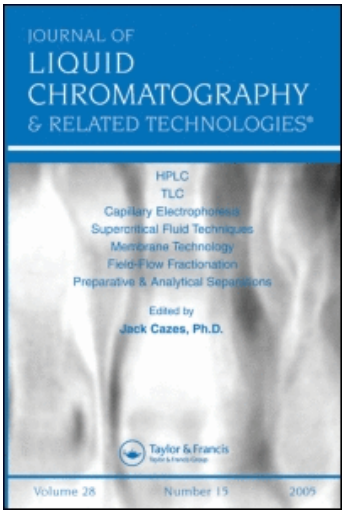


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# SEPARATION AND IDENTIFICATION OF SOME NITROXIDIC DERIVATIVES OF NICOTINIC ACID AND ISO-NICOTINIC ACID BY HPTLC COUPLED WITH ELECTRONIC PARAMAGNETIC RESONANCE (EPR)

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# SEPARATION AND IDENTIFICATION OF SOME NITROXIDIC DERIVATIVES OF NICOTINIC ACID AND *ISO*-NICOTINIC ACID BY HPTLC COUPLED WITH ELECTRONIC PARAMAGNETIC RESONANCE (EPR)

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## ABSTRACT

High performance thin layer chromatography (HPTLC) can be coupled with spectrometric methods in order to improve the selectivity and sensitivity of component identification. The coupling of HPTLC with EPR involves removing each spot together with the stationary phase from the plate, deposition on the quartz capillary, and analysis. This method is used to separate

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and identify some nitroxidic derivatives of nicotinic acid and *iso*-nicotinic acid. The advantage of this method is elimination of the transfer procedure of the substance from the spot, due to the fact that the EPR spectra are recorded directly from the adsorbed-labeled molecules on silica gel. Moreover, this coupling represents a non-destructive method.

## INTRODUCTION

The identification of unknown compounds from a mixture is one of the important problems in chromatography. Planar chromatographic methods provide insufficient qualitative information in order to obtain a reliable identification of compounds using only their retention values. Coupling the thin-layer chromatography (TLC) with spectrometric methods, resulting in an increased amount of information can solve this problem. This information is usually sufficient to identify and to confirm the structure of the analyzed compound, or to identify the compound by comparison with standard substances. The most used combinations are obtained by coupling TLC with ultraviolet-visible (UV/VIS) and fluorescence spectrometry,[1,2] infrared spectrometry (IRS),[3,4] Raman spectrometry (RS),[5] photoacoustic spectrometry (PAS),[6] and mass spectrometry (MS).[7,8]

This paper presents the separation and identification of some nitroxidic derivatives of nicotinic and *iso*-nicotinic acid by high performance thin layer chromatography (HPTLC) coupled with electronic paramagnetic resonance (EPR). Only a few attempts are performed in order to identify the compounds by coupling of EPR with chromatographic techniques, especially in the field of high performance liquid chromatography.[9,10]

## EXPERIMENTAL

### Materials

The solutions of final reaction mixtures were prepared in methanol. All solvents were of analytical grade and were obtained from "Reactivul" (Bucharest, Romania). Chromatography was performed on $10 \times 20$ cm plastic HPTLC sheets pre-coated with silica gel 60 $F_{254}$ (Merck).

### Chromatography

The solutions (0.2 μL) of mixtures were applied as bands to the plates using a micropipet. The plates were developed at room temperature, in a saturated

*N*-chamber, by the ascending technique. The development distance was about 180 mm, and the time required for this was ca. 30 min. The mobile phase was anhydrous methanol–water (96 : 4).

### Electronic Paramagnetic Resonance

Samples (powder probes of adsorbed labeled molecules on $SiO_2$ chromatographic flat) were placed in a 20 mm length, 1 mm id quartz capillary. EPR spectra were recorded at room temperature with a JEOL-JES-3B spectrometer, operating in the X-band (~9.5 MHz) and equipped with a computer-acquisition system. The amplitudes of 12.5 and 100 kHz, the modulation, and the microwave power level were chosen at subcritical values (0.5 G and 20 mW, respectively) to reach the best signal/noise ratio.

## RESULTS AND DISCUSSION

The derivatives of nicotinic and iso-nicotinic acid, 3- and 4-[*N*-4-(2,2,4,4-tetramethyl-piperidinil-1-oxyl)]-carboxamide-1-R (where $R =$ methyl or $R =$ dodecyl)-pyridinium iodide free radicals, were synthesized and investigated as new useful spin labels.[11] The structures of these compounds are presented in Fig. 1.

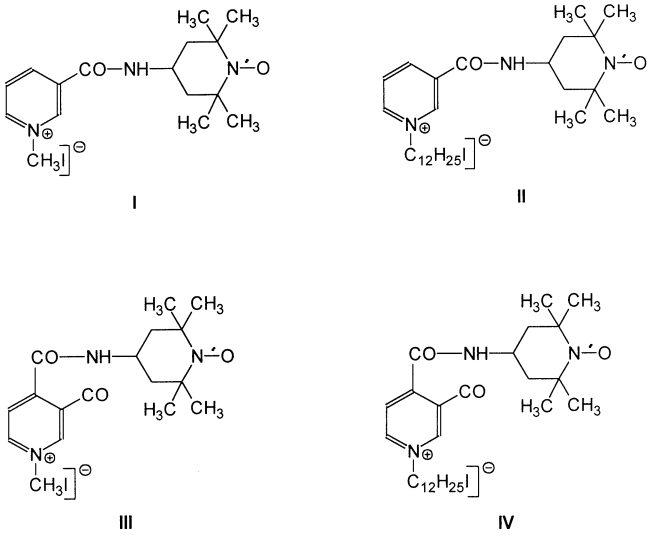


***Figure 1.*** The chemical structures of compounds I–IV.

The synthesis of structures I–IV was performed from the corresponding carboxylic acid and 2,2,4,4-tetramethyl-piperidinil-1-oxyl (aminotempo), in the presence of dicyclohexylcarbodimide. After removing the excess of carboxylic acid through washing with sodium bicarbonate, the reaction mixture was extracted with diethyl ether.

Due to the difficulties in interpretation of the recorded EPR spectra in various environments, it was decided to perform a more precise chromatographic separation of the spin probes. Contamination with starting materials through incomplete reaction and/or partial hydrolysis of compounds I–IV could be taken into account. Furthermore, the EPR spectra of the mixtures of free stable nitroxidic derivatives are misinterpreted because values of hyperfine coupling constants are almost equal or even identical. For these reasons, we tried to separate the components of a paramagnetic system by HPTLC followed by the EPR detection of each spot.

After chromatographic runs, four spots were obtained from each mixture. Each spot was removed from the plate, deposited together with the stationary phase on the quartz capillary, and then analyzed.

The nitroxide radicals are stable free radicals containing a paramagnetic moiety given by an unpaired electron located on the N−O bond. The spin quantum number of the electron $S = 1/2$, and the spin I of $^{14}N$ is 1. Selection rules state that $^{14}N$ has three $(2I + 1)$ energy levels. Thus, the two $S$ electronic energy levels $(\pm 1/2)$ are split into three sub-levels, characterized by a nuclear spin quantum number ($M = \pm 1, 0$), giving rise in the EPR spectra to three absorption peaks of equal probability. The EPR spectra from nitroxide spin-labeled biomolecules are sensitive to rotational motions on the scale of $10^{-11}$ to $10^{-8}$ sec, which is determined essentially by the relaxation processes arising from modulation of the $^{14}N$-hyperfine and $g$-value anisotropy. The rate of motion determines the relative widths of the various resonance signals.

The EPR spectra for the compounds (a–d) from mixture I are presented in Fig. 2. From the EPR spectra of spin probes the following can be concluded:

- It can be seen that in the (a) and (b) cases, the absence of an EPR signal show that the spin probes are not present in the samples;
- The differences observed in the EPR intensities indicate that the amount of the spin probe was appreciably larger in case (c) than in case (d). The case (c) represents the EPR spectrum of reacted nitroxide radical and the case (d) represents the EPR spectrum of unreacted nitroxide radical;
- The EPR spectrum (c) shows a slow rate of motion of spin probes with dominated spin–spin exchange (Heisenberg) effects and dipolar interactions, due to the increase of the density of paramagnetic centers on the surface support.[12]

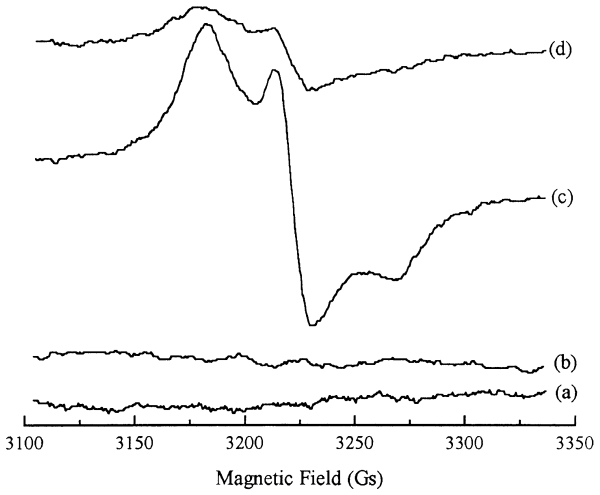


***Figure 2.*** The EPR spectra for the separated compounds (a–d) from mixture I.

The same spectra were obtained for mixtures II–IV. It can be concluded that in each case, the nitroxide radicals of nicotinic acid and *iso*-nicotinic acid derivatives are obtained.

The main advantage of this method is the elimination of the transfer of substance from the spot, because EPR spectra are recorded directly from the adsorbed-labeled molecules on silica gel. Moreover, it can be used for smaller quantities of samples than those used in the liquid chromatography separations. The identification of compounds by the HPTLC coupled with EPR is a non-destructive method, and the substance can be rapidly retrieved after analysis. Later research will take into account the possibility of quantitative determination by on-line coupling of HPTLC with EPR, because this method is faster and cheaper than high performance liquid chromatography.

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