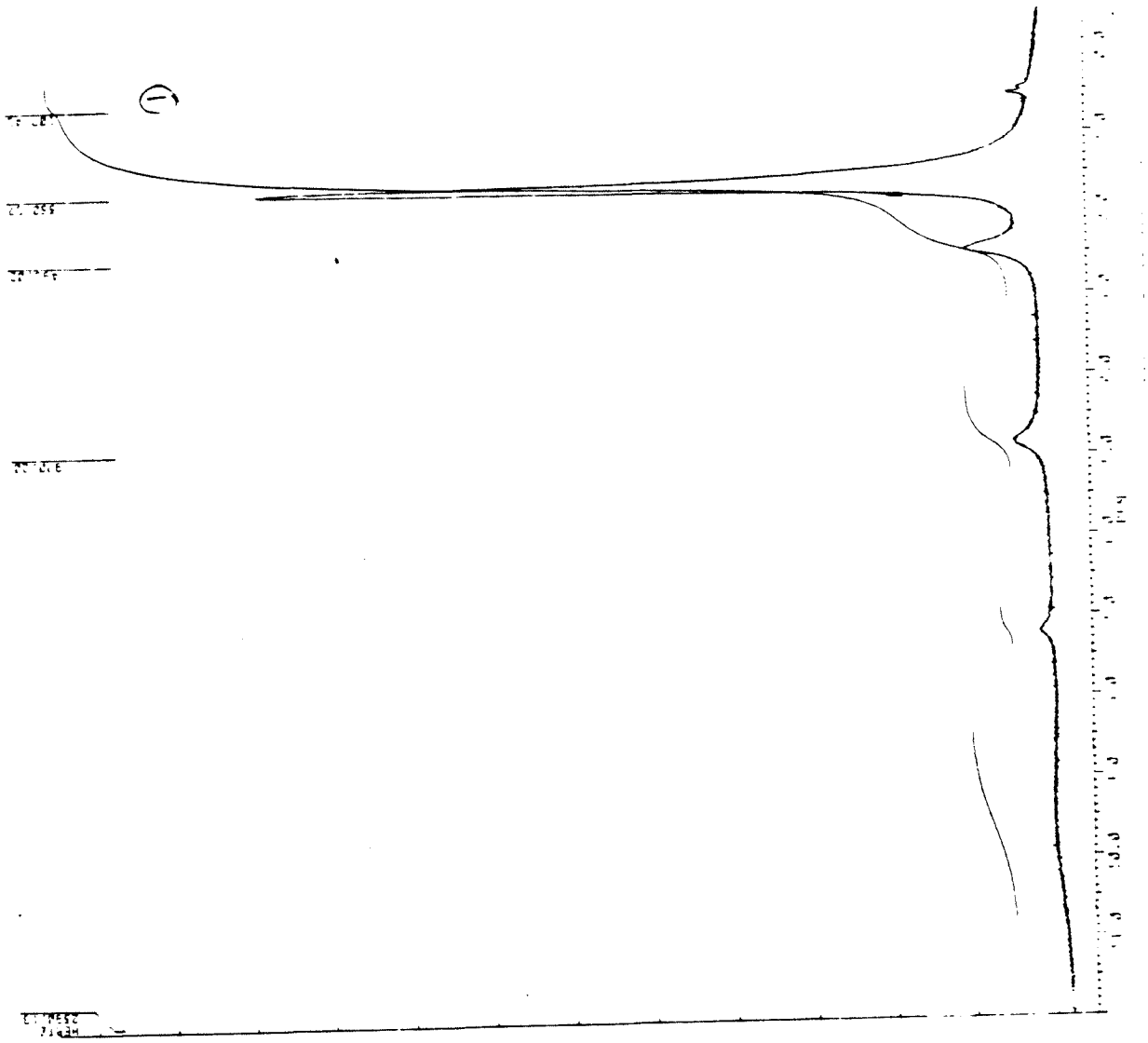


Figure 4.15 NMR spectra of TMPO

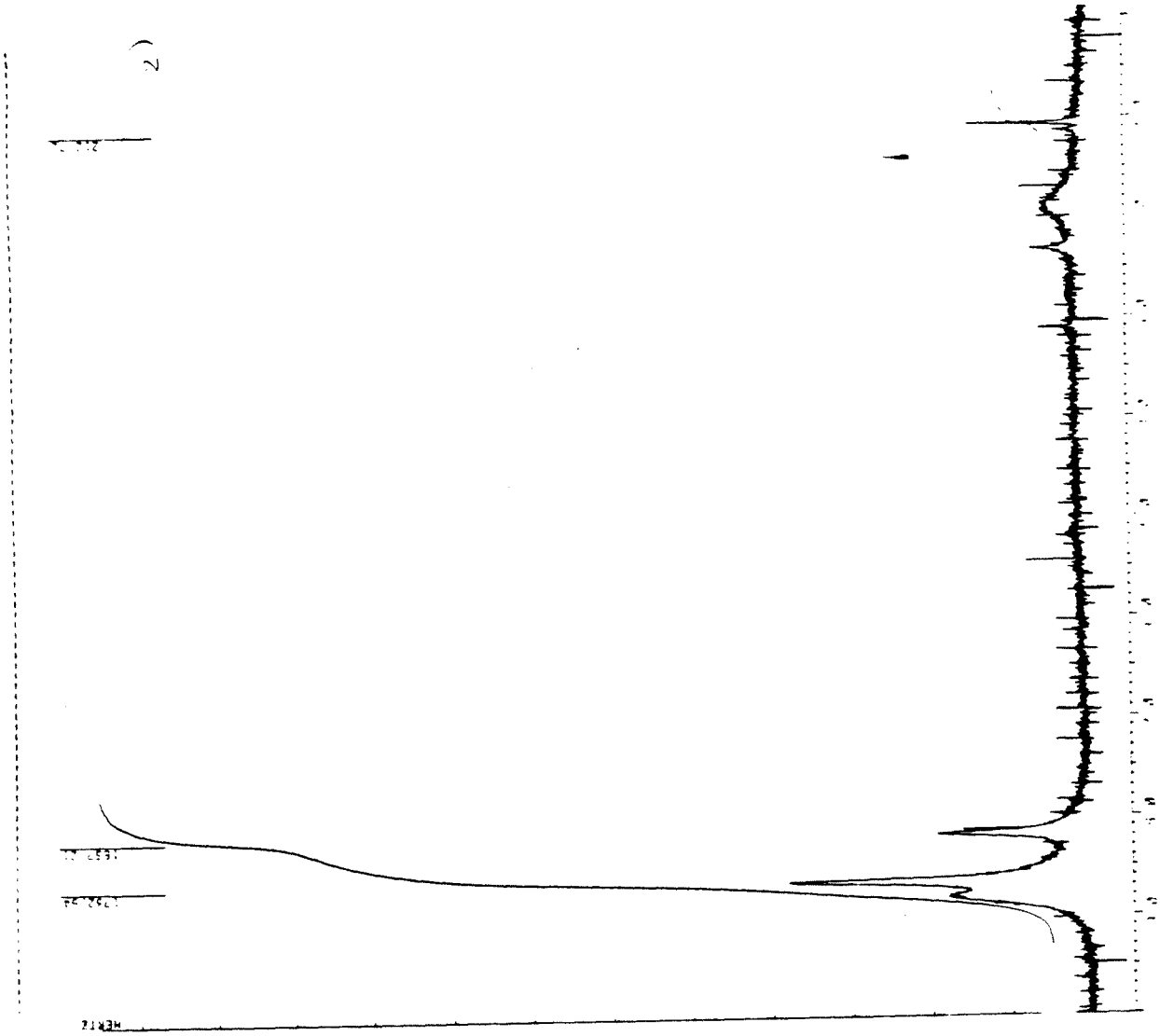


Figure 4.16 NMR Spectra of bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate (Method I)

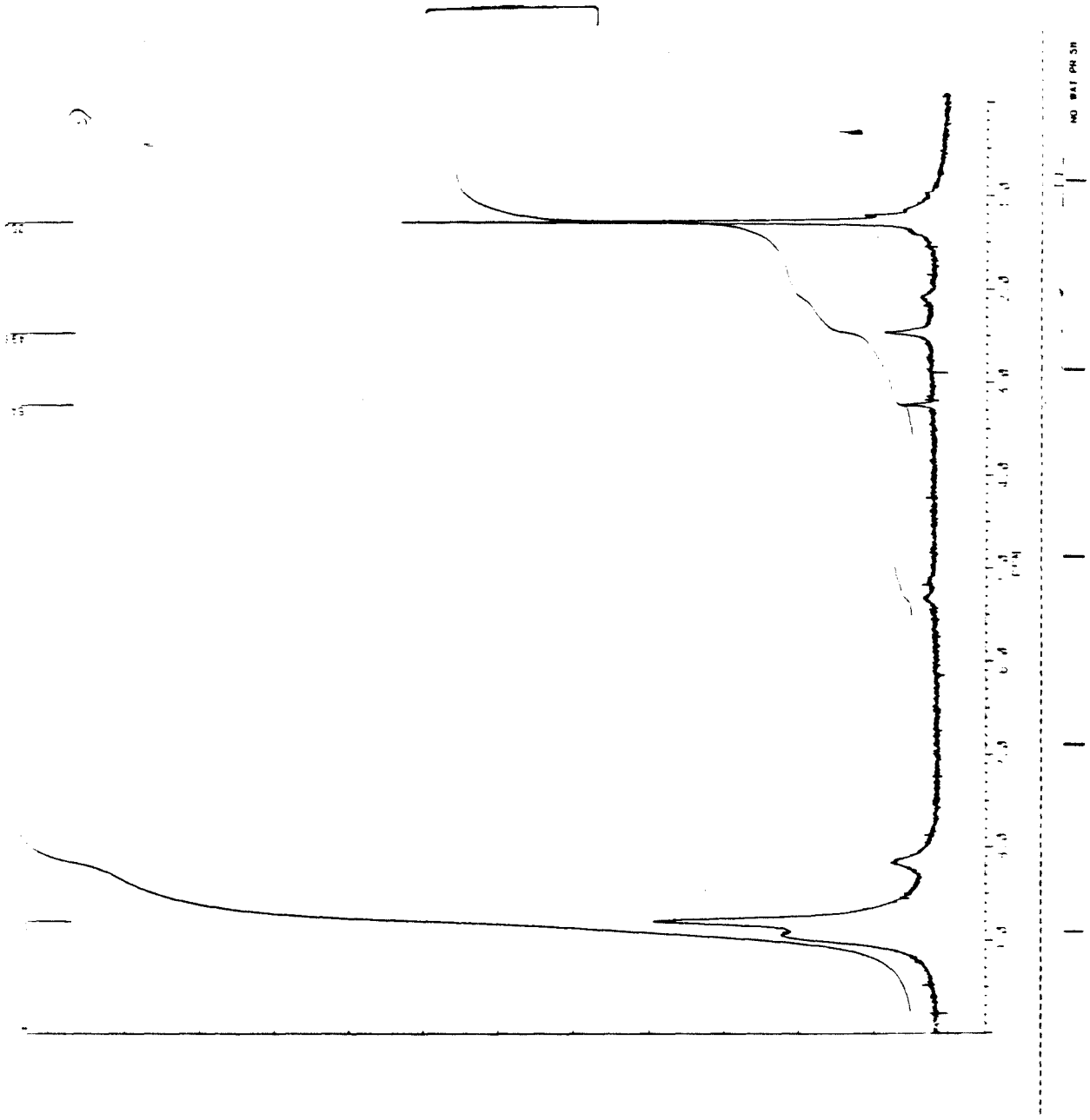


Figure 4.17 NMR Spectra of bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate (Method II)

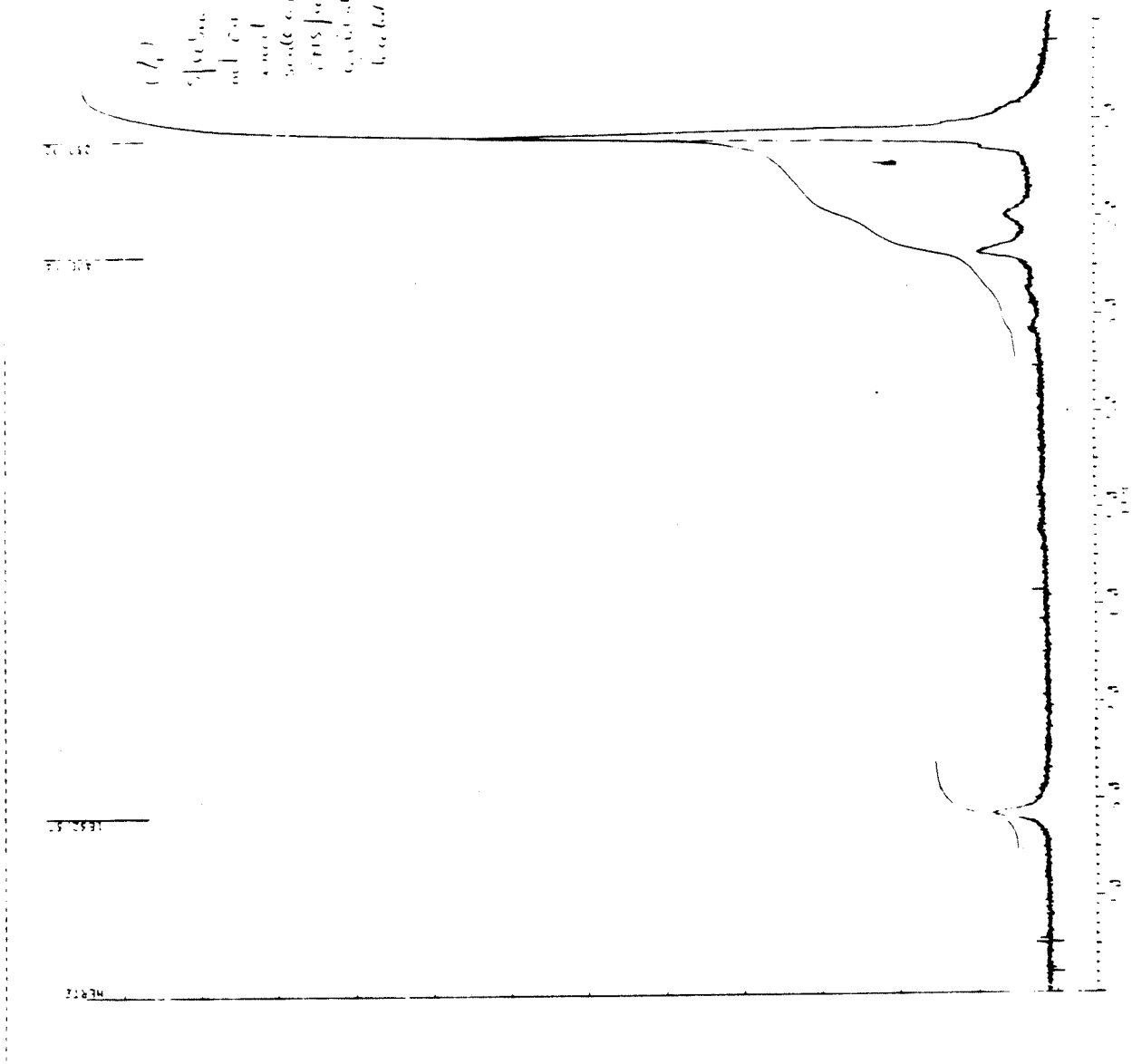


Figure 4.18 NMR Spectra of tetrakis-(2,2,6,6 tetramethyl oxyl 4-piperidyl) pyromellitate

4.6 ESR Analysis

The ESR Spectra attached figs. 4.19 - 4.21 for the bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate (two different routes) and tetrakis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) pyromellitate show clearly the interaction of a free electron spin with two chemically equivalent nitrogen nuclei.

The g, A and linewidth values are given below.

Table V

Compounds	g	A	LW
Bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate(method I)	2.0060	7.7G	3.0G
Bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate(method II)	2.0061	7.8G	2.7G
Tetrakis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) pyromellitate	2.0061	7.6G	3.55G

The samples 1,3 have been deoxygenated by 4-freeze pump thaw cycles; the slightly lesser line width of sample 2 is due to four additional freeze pump-thaw cycles.

From the spectra, therefore, we can consider that the two samples are equivalent proving the presence of two 'N-O' free radicals at the ortho position. ESR spectra of the tetranitroxides at room temperature show wide line width probably merging the exchange interaction of the unpaired electrons with 4 nitrogen nuclei.

They should possess different characteristics features like nine spectral lines at high temperatures due to the effect of conformational electronic exchange through the spatial interaction of all the four nitroxyl groups of the molecule.

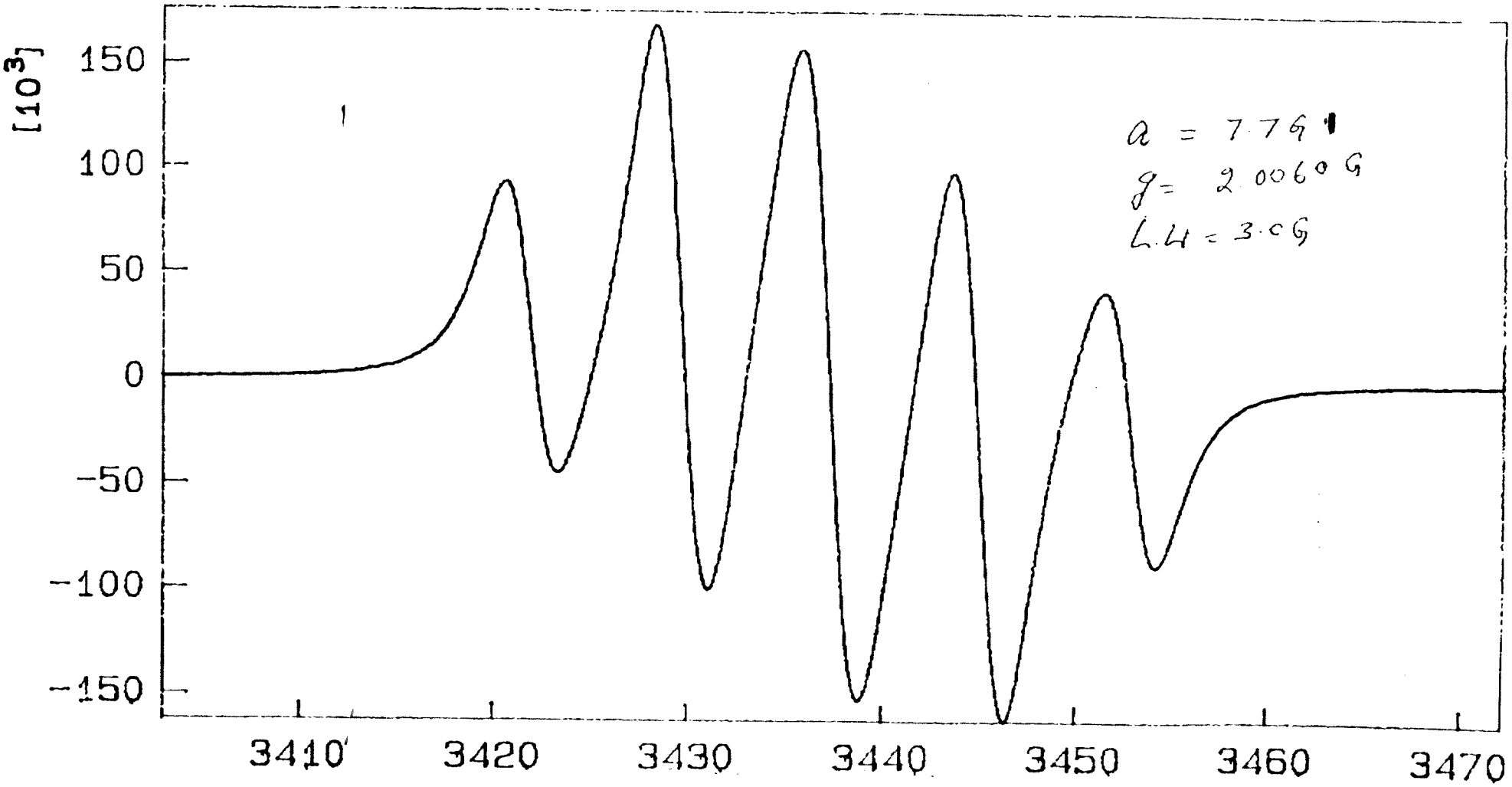


Figure 4.19 ESR spectra of bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate (Method I)

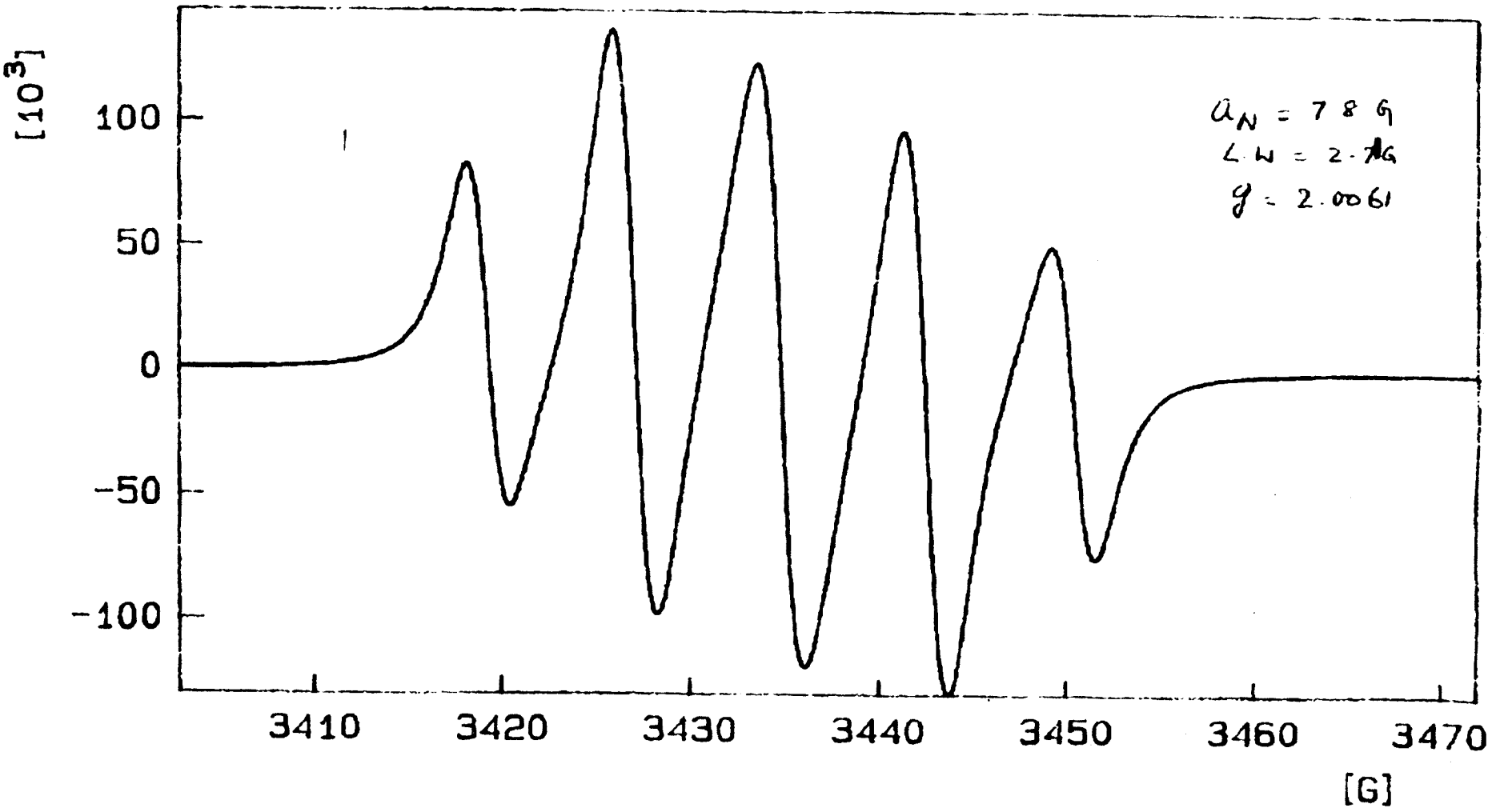


Figure 4.20 ESR spectra of bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate (Method II)

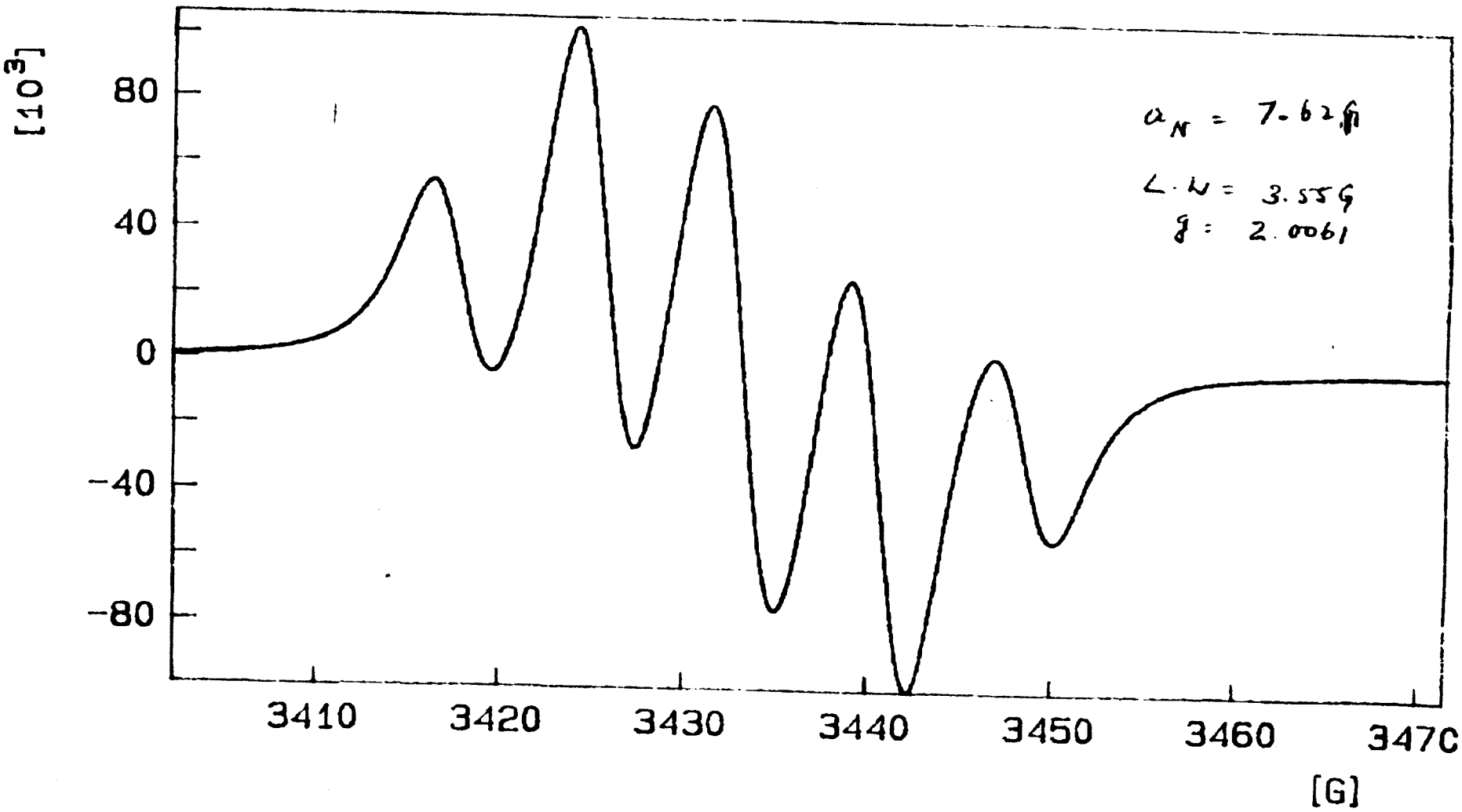


Figure 4.21 ESR spectra of tetrakis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) pyromellitate

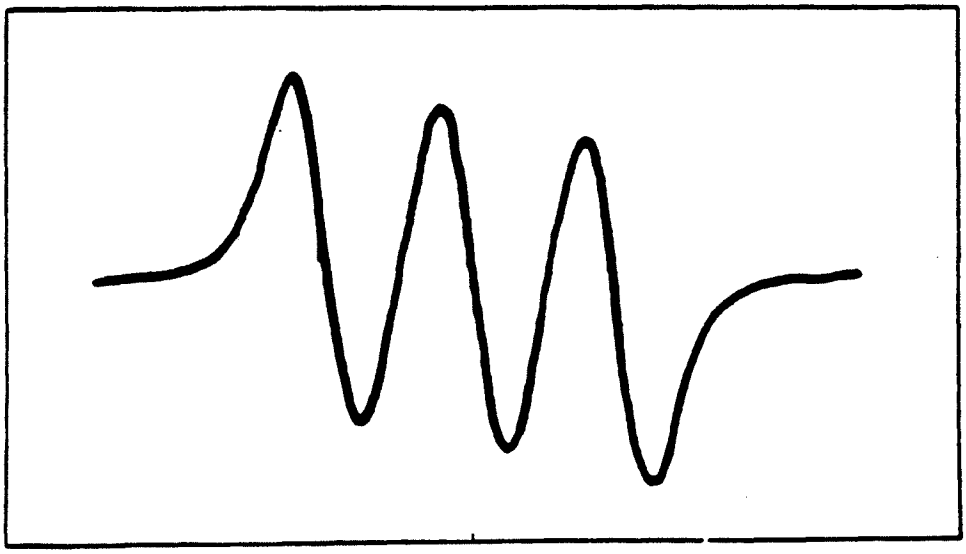


Figure 4.22 E.S.R. spectra of commercial TMPO (Sigma Co.) at 25 °C. Scan Range: 100 G; Modulation Amplitude: 4 G; Receiver Gain: 5; Microwave Power: 2 mW; Field Set: 3240 G; Scan Time: 4 minutes; Microwave Freq.: 9.07813 GHz.

CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

It is found that formation of ester after oxidation is the most feasible method as compared to the oxidation of 'N' after ester formation.

The condensation of the TEMPO with pyromellitoyl chloride should be done with precision and at a very low temperature ~0-4° C. Care should be taken in maintaining this temperature range in order to avoid the formation of the side products, which usually look like a dark slimy residue.

The polymers designed carry different numbers of paramagnetic centers which are in close proximity to each other. With greater molecular weight of six membered ring (piperidine), a given concentration of polyradical nitroxyls is expected to be as effective as a high concentration of an equivalent monoradical or diradical nitroxyls and are also found to have an expanded margin of safety.

These nitroxides are feasible in preparation, have long shelf life and have longer relaxation time compared to inorganic ions (19).

The ESR spectra of the bis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate and tetrakis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) pyromellitate show two equivalent 'N' interacting indicating that two nitroxyl groups are in close proximity to each other. These give a totally different ESR spectra compared to that of the meta or parasubstituted isomers. The meta and the para substituted show only three lines at room temperature where the second and the fourth lines

in the corresponding spectra decrease or vanishes. It is thus found the distance between two nitroxyl groups if less than six carbons, is too much.
Same effect is observed in the expected nine line spectrum of the tetrakis-(2,2,6,6 tetramethyl 1-oxyl 4-piperidyl) phthalate. These isomers need to be heated at higher temperature to get a well resolved spectra.

5.2 Recommendations

Studies on the use of these nitroxides in biological systems and their use as contrast agents for NMR studies is recommended. We recommend designing of derivatized paramagnetic polysaccharides which could be more effective relaxants than the small paramagnetic molecules alone. The soluble and insoluble polysaccharides can be derivatized or spin labelled with TEMPO or other substituted TEMPO. These could serve better than the use of paramagnetic chelated metal derivatives of polysaccharides from the standpoint of drug delivery and residence time in patients..

It is also recommended to introduce more polar functional groups into these molecules by hydroxylation of the carbonyl group into the hydroxyl group. These -OH groups contribute to the intensity by rendering the functional group more polar and thus inducing nitroxyl paramagnetic centers more proximate to water protons and ultimately enhancing the image capability.

These nitroxides prepared should be individually studied for the proton relaxation enhancement capability of nitroxyls taking into consideration the macromolecular environment. Since these could depend on the structure of the nitroxyl agent and the chemical interactions such as the tendency for the protein binding of each nitroxide.

The use of nitroxides in functional biological systems is the ability of cells to reduce nitroxides to hydroxylamines. Studies on the oxidation of the corresponding hydroxyl amines of the compounds prepared should be done enzymatically. The kinetics of these reactions should be studied with respect to biological variables like concentration of the oxygen, the redox state of the cell and the intactness of the membranes.

Also, a kinetic study of the inhibition characteristics of these nitroxides on polymerization should be carried out. These nitroxides with multi nitroxyl centers may possess a step wise radical killing reactivity. Thus the use of these nitroxides as radical terminators and as radical scavengers should be studied.

APPENDIX

Computerized Literature Search

A computerized literature search was done on the use of polynitroxides as contrast agents for MRI using the NERAC Inc. Different databases were tapped.

Most of the references cites the synthesis of polynitroxides. No specific information on mechanism of action was found. The toxicology of the monomers were available but only a couple of citations indicate the toxicity of polynitroxides.

The databases searched were MEDLINE ™ via silver platter CD-ROM, U.S Patent Bibliography, CA search-Physical, SCISEARCH (File 34), SCISEARCH (File 434), BIOSIS (File 55), EMBASE (File 72).

The sources that yielded no information were: Pharmaceutical and Health care Industry News Database (Files 129,130); Biobusiness (File 285); Biocommerce Abstracts (File 286); Chemical Engineering and Biotechnology Abstracts (File 315); Chemical Industry Notes (File 19); Chemical Business Newsbase (File 319); NEXIS access to PTS PROMT, and Newspaper and Magazine Files.

Example Keyword Sequence for Computerized Literature Search

file 55: BIOSIS ™ PREVIEWS -1985 thru 1992

Set	Description
1	Nitroxide*\al
2	ESR\al
3	Electron Spin Resonance\al
4	MRI\al

5	Magnetic Resonance Imaging\al
6	Contrast Agent*\al
7	Cancer Detect*\al

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