

A PERFORMING SYNTHESIS STRATEGY OF LUMINOL, A STANDARD CHEMILUMINESCENT SUBSTANCE

Răzvan PETRE¹, Gheorghe HUBCĂ²

Rezultatele principale ale studiului constau în dezvoltarea unei tehnologii de laborator pentru sinteza luminolului și a intermediarilor săi: α -nitronaftalină, acid 3-nitroftalic și 3-nitroftalhidrazidă. Cromatogramele totale și spectrele de masă au fost comparate cu standardul de referință Merck pentru luminol. Avantajele acestei tehnologii sunt: luminolul sintetizat prezintă o puritate mai mare decât produsul de referință și este mai ieftin.

The main results of the present study reside in the development of an original laboratory technology for the luminol synthesis and its intermediaries: α -nitronaphthalene, 3-nitrophthalic acid, and 3-nitrophthalhydrazide. The total chromatograms and the mass spectra have been compared with a Merck reference luminol. The advantages of this technology are: the synthesized luminol presents a higher purity against the standard and is costly available.

Keywords: chemiluminescence, luminol, chromatography, mass spectrometry

1. Introduction

The light-generating compounds belong to various classes, such as: organic compounds: aromatics (naphthalene, anthracene, phenanthrene, pyrene, perylene, etc.), fluorescein, rhodamine, oxazine, aminoacids (tryptophan, phenylalanine, etc.); inorganic compounds: uranyl (UO_2^{2+}), lanthanides (Eu^{3+} , Tb^{3+}), glasses doped with Nd, Mn, Ce, Sn, Cu, Ag), crystals (ZnS , CdS , ZnSe , CdSe , Gas, Gap, $\text{Al}_2\text{O}_3/\text{Cr}^{3+}$ (ruby)), etc.; organometallic compounds, etc.

Representative chemiluminescent systems with various applications include the following categories: phthalhydrazide, dioxethanes (lophine, lucigenine, acridine, tetrakis(dimethylamino)ethylene, etc.), acridine derivatives, derivatives of oxalic acid (ester oxalates, oxalic anhydride oxamide, oxalic chloride). Among these compounds, luminol (3-aminophthalhydrazide) is a typical chemiluminescent compound, used in special applications: determination of hydrogen peroxide concentration in wastewater treatment, bactericidal agent, bleaching; indirect determination of a large number of natural compounds via

¹ Eng., Scientific Research Center for CBRN Defense and Ecology, Romania, e-mail: razvan_petre74@yahoo.com

² Prof., Department of Bioresources and Polymer Science, University POLITEHNICA of Bucharest, Romania.

enzymatic reactions that generate hydrogen peroxide; determination of thyroxin; acidity determinations of products like milk, wine, fruit juices, fats and oils in highly colour or turbid samples; immunochemical and immunoenzymatic tests; structural and quantitative determinations of proteins; study of leukocyte chemiluminescent emission, with drug testing availability or indirect determination of chemical parameters implying laborious requirement by immunoassay; N-nitrosamine derivatives analysis (nitrosamines are potentially carcinogenic and are produced during processing of nitrite-containing foods); toxic metal contaminants determinations from water and food (Cd, Cr, Pb, Zn, etc.); bacterial contamination determinations from air, water, food and various objects; rapid measuring of organic-phosphorus pesticides residual concentrations in soil and food products; detection of chemical warfare agents and explosives; traces of blood evidence in forensic investigations [1-4].

Luminol has two isomers, with the chemical structures illustrated in Fig. 1. Isomer II is less stable, it does not emit fluorescence in acidic or neutral solutions and in basic solutions, even in the presence of catalysts, nor chemiluminescence. On the contrary, isomer I emits fluorescence in neutral or acidic solutions and in basic solutions, in the presence of an oxidant, and also chemiluminescence. Luminol chemiluminescence reaction is catalysed by a variety of oxidants, such as ozone, hypochlorites, halogens, Fe^{3+} complex salts, oxidized transition metals, singlet oxygen, proteins that contain iron, persulphates and radioactive radiation.

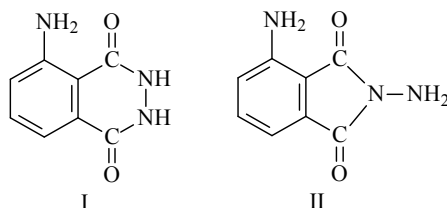


Fig. 1 Luminol isomers.

The present article presents an improved method for luminol obtaining. Advanced luminol synthesis and purification is a topical study due to: on the one hand, current methods are expensive, and, on the other hand, the necessary reagents used during chemiluminescent measurements must have a very high purity. Any impurity conducts to chemiluminescent competitive reactions, with negative extinction effects on the light-generating reactions (quenching).

2. Materials and methods

2.1. Materials

The reagents have been used as received, without any further purification: naphthalene (C_{10}H_8) 98%, nitric acid (HNO_3) 73%, sulphuric acid (H_2SO_4) 95%,

chloroform (CHCl_3) 98% from Merck; sodium carbonate (Na_2CO_3) 98%, chromic anhydride (CrO_3) 99 %, ethyl ether ($\text{C}_4\text{H}_{10}\text{O}$) 99%, tetralin ($\text{C}_{10}\text{H}_{12}$) 99% from Fluka; barium carbonate (BaCO_3) 98%, hydrazine sulphate ($\text{N}_2\text{H}_4\cdot\text{H}_2\text{SO}_4$) 98%, benzene (C_6H_6) 99%, sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4\cdot 2\text{H}_2\text{O}$) 98% from Sigma; methanol (CH_4O) 99%, glacial acetic acid ($\text{C}_2\text{H}_4\text{O}_2$) 96%, hydrochloric acid (HCl) 2N, sodium hydroxide (NaOH) 2N, ammonium hydroxide (NH_4OH) 25% from Chimopar.

2.2. Methods

The laboratory technology for luminol synthesis has been chosen after having considered the following factors involved in the process: reaction yield, reagents availability, costs, completing reaction time, purity of reaction products, number of steps, operation simplicity, temperature influence, handling problems.

There are several pathways to synthesize luminol in function of the first reagent used, accordingly to the scheme presented in Fig. 2. The first common intermediate of these methods is 3-nitrophthalic acid. It can be obtained either by phthalic acid nitration or phthalic anhydride, or by naphthalene nitration, followed by α -nitronaphthalene oxidation.

The nitration of phthalic acid or phthalic anhydride conducts to a mixture of 3- and 4-nitrophthalic acids which are difficult to separate due to similar properties. The literature states that a complete separation of the two isomers cannot be accomplished [5]. Residual amounts of 4-nitrophthalic acid adversely affect subsequent steps of luminol synthesis, contributing to a significant contamination with unwanted reaction products. For these reasons, the second pathway was preferred (i.e., α -nitronaphthalene oxidation), which conducts to a highly pure product. Given the above considerations, the synthesis of luminol that has as starting point naphthalene has been chosen (Fig. 2).

2.2.1. α -Nitronaphthalene synthesis

Naphthalene nitration has been performed by several methods with various nitrating agents. The best results for obtaining of α -nitronaphthalene have been achieved following an original synthesis method.

In the experimental, four different methods from the literature for naphthalene nitration have been tested, whose main features are:

- Piria method: naphthalene nitration with nitric acid at room temperature for 5-6 days; purification consisted in recrystallization from methanol [6];
- David method: naphthalene nitration with sulphonitric acid at 50-60 °C for 7 hours; purification consisted in recrystallization from xylene [7];
- Vogel method: naphthalene nitration with sulphonitric acid at 45-60 °C for 90 min.; purification consisted in recrystallization from diluted alcohol [8];
- Duvalma method: nitration of melted naphthalene with sulphonitric acid at 95 °C for 30 min.; purification consisted in recrystallization from methanol [9].

The best results have been obtained with an original method, related to Duvalma method. New reaction parameters have been set and the final product has been obtained with higher yield and purity above 99%, due to additional purification steps introduced. The synthesis pathway developed is given in Fig.2.

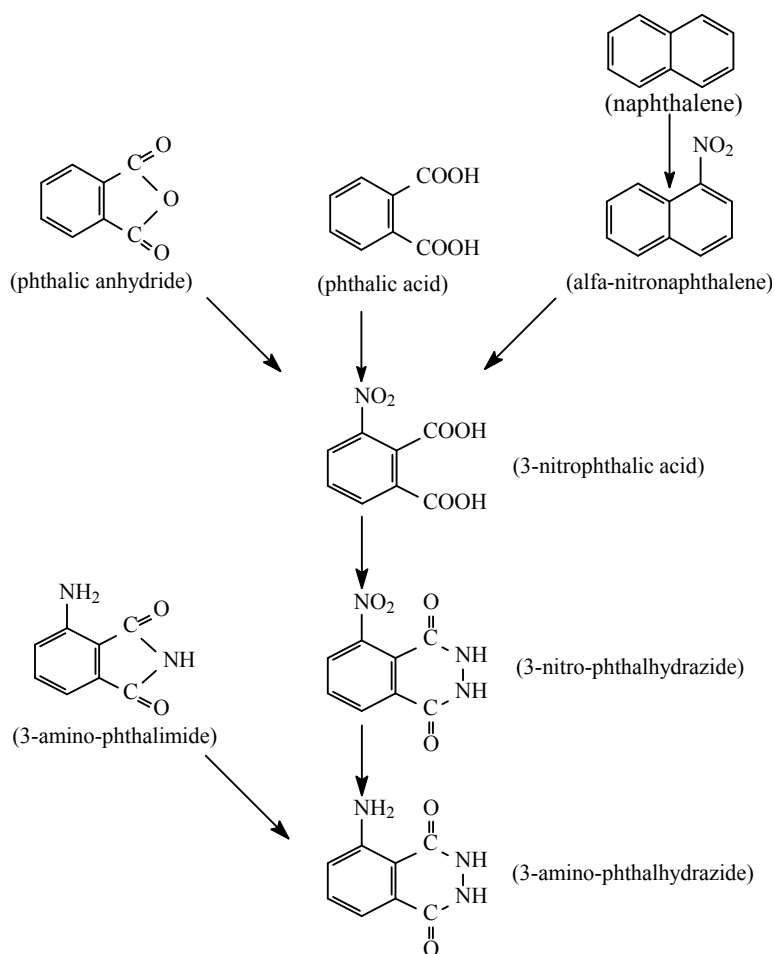


Fig. 2 Luminol synthesis pathways.

Sulphonitric acid has been prepared by adding under continuously stirring, 4 ml of water, 15 ml nitric acid and 17 ml sulphuric acid. 30 g naphthalene (m.p.=80 °C) have been introduced in a 250 ml flask equipped with magnetic stirrer, thermostatic heating bath, thermometer, dropping funnel and condenser. The flask has been heated at 85 °C. The sulphonitric acid has been added dropwise under vigorous stirring, during 10 min. The reaction mixture has been stirred for 25 min. Melted nitronaphthalene has been poured under vigorous

stirring into 200 ml of cold water, thus being washed and pelleted. The yield in α -nitronaphthalene has been 93%. The melting temperature of the raw product before purification was 51-52 °C. Besides α -nitronaphthalene, the following secondary products have been obtained: β -nitronaphthalene, dinitronaphthalene and dinitronaphthol. The acid solution has been separated and 200 ml of water have been added. The mixture has been brought to neutral pH with a 20% Na_2CO_3 solution at 60-70 °C. The solution has been cooled at room temperature and the precipitate has been filtered and dried. Recrystallization from methanol gave α -nitronaphthalene with over 99% purity, accordingly to gas chromatograph system coupled with mass spectrometer (GC-MS) analysis and the m.p.=59 °C.

2.2.2. 3-Nitrophthalic acid synthesis

3-Nitrophthalic acid has been obtained by oxidation of α -nitronaphthalene with chromic anhydride. The results obtained by phthalic acid nitration (Culhane's method [10]) have been unsatisfactory due to the obtaining of a mixture of 3- and 4-nitrophthalic acids, which are difficult to separate. Residual amounts of 4-nitrophthalic acid that cannot be completely removed, lead to spoilage of luminol synthesis.

A high purity 3-nitrophthalic acid has been obtained by the method described by Beilstein [11], consisting in the oxidation of α -nitronaphthalene with chromic anhydride.

5 g α -nitronaphthalene and 35 ml glacial acetic acid have been introduced in a 500 ml flask equipped with magnetic stirrer, cooling bath, thermometer and condenser. 25 g chromic anhydride have been added gradually, under stirring, during 15 min. The reaction mixture was stirred for 90 min. at 70-80 °C, and it was stopped by adding 250-300 ml of water. Exothermal reaction that takes place must be controlled in order not to exceed 80 °C. The mixture was cooled at room temperature and the insoluble impurities (unreacted α -nitronaphthalene), filtered. In order to remove the by-product (nitrophthalide), the solution has been washed with 80 ml chloroform. The separated aqueous solution was treated with 50 g BaCO_3 , at boiling temperature. The carbonate is added in small portions due to release of carbon dioxide, which conducts to foaming. The precipitate, consisting in barium chromate, barium carbonate in excess and barium salt of 3-nitrophthalic acid, has been separated by filtration and washed with water until turning into bright yellow. The precipitate has been treated with 25 ml of 10% Na_2CO_3 solution, for 30 min. at boiling temperature. Sodium salt of 3-nitrophthalic acid was transferred into an aqueous solution. The hot mixture has been filtered and then acidified at 50-60 °C, with 30 ml HCl to pH=1, determining the formation of 3-nitrophthalic acid. The solution has been concentrated by heating to the four folds decrease of volume, and then cooled at 5 °C. The precipitate has been filtered, washed with cold water and dried. The 3-

nitrophthalic acid has been obtained with 64% total yield and 99% purity, accordingly to the GC-MS analysis.

2.2.3. 3-Nitro-phthalhydrazide synthesis

The synthesis of 3-nitrophthalhydrazide using Redemann method [12] has been chosen after the literature analysis and the evaluation of available reagents.

8 g of 3-nitrophthalic acid, 12 ml of water and 2 drops of phenolphthalein solution have been introduced in a 250 ml flask equipped with magnetic stirrer, thermostatic heating bath, thermometer, dropping funnel and condenser. The mixture has been passed to slightly alkaline pH with 25 ml of 2N NaOH solution. The last droplets have been added under stirring, in order to observe the turning point of phenolphthalein. The weak pink colour disappeared when a few pellets of 3-nitrophthalic acid and 5 g hydrazine sulphate have been added. The mixture has been evaporated to dry, taking care to ensure further a good stirring. The completely dry solid has been introduced together with 20 ml decalin in a flask and stirred for 3 hours at 160-170 °C. In the last 30 min., the colour turned from bright yellow to dark yellow. The mixture has been cooled at room temperature and 10 ml of benzene have been added. The precipitate has been filtered and washed with 15 ml of ethyl ether on a sintered glass filter funnel. 12.3 grams of equimolecular mixture consisting in sodium sulphate and 3-nitro-phthalhydrazide have been obtained, being further used directly in the synthesis of luminol. The purification of the resulting reaction mixture has not been necessary.

2.2.4. 3-Amino-phthalhydrazide (luminol) synthesis

The final phase of luminol synthesis has been performed using a Redemann method [13]. The method has as starting point the equimolecular mixture consisting in sodium sulphate and 3-nitro-phthalhydrazide resulted from the previous phase. The mixture has been introduced together with 42 ml of water and 23 ml of 25% NH_4OH solution in a 250 ml flask equipped with magnetic stirrer, thermostatic heating bath and thermometer. The solid phase has been dissolved and 20 g sodium dithionite have been added, stirring the mixture for 7 min. at 60-70 °C and then filtering the insoluble impurities. The red-brownish filtrate has been heated for 30 minutes at 70 °C. During the last 10 min. a yellow precipitate separated. The hot solution was acidified with glacial acetic acid to pH=3. The precipitate turned into bright yellow. The mixture has been allowed to stand overnight and then the precipitate has been filtered. The luminol has been washed thoroughly with cold water and dried. For subsequent purification, the luminol has been dissolved in hot HCl (w:v=5:100 versus water) and decolorizing charcoal has been added. The mixture has been filtered immediately and passed to slightly acidic pH with ammonium hydroxide solution. The pH has been checked with Congo red paper. The mixture has been cooled to room temperature and then the precipitate has been filtered, washed thoroughly with cold water and dried.

The synthesized 3-amino-phthalhydrazide (luminol) had over 99% purity, accordingly to GC-MS analysis and m.p.=328 °C.

2.2.5. Instrumental analyses of the organic compounds synthesized

The analytical determinations of the compounds synthesized have been performed with a GC-MS Carlo Erba QMD 1000, $T_i = 60$ °C, $T_f = 300$ °C; $v = 15$ °C/min., injection volume = 1 μ l, He (99.9999% purity) carrier gas at a rate of 1.5 ml/min. Mass spectrum has been obtained under the following conditions: ionization chamber temperature: 120 °C; current: 150 μ A; temperature of transfer line 270 °C; ionization energy: 70 eV. The Wiley / NIST (National Institute of Standards and Technology) spectral libraries have been used to identify the compounds.

The products have been also analyzed by nuclear magnetic resonance (NMR) with a Varian Gemini 2000-300 MHz spectrometer. Confirmations of the correct assignments of the chemical shifts have been obtained by COSY experiments.

3. Results and discussion

Naphthalene nitration is presented in Fig. 3, together with the reaction by-products.

The chromatogram of α -nitronaphthalene has two peaks: one located at 2.300 retention time, corresponding to the solvent, and the other one located at 13.482 retention time, corresponding to α -nitronaphthalene. The substance purity is therefore proven higher than 99%.

The $m/e=174$ value corresponds to the molecular peak into the α -nitronaphthalene mass spectrum. The $m/e=127$ fragment results from the elimination of the nitro group from α -nitronaphthalene. Subsequently, the elimination of a carbon atom and two hydrogen atoms conducts to a $m/e=115$ fragment, the tropylium cation that is energetically favoured. This one goes to $m/e=77$, the stable phenyl cation, by elimination of two carbon atoms.

^1H -NMR chemical shifts assignments of α -nitronaphthalene analysis are shown in Fig. 4. α -Nitronaphthalene ^1H -NMR spectrum confirms the chemical structure of this compound and its purity (99%), through different types of protons integral values.

The chromatogram of synthesized 3-nitrophthalic acid presents some very small peaks due to impurities and two prominent peaks: one located at 5.001 retention time corresponding to derivatization agent (tetramethylsilane) and the other one located at 15.172 retention time corresponding to derivatized 3-nitrophthalic acid. From the peak areas ratios, it can be concluded that purity of 3-nitrophthalic acid is 99%.

The $m/e=355$ value corresponds to the molecular peak of derivatized 3-nitrophthalic acid with tetramethylsilane into the mass spectrum. The $m/e=340$ fragment results from the elimination of methyl group and subsequently the $m/e=310$ fragment results from the elimination of two methyl groups. The $m/e=149$ fragment has a comparable ratio and results from the elimination of derivatization agent, carboxyl group and an oxygen atom of the nitro group.

The $m/e=147$ value corresponds to a base peak, attributed to a stable cation corresponding to phthalic anhydride, which resulted from the nitro group elimination from 3-nitrophthalic anhydride. The $m/e=73$ value corresponds to the eliminated trimethylsilane radical, and the $m/e=46$ value corresponds to the eliminated nitro group.

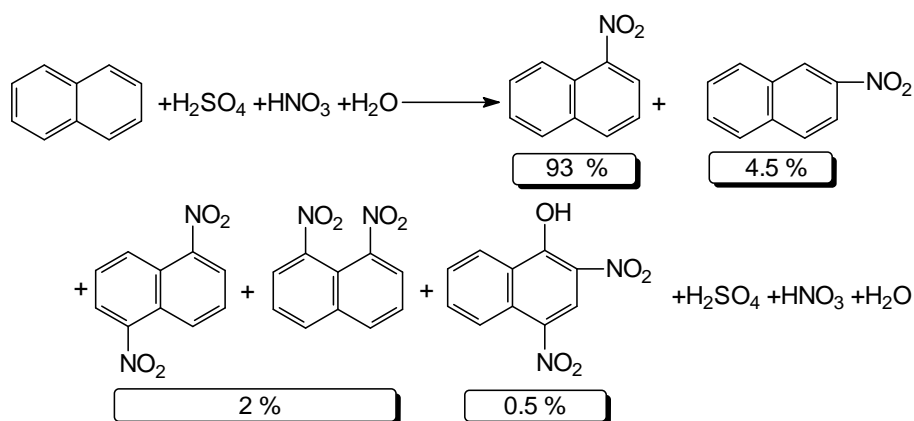


Fig. 3 Naphthalene nitration scheme.

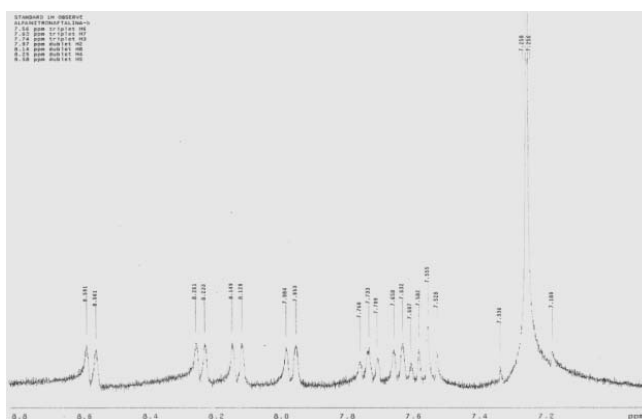


Fig. 4 ¹H-NMR spectrum of α-nitronaphthalene.

The synthesis of 3-nitrophthalic acid is illustrated in Fig. 5. ^1H -NMR chemical shifts assignments of 3-nitrophthalic acid analysis are shown in Fig. 6. The spectrum obtained confirms the chemical structure of the compound and the purity (99%), through different types of protons integral values.

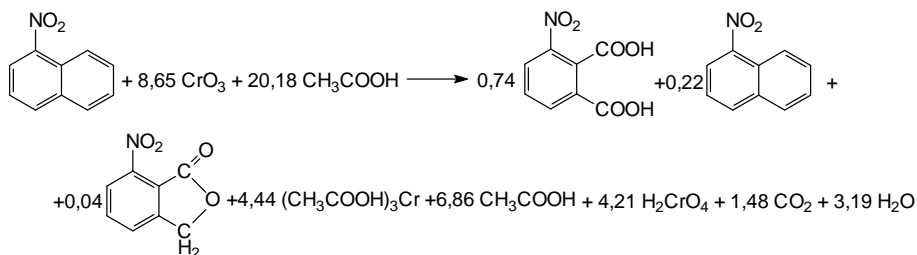


Fig. 5 3-nitrophthalic acid synthesis reaction.

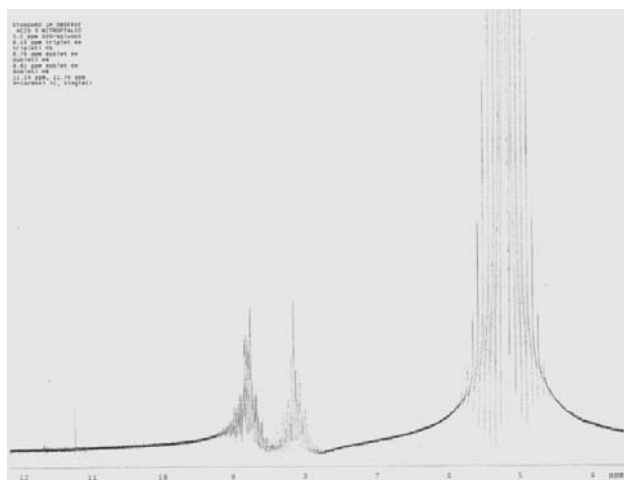


Fig. 6 ^1H -NMR spectrum of 3-nitrophthalic acid

The 3-nitrophthalhydrazide synthesis reaction is presented in Fig. 7, and the 3-amino-phthalhydrazide (luminol) synthesis reaction is given in Fig. 8.

The total chromatograms and the mass spectra have been compared with a Merck reference luminol. The reference compound can be purchased from two providers, Sigma-Aldrich® and Merck Chemicals, at 97% purity.

The luminol chromatogram shows some very small peaks due to impurities and four prominent peaks: one located at 5.131 retention time corresponding to derivatization agent, the second at 15.692 retention time corresponding to luminol with two derivatized groups and the last two peaks located at 16.182 and 16.292 retention times corresponding to luminol with three

derivatized groups and its isomer. Derivatization with tetramethylsilane has been performed because luminol had to be solubilized since a higher volatility is necessary. From the peak area ratio, one may conclude that synthesized luminol has 99% purity (Fig. 9).

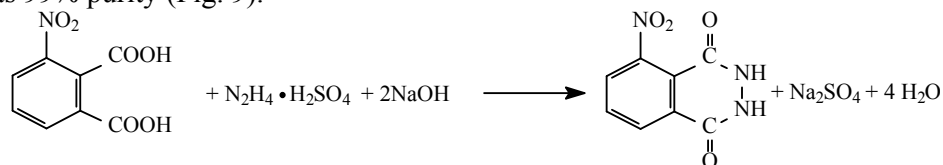


Fig. 7 3-nitrophthalhydrazide synthesis reaction

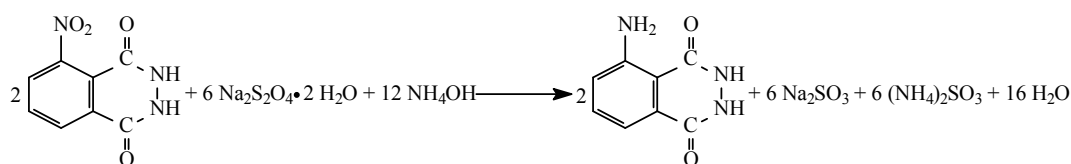


Fig. 8 3-amino-phthalhydrazide (luminol) synthesis reaction.

The chromatogram of the Merck reference luminol presents almost identical retention times with the synthesized luminol. The analysis of comparative chromatograms and peak area ratio indicates that synthesized luminol purity is 99%, while the reference compound (reference luminol) has only 98% purity.

From the mass spectrum of luminol, only representative peaks have been analyzed. The $m/e=393$ value corresponds to the molecular peak of luminol with three derivatized groups. The $m/e=378$ fragment results from the elimination of the methyl group, which passes in $m/e=306$ fragment from the elimination of the derivatized amino group, which subsequently passes in $m/e=290$ fragment from the elimination of a methyl group.

The assigned molar peak value for luminol with two derivatized groups is $m/e=321$. The $m/e=306$ fragment results from the eliminations of the methyl group, which passes in $m/e=248$ fragment from the elimination of the trimethylsilane radical, which subsequently passes in $m/e=234$ fragment due to the elimination of the amino group. The last representative peak, $m/e=73$, corresponds to the eliminated trimethylsilane radical.

3-amino-phthalhydrazide (luminol) analysis through $^1\text{H-NMR}$ is shown in Fig. 10. The spectrum confirms the chemical structure of this compound and the purity (99%), through different types of protons integral values.

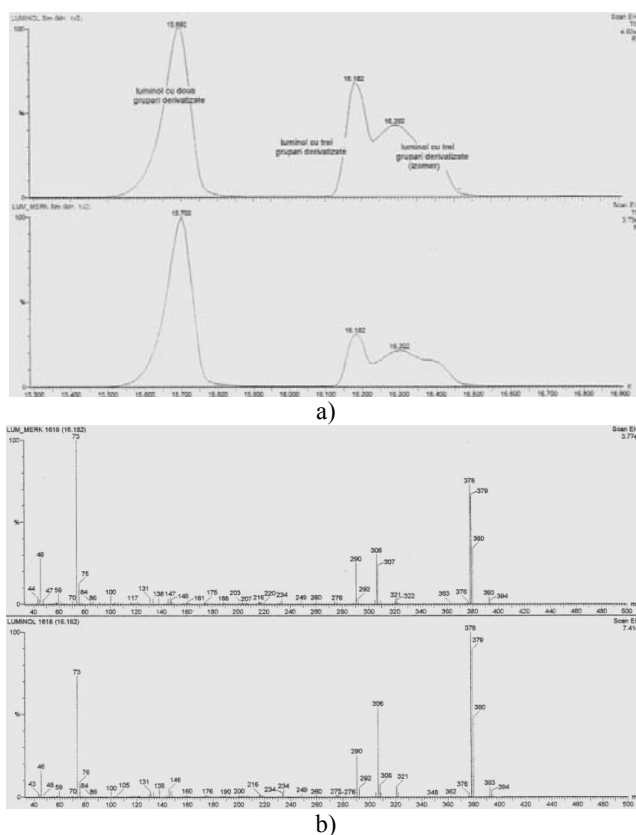


Fig. 9 Comparative chromatograms and mass spectra for synthesized and Merck reference luminol.

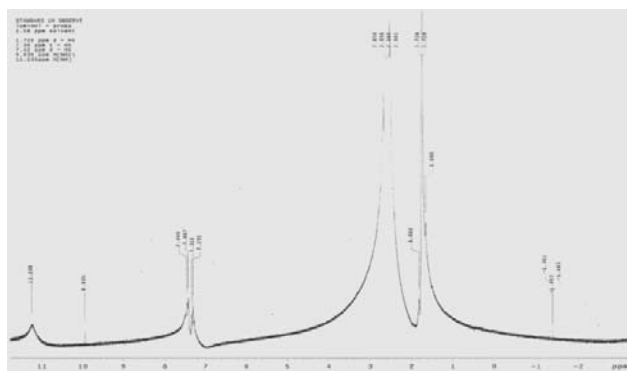


Fig. 10 ^1H -NMR spectrum of luminol

Therefore, the luminol obtained after the completion of presented steps has a 99% purity, appropriate for chemiluminescence determinations.

4. Conclusions

The main results of the present study reside in the development of a laboratory technology for the luminol synthesis and its intermediaries: laboratory technology for α -nitronaphthalene synthesis, for 3-nitrophthalic acid synthesis, for 3-nitro-phthalhydrazide synthesis, for 3-amino-phthalhydrazide synthesis. Every technology approached contains specific instrumental methods of analysis that have been used to confirm the achievement of substances and to determine their purity.

The luminol and the intermediary products have been analyzed through two instrumental methods (GC-MS and NMR).

An original method has been developed, which can be used for the obtaining of luminol with high yield and purity above 99% (as demonstrated by the chromatograms and the comparative spectra for synthesized and reference luminol). The original synthesis strategy described herein conducts to the obtaining of a highly pure product, which can be further used in chemiluminescence measurements.

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