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Review

Forensic application of the luminol reaction as a presumptive test for latent blood detection

Filippo Barni [a,*], Simon W. Lewis [b], Andrea Berti [a], Gordon M. Miskelly [c], Giampietro Lago [a]

[a] *Molecular Biology and Genetics Unit, Carabinieri Scientific Investigation Department of Rome, Viale di Tor di Quinto 119, 00191 Rome, Italy*
[b] *Department of Applied Chemistry, Curtin University of Technology, G.P.O. Box U1987 Perth, Western Australia 6845, Australia*
[c] *Department of Chemistry, The University of Auckland, Private Bag 92019, Auckland, New Zealand*

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Abstract

The forensic application of the luminol chemiluminescence reaction is reviewed. Luminol has been effectively employed for more than 40 years for the presumptive detection of bloodstains which are hidden from the naked eye at crime scenes and, for this reason, has been considered one of the most important and well-known assays in the field of forensic sciences. This review provides an historical overview of the forensic use of luminol, and the current understanding of the reaction mechanism with particular reference to the catalysis by blood. Operational use of the luminol reaction, including issues with interferences and the effect of the luminol reaction on subsequent serological and DNA testing is also discussed.

Keywords: Luminol chemistry; Chemiluminescence; Forensic; Latent bloodstains; DNA typing

Contents

* Corresponding author. Tel.: +39 347 1849158; fax: +39 02 700412617.
E-mail address: filippobarni@tin.it (F. Barni).

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1. Introduction

The emission of light observed when a solution containing luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione or, more simply, 3-aminophthalhydrazide) and hydrogen peroxide is sprayed on dried bloodstains has been utilised by forensic scientists in investigations involving violent crime for more than 40 years. This article reviews the current understanding of the chemistry and mechanism of the luminol reaction as it pertains to the detection of bloodstains. A forensic overview of operational use of the luminol reaction will be given including a discussion of interfering species and the possible detrimental effect of luminol on further presumptive tests for blood and DNA typing analyses.

1.1. Chemiluminescence

Chemiluminescence [1] refers to the emission of light from a chemical reaction, which can occur in solid, liquid or gas systems. The fundamentals of chemiluminescence have been comprehensively reviewed in a number of textbooks and articles in recent years [2–5].

Two main categories of chemiluminescent reaction have been described in the literature, direct and indirect. Direct chemiluminescence can be represented by:

$$A + B \rightarrow [I]^* \rightarrow \text{PRODUCTS} + \text{LIGHT}$$

where A and B are reactants and $[I]^*$ is an excited state intermediate. The luminol reaction is an example of this form of chemiluminescence. In certain cases where the excited state is an inefficient emitter, its energy may be passed on to another species (a sensitizer, F) for light emission to be observed. This is called "indirect chemiluminescence" and is exemplified by the peroxyoxalate (light stick) reaction:

$$A + B \rightarrow [I]^* + F \rightarrow [F]^* \rightarrow F + \text{LIGHT}$$

Once a molecule has been converted to a metastable intermediate in an excited state there are a number of routes by which it can return to the ground state. These routes can be displayed diagrammatically, as in Fig. 1, by an "energy well" diagram, or more simply by the Jablonski diagram, first introduced in the 1930s. The light emission can either be fluorescence or chemiluminescence, if from a singlet state, or phosphorescence if from a triplet state.

The light emitted from chemiluminescent reactions has differing degrees of intensity, lifetime and wavelength with the latter parameter covering the spectrum from near ultraviolet, through the visible and into the near infrared.

For emission to be observed from a chemical reaction, three essential energetic requirements need to be met:

1. There should be an energetically favourable reaction pathway for the production of the excited state species. Of the total number of molecules participating in the reaction a significant number should reach the excited state.
2. The reaction is required to be exothermic, with the free energy change being in the range 170–300 kJ mol^{-1}.
3. There should be a favourable deactivation pathway for chemiluminescence emission, with other competitive non-radiative processes such as intra- or intermolecular energy transfer, molecular dissociation, isomerization or physical quenching kept to a minimum.

The intensity of the chemiluminescence emission from a reaction is dependant upon the rate of reaction and the efficiency of the process generating excited state species. The latter can be described by the chemiluminescence quantum yield, Φ_{CL}, which is defined as:

$$\Phi_{CL} = \frac{\text{total number of photons emitted}}{\text{number of molecules reacting}}$$

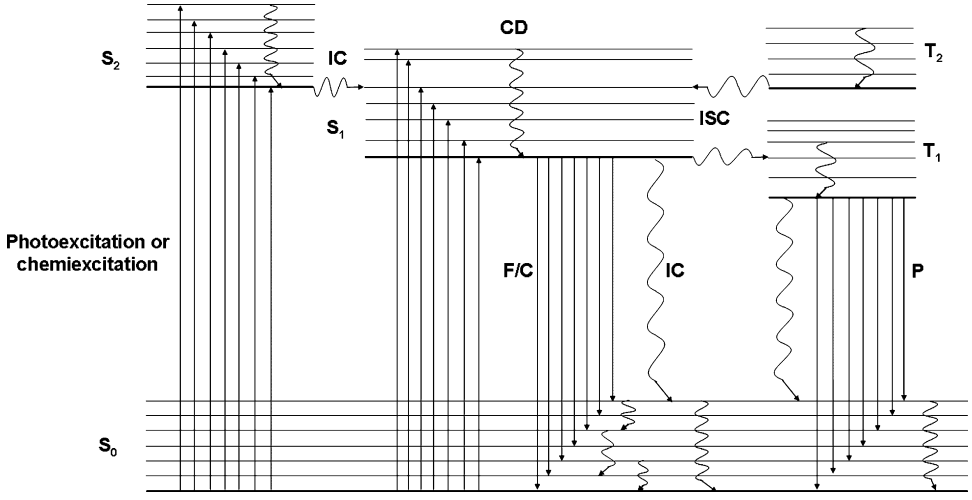


Fig. 1. Jablonski energetic diagram showing energy levels and transitions in a molecular compound: C, chemiluminescence; F, fluorescence; P, phosphorescence; CD, collisional deactivation; IC, internal conversion; ISC, intersystem crossing; S_0, ground singlet state; S_1, S_2, excited singlet states; T_1, T_2, excited triplet states; →, radiative transition; ⇝, non-radiative transition.

Φ_{CL} is the product of three factors: the fraction of excited states produced, Φ_{EX}; the fraction of reacting molecules following the correct chemical path, Φ_R; and the fluorescence quantum yield of the emitter, Φ_F:

$$\Phi_{CL} = \Phi_{EX}\Phi_R\Phi_F$$

Chemiluminescence quantum yields vary widely from 10^{-15} to nearly 1, however most of the reactions used in analysis fall in the range 0.01–0.1 [6]. The use of very sensitive detectors and the almost complete absence of background emission has allowed the monitoring of even inefficient chemiluminescence reactions with quantum efficiencies less than 0.001, such as the oxidative ultra-weak chemiluminescent reactions in living cells [7].

The quantum efficiency and colour of a chemiluminescence emission are greatly affected by the environment in which the reaction takes place. For solution phase chemiluminescence the factors that will affect the reaction are similar to those affecting normal fluorescence and phosphorescence. For example, the chemiluminescence quantum yield and colour of emission for luminol in dimethylsulfoxide and water are 0.05, blue-green ($\lambda_{max} \sim 480$–502 nm) and 0.01, blue-violet to blue-green ($\lambda_{max} \sim 425$ nm), respectively [8,9].

For most analytical purposes it is the chemiluminescence emission intensity (I_{CL}) that is measured, either as an integral over the lifetime of the emission or as a transient response. It is a function of both the efficiency and the rate of the reaction:

$$I_{CL} = \Phi_{CL}\left(\frac{dC}{dt}\right)$$

where dC/dt is the rate of reaction (molecules reacting s^{-1}). Chemiluminescence reactions can occur very rapidly (<1 s) or extremely slowly (>1 day), according to the reaction and the conditions.

1.2. Luminol historical background

Some of the key events in the discovery, study, and use of luminol are shown in Fig. 2. Even though there is some debate as to the first report of the synthesis of luminol [10,11], the German scientist Schmitz has often been suggested as the first to have produced this compound in 1908 [12,13]. Regardless of this controversy, it is now widely accepted that Albrecht was the first to report its involvement in chemiluminescence reactions in 1928 [14] (Fig. 2). Specht, a forensic scientist at the University Institute for Legal Medicine and Scientific Criminalistics of Jena, Germany, first studied in depth the role of hemin, an iron-containing compound derived from heme, in the chemical reaction involving luminol and investigated its potential application in blood detection [15]. This represented the first use of liquid phase chemiluminescence for analytical purposes.

Proesher and Moody [16], investigating both the chemical structure and reaction properties of luminol, correctly predicted the keto-enolic tautomerisation of luminol in alkaline solutions and the fully protonated form in acidic solutions. They concluded that chemiluminescence emission intensity and duration were increased with dried and decomposed blood, aged even for 3 years, with respect to fresh blood. They also observed that luminol solution could be sprayed many times over bloodstains, particularly if dried, allowing a repetition of the chemiluminescence.

McGrath [17] evaluated the specificity of the luminol test on biological fluids and showed that luminol displayed a specificity for blood while appearing insensitive to the other biological fluids studied. Nevertheless, when used as presumptive test for blood identification, he recommended the confirmation of the luminol reaction with other more specific serological tests.

Grodsky et al. [18] proposed a blend of powders made up of luminol, sodium carbonate (Na_2CO_3) and sodium perborate ($NaBO_3{\cdot}nH_2O$) mixed with distilled water. This subsequently became the formula that is most commonly used by today's investigators to detect traces of blood at the scene of a crime. An alternative formulation was proposed by Weber [19] of luminol, sodium hydroxide or potassium hydroxide, and hydrogen peroxide diluted in distilled water. The solution so obtained needed to be kept in a cool place away from direct light and showed a brief lifespan.

Since these early studies in luminol, there have been several other attempts to elucidate the reaction mechanism, with a major effort by Merényi and co-workers in the 1980s culminating in a summary paper in 1990 [20]. Thornton and Maloney

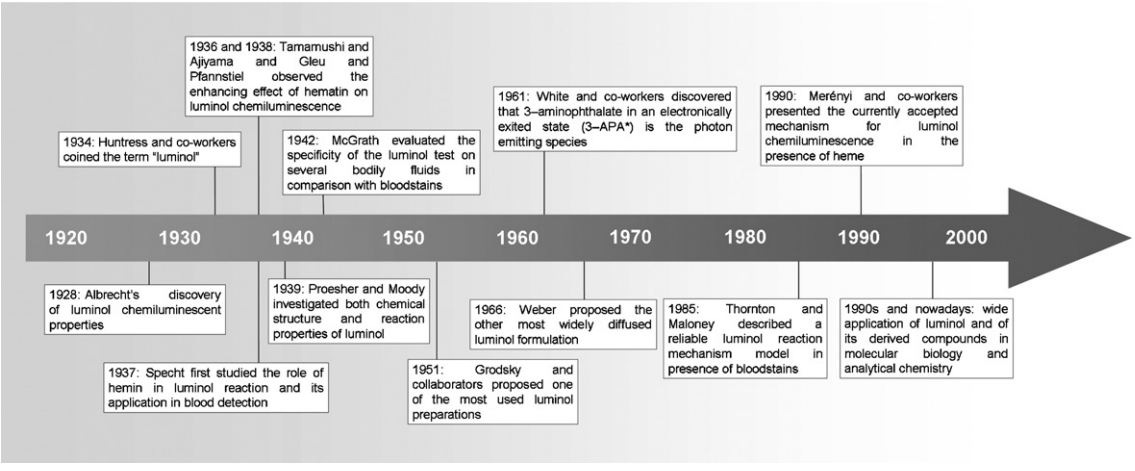
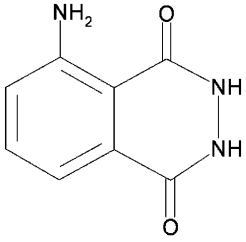


Fig. 2. Luminol timeline from its discovery to the most recent developments.

Table 1
Luminol commonest chemical, physical and toxicological properties [9,32–37]

Names	5-Amino-2,3-dihydro-1,4-phthalazine-dione, *o*-aminophthalyl hydrazide, 3-aminophthalic hydrazide
Molecular and structural formula	$C_8H_7N_3O_2$ 
Molecular mass	177.16 amu
Melting point	319–320 °C
pK_{a1}	6.74
pK_{a2}	15.1
Solubility in water	<0.1 g/100 mL at room temperature
Physical properties	Yellow crystalline solid (grainy crystals)
General properties	Stable at room temperature, sensitive to light, combustible, incompatible with strong oxidizing agents, strong acids, strong bases, strong reducing agents, emits light on reaction with oxidizers (chemiluminescent)
Safety information and potential health effects	The toxicological properties have not been fully investigated in humans; anyway mucosa irritation has been described: eyes, skin, respiratory tract and gastrointestinal tract (with nausea, vomiting and diarrhea). No data available about chronic effects. More information available at The National Toxicology Program (The National Institute of Environmental Health Sciences, NC, USA) website http://ntp.niehs.nih.gov/index.cfm

[13] summarised luminol chemistry from a forensic science perspective in 1985, although their mechanistic argument derived from earlier studies than the Merényi work.

Over the last 20 years luminol has become one of the widest used chemiluminescent reagents for application to molecular biology and analytical chemistry. It has been used as the basis for a multitude of sensitive and selective detection methods including high performance liquid chromatography (HPLC), immunoassay, DNA probes, DNA typing and as substrate in western blot detection [5,21–29]. More recently, also historical and archaeological studies using luminol have been successfully carried out [30,31] disclosing an interesting new application field for luminol-based assays.

2. The luminol reaction

2.1. Luminol chemical and physical properties and chemiluminescence

Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) is a cyclic acyl-hydrazide, and shows the typical reactivity of this class of compounds [32]. Beyond the common chemical, physical and toxicological characteristics which are succinctly described in Table 1 [9,33] and are also available at The National Toxicology Program (The National Institute of Environmental Health Sciences, NC, USA) website (http://ntp.niehs.nih.gov/index.cfm)[1], luminol presents some properties which are especially relevant when it is used for forensic purposes: photo- and thermal-stability and chemical behaviour in protic polar media.

Luminol solutions are sensitive to light and the presence of metal cations; typically they are only stable 8–12 h. Luminol was shown to be thermally unstable, so luminol and its solutions should be protected from high temperature [34].

Two separate pK_a values (6.74 and 15.1), corresponding to loss of the two acyl-hydrazide protons, at (pK_{a1}) and (pK_{a2}) have been found [35–37]. Thus in aqueous solution phase luminol (LH_2) can be found in the fully protonated form in acidic solutions while, when dissolved in a basic solution, above about pH 7, dissociations to the monoanion (LH^-) and dianion (L^{2-}) occur. The fully-protonated and monodeprotonated (monoanionic) forms of luminol can undergo keto-enolic tautomerisation in solution and the solid state [16,32,37] (Fig. 3), although most authors (including us) represent these compounds with the protons on the nitrogens.

Luminol chemiluminescence has recently been reviewed by Barnett and Francis [5]. The light-producing pathway for the oxidation of luminol is a complex multi-step process and is dependent on several factors including pH, temperature and ionic strength of the reaction medium and reactive species that can be present in solution and interact with luminol, metal catalyst or hydroxide ions [5].

White et al. observed that the fluorescence spectrum of an intermediate molecule in the luminol oxidation process named 3-aminophthalate in an electronically excited state (3-APA*) perfectly matched the chemiluminescence spectrum of luminol, thus they concluded that this excited intermediate could be considered the light emitting species upon deexcitation to the ground state (3-APA) [38–40]. This was confirmed in 1965 by Gunder-

[1] The National Institute of Environmental Health Sciences (NIEHS) is one of the National Institutes of Health (NIH) within the U.S. Department of Health and Human Services. The National Toxicology Program is headquartered on the NIEHS campus in Research Triangle Park, NC, USA.

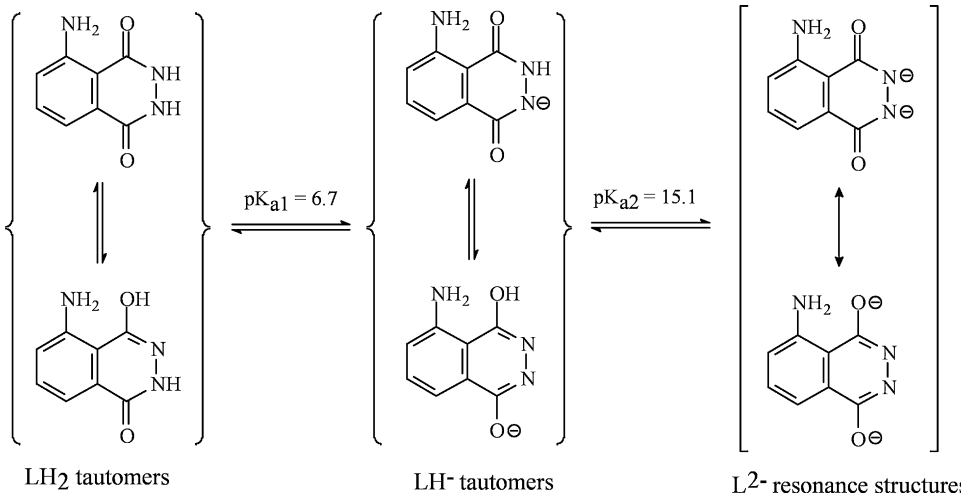


Fig. 3. Luminol protonation and tautomerism in acidic, neutral, and alkaline solution (LH_2, LH^-, and L^{2-} represent the diprotic, monoanionic, and dianionic forms of luminol, respectively).

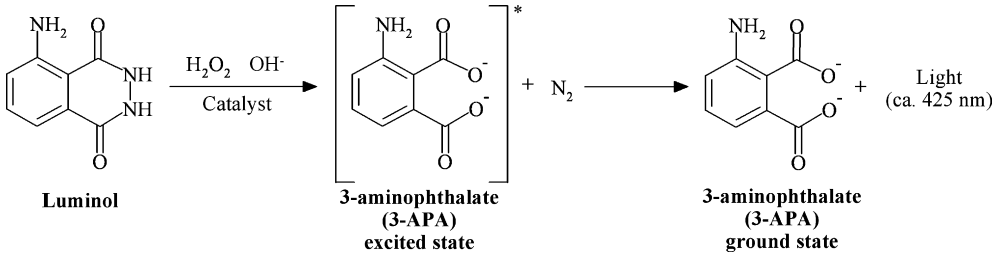


Fig. 4. Luminol chemiluminescence reaction scheme.

mann [41]. In dipolar aprotic solvents such as dimethylsulfoxide (DMSO) containing O_2, or in moderate-strong alkaline protic solvents (pH ∼ 8–11) such as water or lower alcohols and in presence of a strong-mild oxidant (in most cases H_2O_2) and a suitable catalyst such as a metal ion or some kind of oxidoreductase enzyme, the excited 3-aminophthalate dianion (3-APA*) returns to the ground state (3-APA) by releasing energy in the form of light (Fig. 4). When the reaction occurs in protic media, the 3-aminophthalate dianion is produced in almost quantitative fashion [40,42–46].

In aqueous solutions the light observed ranges between blue-violet and blue-green (Fig. 5), although the spectral range of emission is often rather broad and the observed maximum is dependent on several parameters of the reaction [39,44] such as the presence of blood itself which strongly absorbs at 420 nm and may provide an inner-filter effect, thus shifting the observed maximum emission of luminol chemiluminescence to about 455 nm [47].

For the luminol reaction the exact role of the catalyst, which is required when the reaction is carried out in basic aqueous solution, and the reaction intermediates are not completely characterised. It is known that a wide range of other transition metal catalysts and metal-complexes catalyse the reaction and that the optimum conditions of pH for the reaction depends on the identity of the catalyst used and varies between pH 8 and 11 [48] thus suggesting a multiplicity of potential catalysis mechanisms.

2.2. *Hemoglobin and its derivatives: biology and catalytic role in luminol test*

Hemoglobin (Hb) is the oxygen-carrying molecule found in the erythrocytes of all vertebrates and some invertebrates and is

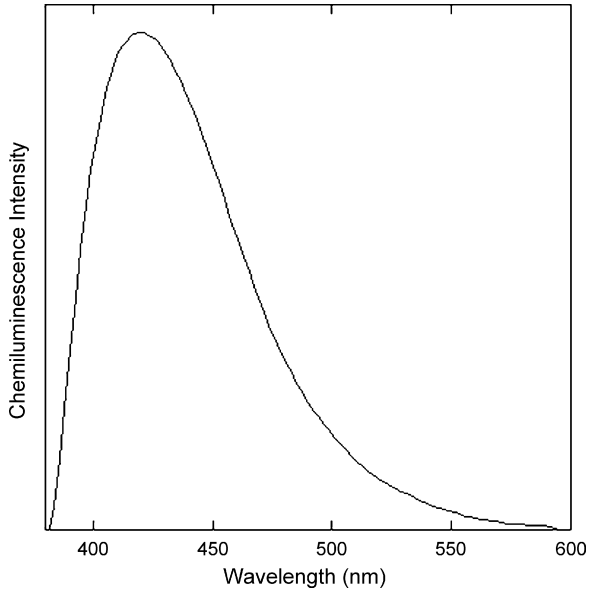


Fig. 5. Typical chemiluminescence emission spectrum for the reaction of luminol with hydrogen peroxide in the presence of hematin.

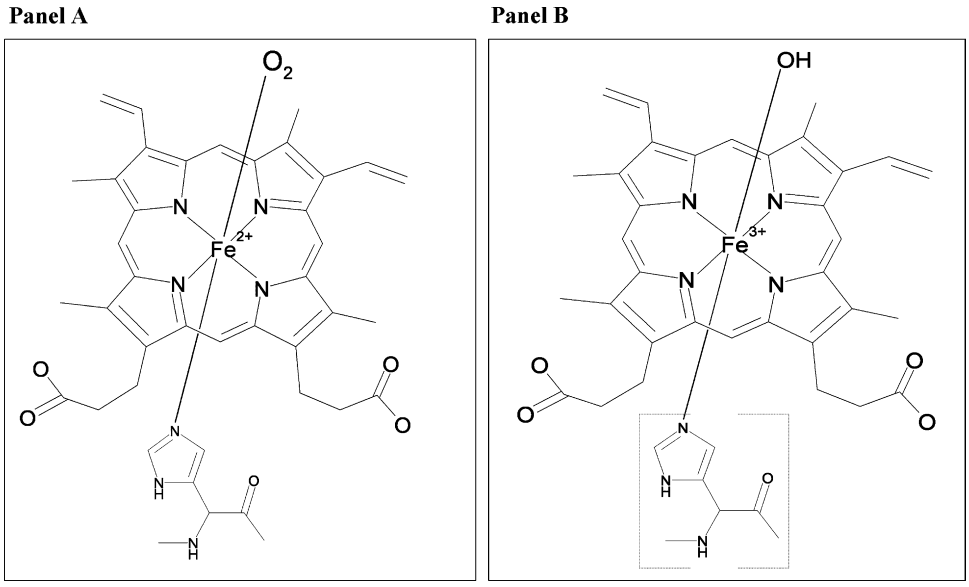


Fig. 6. Iron binding molecules in blood: (Panel A) heme molecular structure in oxyhemoglobin with iron in reduced form (Fe^{2+}) coordinated by O_2 in the sixth coordination position (deoxyhemoglobin has no ligands in this position), and by a histidine on the globin for the fifth coordination position; (Panel B) hematin molecular structure with iron in oxidised form (Fe^{3+}) bonding a hydroxide ion (with the histidine and, more generally, the entire globinic portion usually lost).

responsible for the red colour of blood. Mammalian hemoglobin is a tetrameric hemoprotein composed of four protein portions, named globins each enclosing a prosthetic heme group, consisting of a protoporphyrin IX–Fe^{2+} coordination complex (Fig. 6, Panel A) [49]. The ferrous ion is bound in the middle of the protoporphyrin IX ring by the four pyrrole nitrogen atoms. Of the remaining two axial coordination sites, above and below the planar ring of the porphyrin, one is occupied by a histidine on the globin (fifth coordination position) while the second axial position (sixth coordination position) is available for an exogenous ligand which, in the case of oxyhemoglobin is O_2 (while no ligand is present in this position in deoxyhemoglobin).

Within the body human hemoglobin is protected against denaturation by encapsulation in red blood cells and iron ions are kept in the ferrous state by several mechanisms, both non-enzymatic (globin envelope prevents Fe^{2+} oxidation and erythrocytes reduced glutathione reduces Fe^{3+} to Fe^{2+}) and enzymatic (mainly NADPH-MetHb reductase and NADH cyt b5 reductase of the erythrocytes catalyze Fe^{3+} reduction to Fe^{2+}) so that methemoglobin (MetHb) formation is hampered [50]. Hence, the valence of iron is kept the same upon bonding with oxygen (oxygenation) or losing oxygen (deoxygenation).

Once outside the organism and deposited on a substrate, blood is subjected to a series of degradation processes [10] in which most erythrocytes undergo hemolysis and biological molecules are involved in hydrolytic and/or oxidoreductive reactions primarily catalyzed from their own intracellular enzymes (e.g. aseptic autolysis due to catepsins released from dead cells lysosomes), or from enzymes of microorganisms populating the external environment. Degradation of the polypeptidic portion of hemoglobin takes place, the histidine coordinating the iron ion is generally lost, and spontaneous oxidation of the Fe^{2+} ion contained in the tetrapyrrolic ring of heme prosthetic group to Fe^{3+} ion rapidly occurs since, in this condition, cellular iron reduction processes lack [16,51–53]. If alkaline conditions are present, the Fe^{3+} is coordinatinated by a hydroxyl group ($-OH^-$). The heme prosthetic group containing ferric rather than ferrous iron, with the O_2 being replaced by the hydroxyl group, is named hematin (ferric protoporphyrin hydroxide) (Fig. 6, Panel B) and, correspondingly, the bloodstain shows a chromatic change from a typically red colour to a tawny-brown [49,53]. The processes of loss of the polypeptidic shells of hemoglobin and conversion of heme prosthetic group into hematin are increased when a luminol preparation is sprayed onto the bloodstain due to both the presence of an oxidant and the alkaline environment.

When a luminol formulation is applied on a bloodstain, ferric heme groups are able to catalyze both the decomposition reaction of peroxide and the oxidation of luminol and other substrates by peroxide [54–57]. These reactions are thought to be allowed by the ability of the hydroxy-ferric-porphyrin (OH-Fe^{3+}-P) hematin group to undergo a two-electron oxidation to a hydroxy-ferryl-porphyrin radical (OH-Fe^{4+}-$P^{\bullet}$) (analogous to Compound I in enzymes peroxidases, although the radical centre can then translocate to the globin), which can then return to the ferric porphyrin hematin state in two one-electron reduction steps via the hydroxy-ferryl-porphyrin (OH-Fe^{4+}-P) hematin group (analogous to Compound II in enzymes peroxidases) (Fig. 7) [13,19,57–59]. The catalytic process thus cycles between these three oxidation states of the hemoglobin, with the stable resting state being the ferric hematin. Alternative catalytic cycles and oxidising species have been proposed (e.g. see Thornton and Maloney [13]), but the above cycle is now accepted by the majority of researchers [57,60].

As these ferric heme derivatives show the same catalytic properties and capability of participating in two-electron redox cycles as a group of enzymes called peroxidases widely distributed especially in vegetables, their activity is termed a pseudo-peroxidase or peroxidase-like. This activity is com-

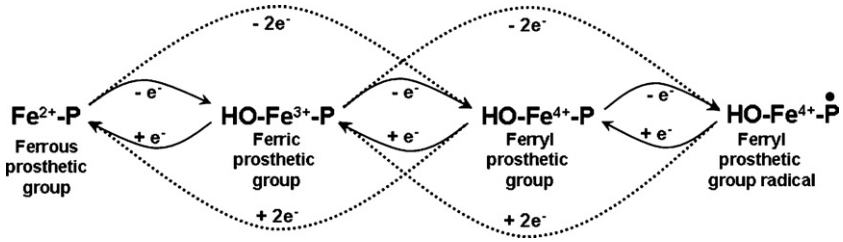


Fig. 7. Iron redox cycling between different oxidation states when hematin is formed and sixth coordination position is occupied by —OH (P, porphyrin).

monly employed as the basis for many presumptive tests for blood including luminol [10,40,61–63].

Thornton and Maloney [13] proposed three other possibilities for the peroxidase-like activity of blood apart from the participation of the heme in hemoglobin. Of these, xanthine oxidase and true peroxidase were thought to be unlikely by Thornton and Maloney as the concentrations of these species in blood is very low. The third possibility, catalase, has a pH optimum of approximately pH 7.0, significantly different from the optimum basic pH of the luminol reaction when used to detect blood stains. They therefore concluded that it is the heme group in hemoglobin which is responsible for the catalysis of luminol chemiluminescence by blood. This conclusion has also been reached by other researchers (Fig. 8) [13,63].

2.3. Redox reaction mechanism

While the identity of the emitter (3-aminophthalate) has been established for many years, the mechanism by which it is produced in an excited state has been the subject of many postulated mechanisms [8,13,20,40,64–68]. A succinct description of the current understanding of the probable reaction mechanism accounting for a number of findings from the aforementioned authors was given by Barnett and Francis in their recent review [5]; based on this work and on the previous research especially by Merényi and co-workers during the 1980s [20,64–68], the currently most accepted mechanism is presented in Fig. 9.

Luminol in strongly alkaline solutions is deprotonated to the monoanionic and the dianionic forms, with the former being prevalent between pH 8 and 14. The deprotonated luminol can be oxidised, most likely by the hydroxy-ferryl-porphyrin radical (OH-Fe^{4+}-P•) and also by the hydroxy-ferryl-porphyrin (OH-Fe^{4+}-P) to form radical intermediates, such as those described in the reaction with the true peroxidase enzymes, which can then react to give an diazaquinone [20,68]. The diazaquinone can then undergo nucleophilic attack from the hydroperoxide ion deriving from the deprotonation of hydrogen peroxide (pK_a 11.7) [20,39,40,47,68]. This is supported by the chemiluminescence intensity being dependant upon hydrogen peroxide concentration, a factor which has been used analytically to determine this species [24,69]. An alternative path involving attack of superoxide (O_2^-) on the radical may also occur, especially under conditions where the radical is in low concentration [20].

The postulated mechanism following addition of peroxide to the diazaquinone (or superoxide to the radical) involves a cyclic addition of oxygen from the added hydroperoxide to the other carbonyl carbon forming a cyclic anti-aromatic endoperoxide whose bonds are particularly weak. The significant amount of readily available energy contained in this species is then gained by cleavage and subsequent reorganisation of these bonds. Since nitrogen is an excellent leaving group because of the relevant strength of its own bonds (and as a gas, it is also entropically favoured), the formation of the dicarboxylate anion by expelling nitrogen gas is favoured. The 3-aminophthalate dianion so formed is in an electronically excited triplet state (3-APA*) (two unpaired electrons of the same spin) [8]. This then undergoes a slow spin-flip process, to an excited singlet state (two unpaired electrons of different spin) which in turn decays to the ground state with the emission of light [38,40]. Evidence for this pathway has been found by studies of diazaquinones which showed that these molecules give chemiluminescence on reactions with basic hydrogen peroxide without the need for catalysts, the emitter being the 3-aminophthalate ion in an electronically excited state (3-APA*) for the diazaquinone derived from luminol, with similar species being observed in the case of related diazaquinones [68].

Light emission is almost instantaneous when luminol is sprayed on hematin, while with blood there can be a build-up

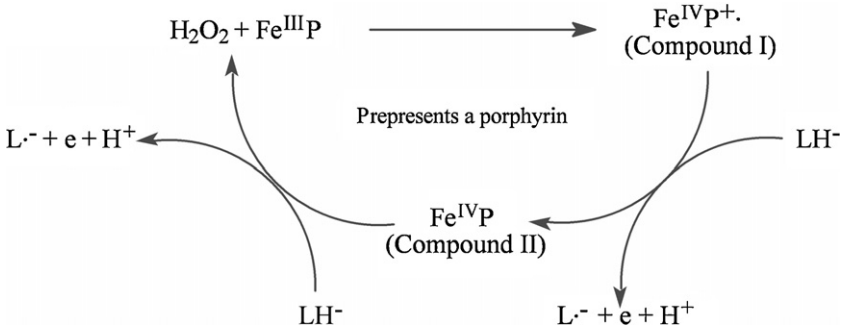


Fig. 8. Redox cycle showing oxidation of luminol by hydrogen peroxide as catalysed by hematin (FeP represents the hematin iron porphyrin).

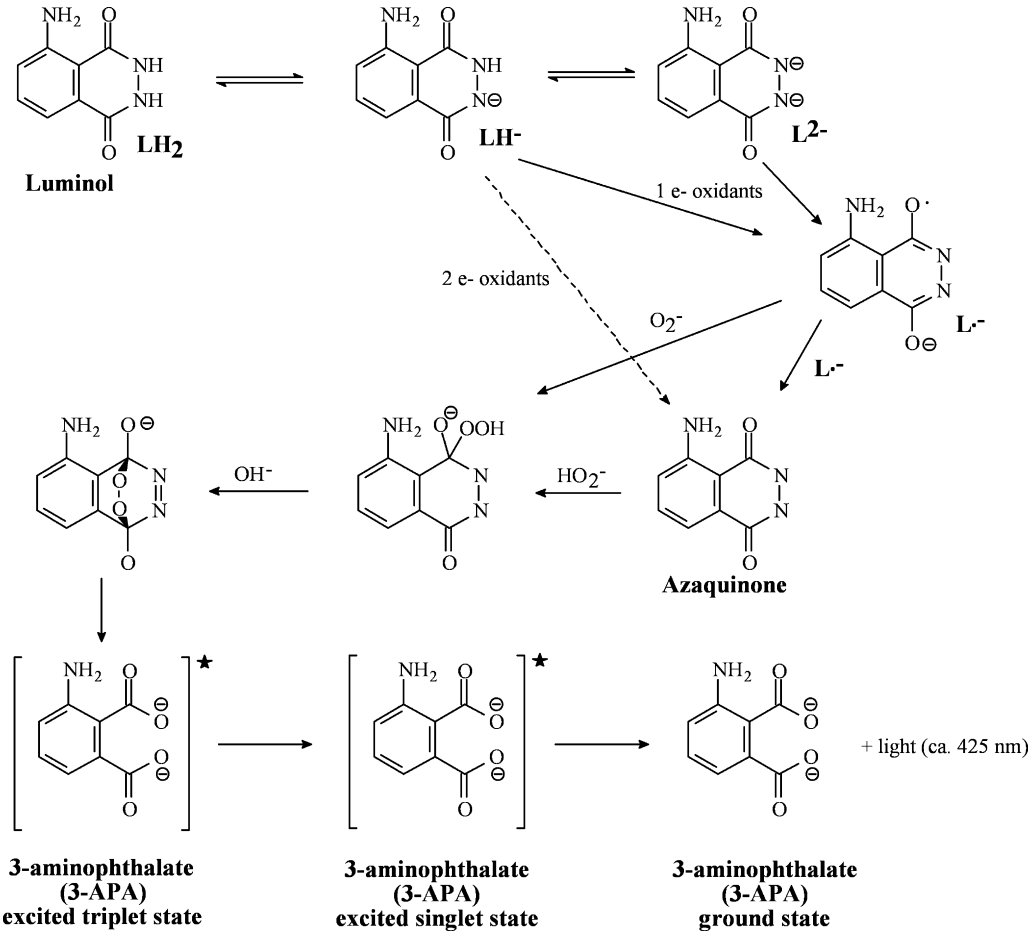


Fig. 9. Postulated reaction mechanism for luminol chemiluminescence.

to a maximum luminescence over a few seconds, followed by a decay in light intensity (Fig. 10). The half-life of the emission is rather variable, depending mainly on both the quantity and the quality of the catalyst, given constant concentrations of luminol, the oxidant and the base [39,44,70]. In most cases the half-life of chemiluminescence from blood has been observed to be about 20–40 s, although detectable emission may be viewed for up to 3 min [47,71].

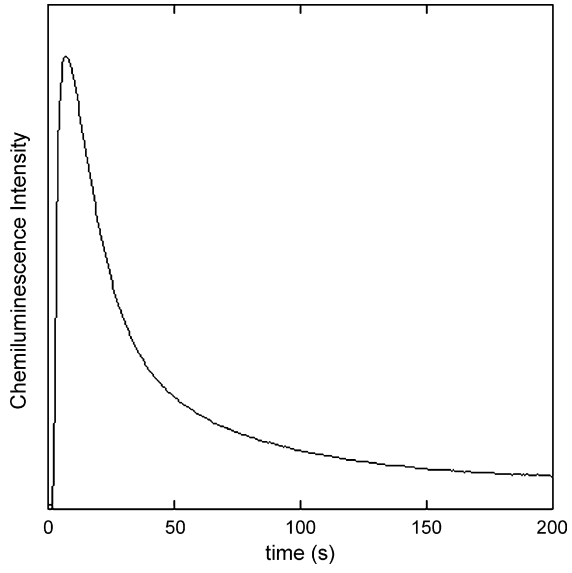


Fig. 10. Time course of the chemiluminescence observed when fresh blood is reacted with luminol and hydrogen peroxide.

In the conditions typical of luminol forensic testing the intensity of the light is primarily proportional to the concentration of the metal ion present, given both the oxidizer and the reductant (luminol) at a constant concentration [5,29].

3. The luminol reaction as a presumptive test for blood

3.1. Operational use of luminol as a presumptive test for blood

Luminol can be used to detect the presence of minor, unnoticed or hidden bloodstains diluted down to a level of $1{:}10^6$ (1 μL of blood in 1 L of solution) [18,63,72]. It can disclose distribution, allowing bloodstain pattern evaluation occasionally enabling the investigators to reconstruct some of the events of a crime by visualizing these patterns [73,74]. Other chemical-based tests widely employed over the years such as fluorescein, tetramethylbenzidine, phenolphthalein (Kastle–Meyer reaction) and leucomalachite green (Medinger reaction), and physical techniques such as the use of Polilight® (Rofin, Dingley, Australia) light source in the forensic detection of blood are useful

under specific circumstances, but do not have the high sensitivity of luminol [75–77].

Several preparations of luminol have been described and in recent years new preparations, using either patented luminol molecule modifications or luminol blood-dependent chemiluminescence enhancers [13,28], have been proposed to improve sensitivity, specificity and duration of the emission. However the two best known formulations for luminol testing, the first described by Grodsky et al. [18] and the second described by Weber [19], continue to be the most extensively used by forensic practitioners due to their good performance, simplicity of preparation, low cost and ready availability of the ingredients. These protocols are summarised in Fig. 11.

Regardless of the preparation, luminol solution is usually directly sprayed in completely dark environments. The light obtained can be photographed or filmed while the luminescent areas are marked in order to allow their detection once the light emission has faded [63,78]. Previous pre-treatment of the surfaces possessing the stains with 2% hydrochloric acid (HCl), recommended by some authors [79,80], seems to decrease sensitivity, raising the background chemiluminescence level [73], and, furthermore, to have detrimental effects for the following DNA typing attempts [63].

Amplification of the luminol chemiluminescent emission by means of intensified cameras has been reported in a forensic context [13,81] but is not in general use at crime scenes.

Due to the potential irritant effects of luminol, harmful effects of the other compounds employed in both preparations, and to the fact that luminol is applied as an aerosol, particular care has to be taken in its use (Table 1). Suitable protective equipment composed of goggles, respirators, gloves and protective clothes should be used by the operators when luminol is sprayed and the area investigated should be aerated after luminol application. The number of people assisting the operations should be limited to those strictly necessary [63].

Subsequent to bloodstain location and photograph documentation is the collection of the stains for further laboratory testing. The collection method depends on the nature of the substrate on which the stain is located. For unmovable objects such as tiles, walls or cars the best procedure consists of swabbing the surface with cotton swabs or other highly absorbing support. Many laboratories use the oral-swabs commonly employed to collect reference samples in crime cases. This concentrates all of the available stain in a small area of the swab. Alternatively, in some cases, the stains may be removed from the surface by scraping off with a scalpel and collecting the removed material.

When the stains are diffuse on a wide unmovable area, e.g. on a wall surface, it is possible to employ an adsorbent card containing preservative substances which protect the bloodstains (proteins and DNA as well) from bacterial and fungal hydrolytic and oxidative degradation [63,82,83].

For movable objects such as furniture components, small panels, carpets and tools, the best and most conservative procedure consists in the collection of the complete object from which the stains will be recovered in the laboratory.

Regardless of how the stains are collected, the essential requirements to be met are the recovery of the available blood, the collection of a control sample in a tested area not exhibiting chemiluminescence and the complete drying of the support used for blood collection in order to avoid the microbial and fungal degrading action.

Panel A

1.	Weigh out 3.5 g of sodium perborate (you may store it for an indefinite time in a glass or plastic container).
2.	Add 0.5 L deionized water (never use common undemineralised water).
3.	Stir and mix until complete dissolution of sodium perborate.
4.	Add, in sequence, 0.5 g of luminol and 25 g sodium carbonate (you may store them for an indefinite time in a glass or plastic container, luminol requires protection from light).
5.	Stir and mix until complete dissolution.
6.	Decant solution into a vaporizer or sprayer and use immediately.

Panel B

1.	Weigh out 8 g of sodium hydroxide and completely dissolve them in 0.5 L of deionized water to obtain a 0.4 N solution (stock solution A).
2.	Measure 10 mL of 30 % hydrogen peroxide and add them to 0.49 L of deionized water to obtain a 0.176 M solution (stock solution B).
3.	Weigh out 0.354 g of luminol and completely dissolve them in 0.0625 L of 0.4 N sodium hydroxide solution to obtain a final volume of 0.5 L (0.004 M) (stock solution C).
4.	Store the three stock solution in glass or plastic containers at 4° C, away from direct light.
5.	Prepare the test solution by mixing 0.01 L of each of the three stock solutions to 0.07 L of deionized water to obtain 0.1 L of final working solution.
6.	Decant solution into a vaporizer or sprayer and use immediately.

Fig. 11. Commonest forensic luminol formulations. (Panel A) Grodsky et al. luminol formulation protocol (1951) [18]. (Panel B) Weber's luminol formulation protocol (1966) [19].

3.2. Factors influencing the use of luminol

While luminol preparation and application is rather simple, interpretation of results is more challenging. Interpretation of luminol chemiluminescence characteristics and patterns at the crime scene should take into consideration the physical structure of the substrate upon which the bloodstains are found, the chemical composition of the substrate possessing the stains and any other substances present on the substrate.

3.2.1. Physical nature of substrate

The first issue confronting the forensic practitioner when using the luminol test at a crime scene is a consideration of the physical nature of the substrates possessing the stains [51,63,73]. Substrates can be divided roughly into two groups: absorbent materials and non-absorbent materials.

Absorbent materials encompass substrates with irregular porous surfaces such as wood-finish panelling, walls, and interstitial spaces between tiles or wood objects which, due to the grooves or cracks onto the surface, show superficial absorbent properties and are able to keep blood remains, even after vigorous scrubbing, for a long time [63,73]. In this group can be included also substrates with much greater absorbing properties such as carpeting, leather clothes, fabric clothes, roof-linings, blankets, etc.

Absorbent materials represent fairly easy surfaces to analyze because they often can retain significant amounts of blood, maintaining it in relatively undegraded form even for many years, thus giving intense reaction with the luminol test. This is primarily due to rapid drying of blood, especially in domestic or covered environments, thus preventing its degradation by environmental biological agents such as bacterial hydrolytic enzymes. Moreover, these substrates can protect blood from physical or chemical environmental agents such as solar rays, moisture and water, or cleaning attempts after the crime has been committed [63,73].

Due to the structure and to the relatively large quantity of blood that may be absorbed, absorbent materials are resistant to cleaning with bleach and/or soapy water. It is also possible to spray multiple applications of the luminol reagent without the risk of excessively diluting the stains in order to best visualize and to successfully photograph the bloodstain pattern [63,73].

Non-absorbent substrates such as non-textured linoleum, vinyl, tile, glass, metal and many others, present more difficulties both in the reagent application and in the quality of chemiluminescence. These substrate surfaces are unable to effectively retain and store blood and, moreover, cannot prevent its degradation especially by physical and chemical agents. As clearly demonstrated by Lytle and Hedgecock [73], these surfaces are fairly easy to completely clean and a mild washing attempt by water and soap lead to the removal of the bloodstains yielding almost non-existent reaction with luminol.

A further complication is that the application of luminol solutions to non-absorbent surfaces can lead to the bloodstain pattern running, due to the limited retention of the resulting solution by the smooth surface. This can lead to complete loss of the bloodstain pattern [63]. Particular care should therefore be used when dealing with these substrates, particularly when they are non-horizontal, in order to avoid the loss of the stains. Investigators should first use a minimum amount of luminol solution by rapidly spraying, preferably with a nebulizer, the suspected area, and avoiding further applications, quickly photograph the emission [51,63,73].

3.2.2. Influence of interfering substances

There is a wide range of environmental and pharmaceutical, domestic and industrial substances which are able to affect luminol blood-induced chemiluminescence. This may be due to catalytic activity, their redox properties, or their chemical reactivity with the luminol mixture or with iron in the bloodstains. Examples of such chemicals are the components of several commonly occurring materials such as soils, detergents, bleaches, carpet, metal objects, tools, plastic panels, wood, and vegetable compounds.

Compounds which may suppress luminol chemiluminescence are summarised in Fig. 12. Ligands with an high affinity/reactivity for a specific oxidation state of iron such as sulfide (ferryl ion ligand) or cyanide (ferric ion ligand) or compounds acting as anti-oxidising species (standard reduction potential, $E^{0\prime} < E^{0\prime}$ *luminol*) such as ascorbate, phenolics, anilines and thiols [84], may act as molecular traps subtracting either the catalyst (iron ions) or the reductant (luminol),

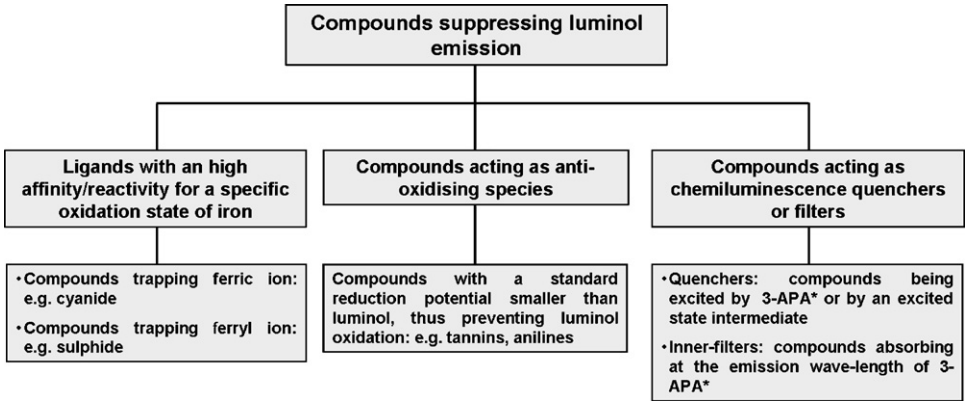


Fig. 12. Classification of compounds suppressing or reducing luminol chemiluminescence.

or may prevent luminol oxidation, respectively. Also quenching (intermolecular electronic energy transfer) or inner-filter effects (molecule absorbing at the emission wave-length of the emitter) from other molecular constituents of the bloodstains (heme itself, O_2, several aminoacids, etc.) or, more likely, of the substrate possessing the evidence should be regarded as possible interferents due to their ability to decrease the observed chemiluminescent/fluorescent intensity [85].

However, in practice, most of these substances are unlikely to come into contact with blood, with some relevant exceptions such as polyphenolic derivatives [69,86,87] like tannins which are widely present in wood. Thus these species do not generally significantly impact on the forensic use of luminol, and false-negative results have not been described in the forensic literature.

The most problematic chemicals for a correct interpretation of luminol test results are those which provoke intensification or a generation of a chemiluminescence emission even if blood is not present, leading to false-positive results. Due to the possible presence of these substances at the crime scene, the luminol test must not be considered sufficiently specific to permit an unequivocal identification of blood [15,18,51,88,89].

Those compounds which generate luminol chemiluminescence, or enhance the luminol emission in the presence of bloodstains can be divide into three major categories (Fig. 13):

1. compounds showing a catalytic true peroxidase or peroxidase-like activity;
2. compounds with a high oxidizing capacity towards luminol;
3. compounds with a complex chemical composition with an undefined action mechanism towards luminol mixture.

The first group encompasses inorganic or bioinorganic species and undoubtedly is the major source of luminol interferences as these compounds often show excellent catalysing properties in redox reactions such as that involving luminol oxidation and are widely distributed in the environment and in plants. In general three main types may be characterized in this group: free metal ions, in most cases included in inorganic compounds such as rust or soils; biological complexes between metal ions and organic components (such as metal–porphyrins, and including bacterial or plant pigments) often within protein structures; enzymes belonging to the oxidoreductases class such as horseradish-peroxidases.

Iron compounds, especially in the form of Fe^{2+} and Fe^{3+}, are constituents of many inorganic and biological species abundantly distributed in the environment [90–92]. In soils and sediments, iron is the dominant redox-active element by virtue of its abundance and favourable reduction potential located midway in the aqueous regime. Iron is the fourth most abundant element on the earth's crust and is present in several minerals such as hematite (Fe_2O_3), magnetite (Fe_2O_3), siderite ($FeCO_3$) or pyrite (FeS_2) which may serve as large reservoirs of electron-buffering capacity in soils and whose surfaces catalyze reactions that may proceed only slowly, if at all, in bulk solution [93].

In aqueous aerobic environments and at neutral pH iron can be found in highly insoluble crystalline and amorphous hydroxide and oxide forms [94] including such substances as rust (a mixture of iron oxides and hydroxides with a variable hydration degree and structural formula [$Fe_2O_3 \cdot nH_2O$]), and these compounds can also act as catalysts for the luminol reaction [95,96]. Also many metal objects and baked clays contain iron.

Similarly several other metallic ions such as cobalt, chromium, nickel, copper, and manganese, which are also found in soils or metal objects and some chemical products, have been reported, in various experimental studies, as capable of producing visible chemiluminescence when exposed to the luminol solution [42,83,97–99].

Ferric or ferrous ions and other metals ions especially cobalt, copper, and manganese, are present in some biological molecules including redox active prosthetic groups. Examples include the heme proteins peroxidases, catalase, cytochromes, and non-heme biomolecules such as the iron–sulfur cluster enzyme aconitase and the electron transfer proteins rubredoxins and ferredoxins [100,101]. Storage and transfer proteins like ferritin or hemosiderin, the main storage forms of iron in mammals, as well as transferrin, the iron transferring protein into the blood, contain significant concentrations of ferric ions. In ferritin or hemosiderin iron is incorporated in the min-

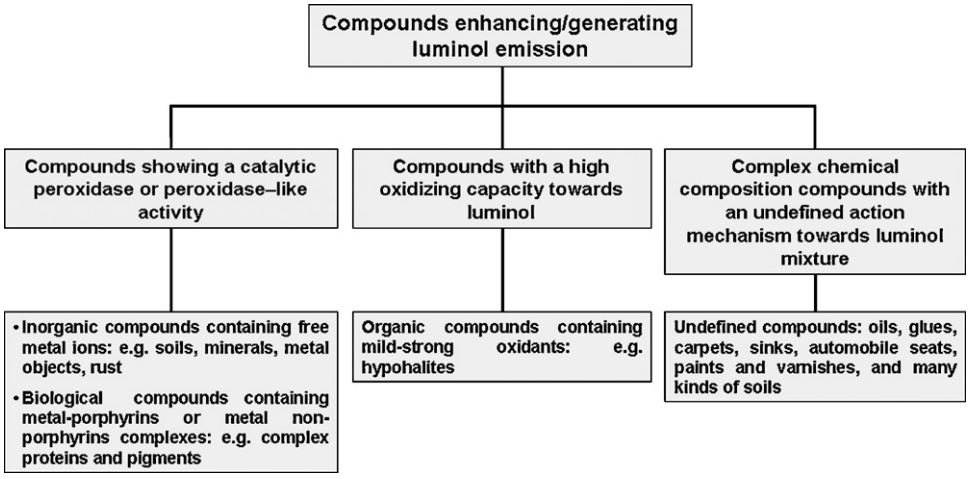


Fig. 13. Classification of compounds provoking or enhancing luminol chemiluminescence.

eral ferrihydrite form, $[FeO(OH)]_8[FeO(H_2PO_4)]$, whereas in transferrin it is coordinated to the polypeptidic shell aided by a carbonate anion cofactor [102]. Other major examples of metal-containing biomolecules in living organisms include the enzyme cofactors cobalamin or vitamin B_{12} (containing Co^{2+}) [103], and plants and bacterial pigments containing Mg^{2+} or Mn^{2+} [90,104]. These compounds intrinsically possess, in suitable conditions, redox properties of key importance for their chemistry and biology [49,105] and are therefore widely distributed in animal, plants and microorganisms.

Heme containing proteins represent a serious challenge for luminol test interpretation as they are capable of efficiently catalysing the luminol oxidation reaction. A hemoprotein is a protein containing a heme prosthetic group, either covalently or non-covalently bound to the protein itself [49]. The iron in the heme is capable of undergoing oxidation and reduction cycles usually involving a reversible change from +2 to +3 oxidation states and vice versa, though stabilized ferryl, Fe^{4+}-containing compounds, are well known as reaction intermediates in the peroxidases [90,104]. Heme-proteins are found in such diverse roles as transport (hemoglobin, myoglobin, transferrin, neuroglobin, cytoglobin, and leghemoglobin) [106,107], catalysis (peroxidases), active membrane transport and electron transfer (cytochromes) [108].

Peroxidases, found in bacteria, fungi, and animals but most widely distributed throughout the plants (e.g. horseradish peroxidase and turnip isoperoxidases), are heme-containing enzymes (heme b type) belonging to the class of oxidoreductases which catalyze the oxidation of a substrate by hydrogen peroxide [109–111]. Due to their biochemical properties peroxidases have been extensively investigated and used for a plethora of analytical applications especially in biochemistry and molecular biology. When the reducing compound is luminol, peroxidases are capable of catalyzing its oxidation with a much higher efficiency than any other catalytic species [49,105] and, for this reason, can produce significant levels of chemiluminescence, which can be misinterpreted as blood-dependent [73,112,113].

Of particular relevance in the forensic use of luminol are the interfering effects from plant peroxidases which are mostly abundant in fibrous plant material from fruits and vegetables (including the pulp and juice of fruits) but are also found with chemical variations among photosynthetic microorganisms [69,114]. In the presence of these enzymes, and to a lesser extent with other plant compounds such as the Mn^{2+} in Photosystem II [115] and/or the whole chloroplast [116], the luminol test can produce false positive indications often showing undetectable differences in light emission characteristics from that seen with blood [63,114].

The peroxidase-catalyzed chemiluminescent oxidation of luminol involves the initial formation of highly oxidising species upon reaction of the oxidant (e.g. H_2O_2) and peroxidase. In a similar but better understood fashion to the above described hematin catalytic mechanism in bloodstains, the ferric-porphyrin prosthetic group of peroxidases undergoes a redox cycling between ferric, ferryl, and ferryl radical cation states while luminol (mono or bideprotonated form) is converted to a radical form which undergoes further reaction by the mechanisms suggested earlier to yield the electronically excited 3-aminophthalate dianion [49,70,117–120].

Commonly encountered examples of the second category of interferents, namely compounds with strong oxidizing properties towards luminol, are sodium hypochlorite, potassium permanganate and iodine. These species are present in many household and industrial chemical solutions, including insecticides, cleaning agents, disinfectants or antiseptics [51,95,121–123].

Hypohalites of chlorine and bromine (hypochlorite, hypobromite) and related oxidants such as *N*-bromosuccinimide, 1,3-dibromo-5,5-dimethylhydantoin and chloramine-T, are well known as oxidants in chemiluminescent reactions. For example, the red glow accompanying the reaction between hypochlorite and hydrogen peroxide, was first reported by Mallet in 1927 [124]. The activity of these compounds as oxidants in chemiluminescence reactions has recently been comprehensively reviewed by Francis and co-workers [125] as powerful reagents in a wide range of analytically useful reactions because of their relevant oxidising properties.

Hypochlorite (OCl^-), a common component of domestic and industrial bleaches, was one of the first reagents used to induce the brilliant blue emission accompanying the oxidation of luminol [1] and it has been the subject of several qualitative and quantitative studies [126–129]. Hypochlorite is classified as a medium-strong oxidant with a standard reduction potential ($E^{0\prime}$) of 0.841 V (referring to the reduction reaction $OCl^- + H_2O + 2e^- \rightarrow Cl^- + 2OH^-$) [130] and is capable of amplifying the chemiluminescence emission in luminol oxidation by hydrogen peroxide when both the compounds are present in the reaction medium [130]. In 1991, Arnhold et al. [69] found a linear relationship between concentration of hydrogen peroxide and light intensity in the concentration range 5×10^{-8} to 7.5×10^{-6} mol/L with a maximum amplification level (550-fold) at 7.5×10^{-6} mol/L H_2O_2. The increased chemiluminescence of the luminol reaction in the presence of hydrogen peroxide and sodium hypochlorite is probably due to the hypochlorite being able to generate the diazaquinone intermediate efficiently, with this then rapidly reacting with peroxide. The chemiluminescence spectra of these reactions showed a wavelength maximum at 431 nm independent of the concentration of hydrogen peroxide. This value was similar to published chemiluminescence emission maximum for luminol oxidation without sodium hypochlorite (425 nm) in other experimental systems suggesting that hydrogen peroxide was a necessary component in the chemiluminescent oxidation of the luminol by sodium hypochlorite [44,131].

Investigations by Brestel [132] and Gorova et al. [133] showed that the luminol redox reaction involving sodium hypochlorite had a pathway similar to that described for other oxidising species such as sodium perborate or hydrogen peroxide as described in a previous section, although Eriksen et al. suggested that hypochlorite can form the diazaquinone without the intermediate formation of a radical [64]. Hypochlorite is thus one of the most important examples of substantial interfering substances, as it is widely distributed throughout the domes-

tic environment and can cause positive interference with the common luminol sprays including Grodsky's or Weber's formulations [18,19]. In addition, it may be used in an attempt to clean up a crime scene and remove blood evidence via its oxidation and physical elimination [134,135].

The final category of interfering substances covers a range of compounds which can be found in materials such as domestic and commercial oils, various glues, carpets, sinks, automobile seats, paints and varnishes, and many kinds of soils [63,114,135]. These substances are often able to catalyse the chemiluminescence almost as effectively as does the blood, but, due to their complex chemical composition, the exact mechanism underlying these interferences is not yet completely understood.

3.3. Interpretation of luminol test results

Luminol emission pattern interpretation can involve a qualitative statement of the luminescence pattern characteristics and an evaluation of emission spectra characteristics (maximum emission wavelength and emission intensity) [114,134,136]. In addition attempts can be made to inhibit or at least reduce the interferences of chemiluminescence deriving from the reaction of luminol with substances other than blood by using chemical species followed by an emission intensity measurement [47,71]. The latter approach however has only been employed successfully for hypochlorite bleach-induced chemiluminescence.

Generally visual examination is used when the luminol test is employed in a forensic situation, rather than instrumental detection of the luminescence. An experienced practitioner may distinguish the true blood-catalyzed chemiluminescence from that produced by other substances by the evaluation of parameters observable to the naked eye such as emission intensity, duration and spatial distribution. However this approach may also lead to misinterpretation, due to a subjective, informal and non-quantitative evaluation, for example, because its intensity is qualitatively much weaker than that expected for blood. In other circumstances an emission of similar intensity may be thought to derive from diluted bloodstains and is accepted. Therefore, caution should be exercised when using the test. Any confusion which may arise over a stain can usually be resolved by an intelligent observation and, if necessary, by further testing [73], for example, by using a different presumptive test for blood, such as the immunochromatographic test for the confirmation of human blood presence Hexagon OBTI (Human GmbH, Wiesbaden, Germany) [137].

In practice false positives with metals are rarely a problem as these can usually be anticipated or resolved by careful observation of the crime scene. Interfering solid substances such as metal objects or surfaces that are coated homogeneously with these substances (e.g. some varnishes and paints) generally show different and distinguishable emission patterns with respect to both the spatial distribution and, often, the emission intensity of luminescence. Upon reaction with metals both emission kinetics and intensity of chemiluminescence are rather characteristic: the emission will be twinkling and intense but short, while a luminol reaction with blood will produce an intense, long-lasting, even glow. Moreover, an interfering chemiluminescence, especially from solid object such as a water valve, a knife, a copper pipe, a floor, a carpet or a soil, will reproduce the shape, the composition, the contours and the dimensions of the object while luminol emission patterns with blood will appear as spatters, wipes, smears, drag marks or even footwear impressions.

The presence of hypochlorite-based bleaches on non-porous surfaces being sprayed is sometimes recognizable and can be identified by an experienced forensic practitioner as it leads to bright flashes of chemiluminescence as opposed to the more gradual development of chemiluminescence by blood.

One operational advantage of the luminol test is the ability to highlight the presence of scattered, very small droplets of blood by the individual 'sparkles' of blue chemiluminescence produced by each droplet. This makes this test easier to interpret then the other three common presumptive tests for blood (the benzidine, phenolphthalein and leuco-malachite chromogen tests) [51,77].

In theory, a quantitative evaluation of emission spectra (maximum emission wavelength and emission intensity) could be used to reduce the interferences of chemiluminescence deriving from the reaction of luminol with substances other than blood by using chemical species. Recently, Quickenden and Creamer [114] and Quickenden and Cooper [134] in a series of studies have used instrumental methods to examine the emission of light from the luminol test in order to investigate the potential to discriminate between true-positive and false positive results, and occasionally false negatives. These studies included investigating the potential for distinguishing between human hemoglobin and other species on the basis of spectral shifts of the wavelength of maximum emission. They also carried out comprehensive studies of the luminol chemiluminescence emission elicited by a wide range of common potentially interfering substances such as vegetable and fruit smears, pulps and juices and household/industrial chemicals such as cleaning agents, insecticides, glues, paints and varnishes. Of the 250 substances examined, they identified only a small number which produced chemiluminescence comparable to that of hemoglobin (Table 2): turnips, parsnips, horseradish, commercial bleach (sodium hypochlorite), copper metal, some furniture polishes, some enamel paints and some interior fabrics from automobiles [136].

Creamer et al. also studied [135] the serious issue of the hypochlorite interference effect with the luminol test [126]. They observed that when a person attempts to remove bloodstains by washing the area with water or sodium hypochlorite solution, depending on the thoroughness of the clean, the effect on the luminescence spectrum could range from the complete absence of emission to various combinations of blood-initiated emission and hypochlorite-initiated emission (each peaking at its separate respective wavelength) which might be expected if the cleaning process is not complete.

Finally the same group examined in a recent study a specific kind of crime scene, namely the interior of an automobile, taking into consideration both the effect of potential interferences from the internal fittings of the vehicle but also the effect of high temperature within the vehicle of the efficacy of the test [138]. The effects of attempts to wash hemoglobin from the interior of the vehicles tested using a variety of cleaning methods were also

Table 2
Spectral measurements showing the major interferences with the luminol test for blood detection expressed as mean peak wavelength shift from hemoglobin (nm) and mean intensity (percent of hemoglobin value); errors shown are 95% confidence intervals in the mean values

Interfering substance	Mean peak wavelength shift from hemoglobin (nm)	Mean intensity (% of hemoglobin value)
Copper metal	2 ± 10	106 ± 10
Matte-finish enamel paint (Dulux®)	9 ± 4	100 ± 10
125 g/L NaClO aqueous solution	9 ± 4	84 ± 22
Gloss acrylic spray paint (Taubman®)	22 ± 3	81 ± 34
Turnip pulp	3 ± 4	74 ± 35
Parsnip pulp	8 ± 5	56 ± 23
Roof lining (1992 Ford Laser®)	13 ± 7	22 ± 11
Horseradish pulp	3 ± 4	20 ± 12
Wooden-furniture polish (Goddard's®)	11 ± 23	20 ± 4

From Ref. [136]. Copyright © 2003 John Wiley & Sons Ltd. reproduced with permission.

investigated. It was found that there was little interference from materials within the three automobiles tested, although some surfaces did elicit weak luminol chemiluminescence. Attempts to remove hemoglobin with water alone were not successful, however soapy water or a proprietary car cleaner removed a significant proportion of the hemoglobin from the tested surface (ca. 90%). Soap and water and cleaners produced better results than plain water because they have the ability to solvate the globin proteins more effectively.

The effect of increased car interior temperature lead to improved sensitivity and chemiluminescence emission intensity of the test and this was hypothesized to be due to thermal conversion of hemoglobin to methemoglobin in the presence of molecular oxygen.

3.4. Improvements to luminol formulations

To minimize luminol interferences and/or to increase the yield of the chemiluminescence emission other approaches have been investigated over the years. These include the use of derivatives and analogues of luminol, a variation in the order of mixing of the reagents, the pre-treatment of the substrate to be tested with chemical substances and, finally, the addition to the luminol mixture preparation of chemical additives which selectively react with the putative interfering species reducing their availability for the reaction with luminol.

Ewetz and Thore in 1975 described a modified luminol-perborate assay in which experimental samples were pre-treated with a solution of 0.1 M NaOH before adding a luminol preparation without perborate, followed by treatment with a solution of perborate [88]. They observed a reduced light emission from isolated Fe^{2+} ions to a low constant value independent of concentration, whereas in hematin compounds the light emission was stoichiometrically related to these molecules allowing identification of the two emission profiles based on the reaction kinetics. This effect could be probably due mainly to the dissociation of globin portion of the proteins tested from the prosthetic group following the pre-treatment with NaOH [139]. The incubation with an alkali solution exposed the hematin making it available for the luminol reaction while free Fe^{3+} is mostly complexed with OH^- ions to form $Fe(OH)_3$ which is poorly soluble. Despite this pioneering study being primarily aimed at defining a quantitative analytical assay for the selective determination of hematin compounds in environmental chemistry, it was one of the first attempts to increase the luminol test selectivity by changing the traditional test procedures. However this approach has never been successfully applied to the forensic field but was restricted only to laboratory based analytical applications.

More recently, several advances in the identification of chemicals interfering with the chemiluminescent reaction of luminol with hypochlorite have been made, and these provide insight into how hypochlorite interferences might be reduced in a forensic context. Most notably, Arnhold et al. [86], investigating the inhibitory action of some biological species towards chemiluminescent reaction in the luminol–H_2O_2–NaOCl system under biological pH conditions, found that several of these species could directly interact with NaOCl. Most substances tested such as thiourea, cysteine, human serum albumin, ascorbic acid or methionine, acted as competitors with luminol for the interaction with NaOCl due to either thiol or amino groups, the former being easily oxidized by NaOCl, the latter reacting with NaOCl to form chloramines. In both cases these functional groups were able to scavenge NaOCl, subtracting it from the reaction with luminol. Again, despite the interesting findings no effective attempts to use chemical additives to increase the selectivity of luminol forensic formulations (Grodsky's or Weber's formulations) have found widespread application.

More recently however the successful use of a chemical species preventing luminol emission by interfering compounds has been reported by Kent et al. in their studies into reducing the effect of hypochlorite-containing bleaches [71]. In previous analytical chemistry papers, Gray et al. [140], Margerum et al. [141], and Antelo et al. [142,143] had described the reaction of amines with hypochlorous acid to form chloramines according to the following equation:

$$RR'NH + HOCl \rightleftharpoons RR'NCl + H_2O$$

where R, R′ are H or alkyl, and had showed that the reaction rate between hypochlorite and amines is pH dependent and depends on the basicity of the amine.

Kent et al. investigated whether primary and secondary amines could inhibit the chemiluminescence due to hypochlorite under the alkaline conditions typical of forensic luminol tests (Grodsky's or Weber's formulations), and whether the presence of amines had an effect on the heme-catalyzed luminescence of luminol [71]. The authors observed an inhibition of bleach-induced chemiluminescence by amines, the effect being increasing with the alkalinity of the amines. The best inhibition effects (almost complete inhibition) were obtained with strongly basic amines such as 1,2-diaminoethane since these were the most effective competitors for hypochlorite under the conditions of common forensic sprays (those reported by Grodsky and Weber

both lead to pH > 10). The amines did not interfere significantly with the hemoglobin-catalyzed oxidation and only slightly reduced the chemiluminescence observed from blood still assuring a satisfactory intensity and longevity of light emission, sufficient to be effectively used in a forensic context. A major disadvantage to this approach to reduce hypochlorite effect on luminol emission was the toxicity of amines involved. In a follow up study, King and Miskelly confirmed the above results but they also found that the far less toxic amino acid glycine was very nearly as effective as an additive as the amines [47].

In the above studies it was also noted that if the bloodstain could be left to air for a period of 1–2 days, the hypochlorite would decompose and thus no longer interfere with a standard luminol treatment. This effect has also been reported by Creamer et al. in 2005 [135] who found that the interference effect by bleach decreased if the area to be sprayed were left for several days allowing to the bloodstains to thoroughly dry, as the hypochlorite decomposes, thus dissipating its effect on luminol emission.

Lastly, a new luminol-based formulation (patented) called Bluestar® Forensic (ROC Import Group, Monte Carlo, Monaco) was recently developed in an attempt to eliminate some inconveniences (especially low emission intensity, brief lifespan of the emission and shortness of the solution life) associated with luminol sprays. Recent papers have compared the performance of Bluestar® Forensic luminol spray to typical luminol preparations (Grodsky's and Weber's formulations) [144,145]. These papers concluded that Bluestar® Forensic provided convenience of preparation (easy to mix in the field), that chemiluminescence was sufficiently intense and long-lasting that it could be visualised in the presence of some ambient light, and that emission intensity was still reasonable when a bloodstain was resprayed. Moreover, Bluestar® Forensic was described as a more stable formulation since it could be used for several days after mixing, possibly due to it containing urea peroxide as a stable oxidant. It is claimed that Bluestar® Forensic is not destructive to DNA, whereas other Bluestar® Forensic formulations (for hunters and for training) can adversely affect DNA analysis (for more information, please visit the website http://www.bluestar-forensic.com).

3.5. Sample collection and effect on serological and DNA analyses

Once a bloodstain has been located and photographed it can be sampled for further serological and genetic analyses [63,73] as described in an earlier section. Once the blood residues have been collected the forensic biologist may proceed in two ways: further presumptive test for blood detection may be performed in order to confirm the human hematic nature of the stains or, more likely, direct DNA extraction aimed at DNA typing procedures may be carried out [63,72].

As the luminol test has been employed to detect blood stains that otherwise would not have been revealed due to the limited amount of the blood present, in most cases it is preferred to directly perform DNA typing procedures in order to avoid a partial loss of the already small amount of blood. Nevertheless over the years many attempts have been made to better characterize the recovered bloodstains by using both additional presumptive tests and DNA typing analyses.

A major advantage of the luminol test is the lack of significant damage to the genetic material, especially when modern PCR techniques are employed to analyze microsatellite DNA. Only moderate adverse effects have been noted over the years when other DNA testing procedures or serological markers were commonly used for identification purposes.

Early studies by Specht [15], Proesher and Moody [16], and McGrath [17] in the first half of the 20th century demonstrated the absence of interfering effects between luminol solution and other confirmatory tests performed after the luminol test.

Lytle and Hedgecock in 1978 [73] mentioned the non-destructive and preservative properties of the luminol solution towards other serological assays as it did not prevent subsequent identification tests or ABO blood grouping analyses. However, they did report an interference with the electrophoretic analyses aimed at typing of enzymes, such as erythrocyte acid phosphatase and phosphoglucomutase, which were important at that time.

Duncan et al. [146], investigating common fingerprint developing agents, showed that luminol had no destructive effects on catalytic examinations, crystal tests for hemoglobin, species test or elution method for the detection of blood group antigens, but again noted that it could seriously affect electrophoretic typing of enzymes.

Grispino in 1990 [147], employing a luminol preparation according to Specht [15] and a modified formulation of this original solution followed by several blood confirmatory tests, found no significant detrimental effects on presumptive tests, Takayama confirmatory test or species tests. However, a diminished ability of ABO blood grouping by absorption elution and a complete loss of electrophoretic band patterns in blood enzyme typing were observed, likely due to the denaturing action of the luminol mixture.

A comprehensive study by Laux in 1991 [148] confirmed and extended these results.

The minor detrimental effects noted by these authors were likely due to the capability of the luminol preparations (and not necessarily to the luminol molecule itself) to react with DNA or proteins. The presence of mild-strong oxidizing compounds such as perborate may provoke oxidative damage to proteins [149,150] and, also, on pyrimidine and purine nitrogenous bases leading to the fragmentation of the DNA double helix [150]. The very high pH used for the luminol test (pH ∼ 11) may lead to alkaline hydrolysis of both peptide bonds in proteins [151] and *N*-glycosidic bonds between the 2-deoxyribose and the nitrogen base of DNA leading to an abasic site where the phosphodiester bond on the polydeoxyribosephosphate strand may undergo subsequent hydrolysis [150,152].

Hochmeister et al. [153], testing for the first time the effects of presumptive reagent such as luminol, benzidine, phenolphthalein, orthotolidine, leumalachite green, and other chemicals on a subsequent DNA typing procedures and on semen stains found that evidentiary body fluid stains treated still could be successfully typed by restriction fragment length polymorphism (RFLP) procedures.

In the last 15 years, RFLP have been replaced by polymerase chain reaction (PCR) coupled to the employment of microsatellite short tandem repeats (STRs) DNA [154–156] which allows forensic biologists to obtain DNA typing results from minimal amounts of biological material, previously insufficient for a successful RFLP procedure [157].

Cresap et al. [158], investigating the effects of both luminol solution and Coomassie Blue on DNA amplification by PCR reported that the PCR procedure was completely unaffected by these assays in a wide range of blood concentrations.

Since the studies of Cresap et al., the results obtained in a number of studies clearly indicated that it was possible to recover adequate amounts of DNA suitable for STRs typing by the PCR technique from luminol-treated bloodstains. Luminol did not adversely affect, at least in a detectable manner, either microsatellite DNA stability or DNA extraction methods or PCR chemistry. For example, in a comprehensive paper of 1999 Gross et al. [159] found that the standard treatment according to Grodsky et al. [18] had no detrimental effects on the PCR testing, with the DNA yield and the ability to type the bloodstains using PCR-based technologies being mainly dependent on the nature of the substrate and the method of cleaning.

Fregeau et al. [72] investigated whether the commonest blood enhancement reagents could interfere with the subsequent DNA extraction procedures and with AmpF*l*STR® Profiler Plus™ (Applied Biosystems, Foster City, CA, USA) fluorescent STRs DNA analysis. Fresh or aged bloody fingerprints on various porous and non-porous surfaces were extracted and typed after short-term exposure (less than 54 days) to a range of chemicals including luminol on linoleum, glass, metal, pine white-painted wood, some kinds of clothing and paper. DNA yields before and after treatment indicated a reduction in the quantity of DNA recovered from bloody fingerprints by a factor of 2–12 probably because of the effect on the integrity of the DNA molecules which could potentially compromise DNA typing analysis in the case of small stains. Nevertheless they noted no adverse effects on the PCR amplification of the nine STRs systems surveyed or of the gender determination marker Amelogenin when chemical enhancement of bloodmarks using any of the selected compounds was conducted for a term below 54 days of exposure. This study demonstrated that PCR STRs DNA typing procedures were robust and provided excellent and effective results even when used after exposure to different enhancement chemicals.

In a contemporaneous study, Della Manna and Montpetit [160] investigated the capability of routinely isolating and recovering amounts of DNA suitable for PCR typing from luminol-treated latent bloodstains. They noted that adequate amounts of DNA suitable for PCR typing at all of the Promega PowerPlex® 1.1 (Promega Corporation, Madison, WI, USA) loci upon post luminol treated bloodstains could be effectively recovered.

4. Conclusions

Luminol has always been considered by the international forensic community a fascinating but, at the same time, an "obscure" chemical compound. Despite of its "age" and the numerous attempts to reveal the chemical mechanism of light emission, the interferences from substances other than blood and how the reaction could be improved for forensic purposes are still not completely understood even in experimental controlled systems. Nevertheless, despite these issues, luminol continues to provoke great interest and to represent a challenge because it has revealed itself in practice to be an affordable, sensitive and simple detection system for invisible bloodstains detection with few detrimental effects on the subsequent DNA recovery and typing. However, the undoubted chemical complexity of the emission reaction and the presence of several substances interfering with the reaction and potentially leading to incorrect results, should oblige the forensic practitioner to know these disadvantages in order to carefully deal with them and to properly use the "cold light" test at the crime scene.

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