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NINHYDRIN-BASED FORENSIC INVESTIGATIONS: II. CYANIDE ANALYTICAL TOXICOLOGY

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Abstract

Recently, a novel ninhydrin reaction with cyanide has opened new ways to detect this poison in the body fluids and the environmental samples. Mainly, detection of cyanide with ninhydrin at a crime scene could be of prime interest for forensic investigators. Besides, the reaction conditions are very common, except the need to avoid oxygen and oxidizers. The ninhydrin-based assay proved to be highly sensitive and selective. Therefore, we investigate in this review the possible forensic applications of ninhydrin-based cyanide assay.

Keywords: *forensic science; ninhydrin; cyanide determination*

Introduction

The chemistry and applications of ninhydrin reactions with a variety of substrates has undergone and continue to undergo major developments over the course of nearly 100 years (Friedman, 2004). Following its discovery by Siegfried Ruhemann in 1910, ninhydrin rapidly became a practical analytical tool. In 1954 it was found to be an important reagent to develop fingerprints on porous surfaces (Drochioiu *et al*., 2011). Since its use in forensic chemistry, many efforts have focused on improving the reagent (Hansen & Joullie, 2005). Ninhydrin was successfully used for determining the biogenic amines histamine (Friedman & Noma, 1981), and phenylethylamine (Friedman & Noma, 1986), lysine (Friedman et al., 1984; Finley & Friedman, 1973), tryptophan (Friedman & Cuq, 1988; Friedman & Finley, 1971), non-protein amino acids (Bell, 2003), sulfur amino acids (Friedman *et al*., 1979), etc.

Sanford Moore and Wiliam Stein automate the amino acid chromatography in 1948 enabling rapid assays of all amino acids in a protein hydrolysate at nanomole levels (Moore, 1968). Ninhydrin is also widely used with paper and silica gel plates (Laskar *et al*., 2001). The complexation of Ruhemann's purple with certain metal ions enhances the sensitivity of the analyses by allowing estimation of the resulting chromophore by fluorescent, luminescent, phosphorescent, and laser techniques (Friedman, 2004). The development of fluorogenic ninhydrin reagents (Boppana & Rhodes, 1990; Wimalasena *et al*., 2003) and their use in forensic science (LaPorte & Ramotowski, 2003; Schwarz & Frerichs, 2002). The ninhydrin reaction facilitated the analysis, isolation, and characterization of many antibiotics, bacterial toxins, and microbial products containing ninhydrin-reactive amino groups (Frutos *et al*., 2000; Gallo-Martinez *et al*., 2002; Jeannotte *et al*., 2003).

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So-called "nonclassical" ninhydrin reactions do not involve formation of Ruhemann's purple and they are used to measure cysteine, protein-bound tryptophan, pipecolic acid, and sialic acid (Friedman, 2004). Condensation of ninhydrin with aldehydes and primary amines affords highly fluorescent ternary products (Samejima et al., 1971). Some derivatives such as quinoxaline, guanide, or dimethyldihydroresorcinol ones were prepared starting from ninhydrin (MacFadyen, 1950). Cyanide also reacts with ninhydrin in alkaline solution to form a blue-colored product with a λ_{max} of 590 nm.

This reaction can be used to measure low levels of cyanide in industrial effluents (Nagaraja et al., 2002). Nevertheless, the process whereby the blue compound is formed from ninhydrin has remained obscure. A large body of research is still needed to establish the reaction conditions and sampling in order to use ninhydrin-based cyanide assay in forensic sciences.

Therefore, this work deals with the forensic applications of cyanide reaction with ninhydrin and its mechanism. The formation of a stable and separable ninhydrin-cyanide compound has also been discussed.

Cyanide poisoning

Cyanide poisoning presents one of the most difficult challenges in disaster medicine. However, although an efficient poison, cyanide has limited capacity as a toxicant (Zemlyak *et al*., 2009). Survival following cyanide exposure with appropriate supportive treatment has been reviewed (Baud, 2007). Cyanide is one of the most lethal poisons known (Geddes et al., 2005). The high toxicity of cyanide lies in its ability to inhibit oxygen uptake by cells, binding with the ferric iron in cytochrome oxidase, blocking the oxidative process of cells. While cyanide poisoning is rare, it can occur from smoke inhalation from both residential and industrial fires (Ishii *et al*., 1998). Lethal cyanide blood levels for fire victims in the cyanide concentration range 23-26 μM(Geddes et al., 2005).

Cyanide is well-absorbed in animals and humans via digestive, respiratory, and cutaneous routes. Cyanide causes energy depletion in the cell and destroys the cytoskeleton (Zemlyak *et al*., 2009). Concomitant inhalation of hydrogen cyanide and carbon monoxide has also been reported to be largely responsible for the toxicity of fire smoke (Bhattacharya et al. 2002).

Cyanide toxicity is attributed to inhibition of cytochrome c oxidase (Leavesley *et al*. 2008). Cytochrome c oxidase is essential to respiration, providing ATP from respiration that powers all aerobic organisms (Collman *et al*., 2008). Cyanide ions inhibit succinate dehydrogenase (respiratory chain complex II) in the mitochondrial inner membrane (Alexi *et al*. 1998) and cytochrome c oxidase (complex IV), respectively.

There have been some cases of cyanide toxicity following treatment with sodium nitroprusside (Quinlan *et al*., 2008). However, the treatment with sodium thiosulphate, sodium nitrate and haemodialysis results in the elimination of cyanide from the circulation. Cyanide is present as cyanogens in various plants such as cassava and may induce toxicity in humans and animals eating these foods (Kaewkannetra *et al*., 2009).

Cyanide determination

The extreme toxicity of cyanide requires sensitive methods for its detection and quantitation, especially in blood and body fluids (Lindsay *et al*., 2004). Several chemical and physicochemical methods for the detection of cyanides, such as potentiometric, chromatographic, spectrophotometric, flow injection and electrochemical analysis have been proposed (Lu *et al*., 1995; Ng *et al*., 2000). Generally, spectrophotometric methods for the determination of cyanide in waste water and biological fluid are preferred (Ansari *et al.,* 2008; Drochioiu *et al*., 2000). Cyanide reacts with picric acid to form isopurpurin, which can be determined spectrophotometrically (Drochioiu *et al*., 2000; 2003a). On adding resorcinol, the sensitivity of the assay was increased (Drochioiu *et al*., 2003a; 2003b). In fact, cyanide reactions are quite unexpected (Drochioiu *et al*., 2011a; 2011b). A method describing determination of cyanide in blood by head-space gas chromatography with electron capture detector was reported by Felby (2009). Free cyanide in drinking water was determined by ion chromatography with pulsed amperometric detection (Christison & Rohrer, 2007). Currently, a sensitive method based on cyanide conversion to cyanogens chloride by reacting with chloramine-T (Epstein, 1947) and the determination of the formed cyanogens chloride by gas chromatography with electron capture detection is used (Valentour *et al*., 1974). Another method involving headspace single-drop microextraction (SDME) with in-drop derivatization and CE was developed for the preconcentration and determination of free cyanide (Jermak *et al*., 2006). The main advantage of this method is that sample clean-up, preconcentration and derivatization procedures can be completed in a single step.

The isolation of cyanide from blood is mostly performed by head-space extraction (Maseda *et al*., 1989; Odoul *et al*., 1994), or microdiffusion with subsequent extraction with an organic solvent has usually been used (Valentour *et al*., 1974). However, classical König method with its modifications is still in use or the determination of hydrogen cyanide in air (Pitschmann *et al*., 2010).

The reaction with chloramine-T can take place during the extraction, after the head-space sampling on a pre-column packed with chloramine-T (Maseda *et al*., 1989) or by reaction with chloramine-T hold in a glass tube inside the head-space vial before the head-space sampling (Odoul *et al*., 1994). Using nitric acid instead of phosphoric acid (Maseda *et al*., 1989) has the advantage of releasing hydrogen cyanide from blood (Felby, 2009). Spectrophotometric determination of trace concentrations of cyanide ions based on the nucleophilic addition of cyanide to imine group of the new reagent 4-hydroxy-3-(2-oxoindolin-3-ylideneamino)-2-thioxo-2*H*-1,3-thiazin-6(3*H*)-one was reported by Hamza *et al*. (2010). An interesting spectrophotometric determination of cyanide using cobalt (II) phthalocyanine tetracarboxylate as a chromogen was proposed by El-Nemma *et al.* (2010). A Raman method (Presmasiri *et al*., 2001; Tessier *et al*., 2002) for selective cyanide detection based on evaporated cuprous iodide substrate was also developed (Reddy *et al*., 2010). The trace amount of cyanide can be determined with a kinetic spectrophotometric procedure (Abbasi *et al*., 2010). Another simpler method is based on the use of p-aminoacetanilide (Parmar *et al.,* 2010). Cyanide was determined with a method based on its reaction with aquacyanocobyrinic acid heptamethyl ester reagent at pH 9.5 to give dicyanocobester (Hassan *et al*., 2007).

The increase of the absorption bands of the reaction product at 368 and 580 nm and the decrease of the reagent band at 353 nm are linearly proportional to the cyanide concentration. Cyanide can be determined with a detector tube for phosgene, hydrogen cyanide and cyanogen chloride by tristimulus colorimetry (Pitschmann *et al*., 2008). The inhibition of catalase activity by cyanide was used to determine the trace levels of this poison (Drochioiu *et al*., 2004b). Enzymatic determination of anions was reviewed by Yablotskii *et al.* (2010). Badugu *et al*., (2005) reviewed quinolinium based boronic acid in the determination of anions, including cyanide, and compared toxicities and method feasibilities of various anions.

The selective sensing of cyanide anions in water has been studied using a hybrid biomaterial composed of a mesoporous TiO_2 film of crystalline nanoparticles and the protein hemoglobin (Poland *et al*., 2006).

Preliminary work on a headspace method for HCN revealed very poor sensitivity due to adsorption of the HCN onto instrument surfaces (Turner & Shuker, 2006). Therefore, thisapproach should be abandoned in favour of the colorimetric procedure. In the case of cyanogens in cassava, the sample is extracted into acidic medium, buffered and the glycosidic cyanogens hydrolysed enzymatically to cyanohydrins.

Cyanide reactions with ninhydrin

Cyanide reacts easily with various compounds and metal ions such as iron, nickel, copper etc. (Drochioiu *et al*., 2000; Noorozifar, 2007). Cyanide ion reacts also with ninhydrin (2,2-dihydroxy-1,3-indanedione) to afford 2-cyano-1,2,3-trihydroxy-2*H* indene, which is blue in color at high pH and red-colored in the presence of sodium carbonate. Besides, hydrindantin seems to be an intermediary of reaction. The influence of oxidizers such as bromine, oxygen, iodine etc., and metal salts such as those of copper, silver and mercury on the reaction of ninhydrin with cyanide has been investigated. These may hinder it due probably to the degradation of hydrindantin-like compounds or the formation of complexes with ninhydrin.

Ruhemann treated ninhydrin, **1**, with hydrogen cyanide, and obtained a deep red intermediary, which was readily decomposed to afford brown needles of **2**. No red intermediate was isolated. Besides, a red color of ninhydrin appears on heating it to 120 °C or solving ninhydrin in concentrated sulfuric acid (MacFadyen, 1950). The red color was assigned an indene structure.

Compound **2** decomposes at 148 ^{o}C with evolution of hydrocyanic acid to give *o*-carboxyphenylglyoxal, **3**. Ninhydrin, on standing at room temperature in alkaline solutions also gives **3** or even *o*-carboxymandelic acid, **4,** at higher pH values (Figure 1).

The conversion of ninhydrin to hydrindantin, **5**, has previously been reported to be catalyzed by cyanide ion (Bruice & Richards, 1958). Also, **5** proved to be unstable with increasing pH converting to 1,3-dihydroxy-2*H* indene-2-one, **6** and ninhydrin, **1** (MacFadyen, 1950). In addition, the red color of **6** was assigned to the monovalent anion, and the blue color to the divalent anion, **7**. Therefore, it was considered that **6**, which is deep red in color, reacts with cyanide to afford **2**. Nevertheless, only the indene structure of **6** was accepted so far, and no indication about the indene derivative with CN group in the molecule has been found (Ruhemann, 1910).

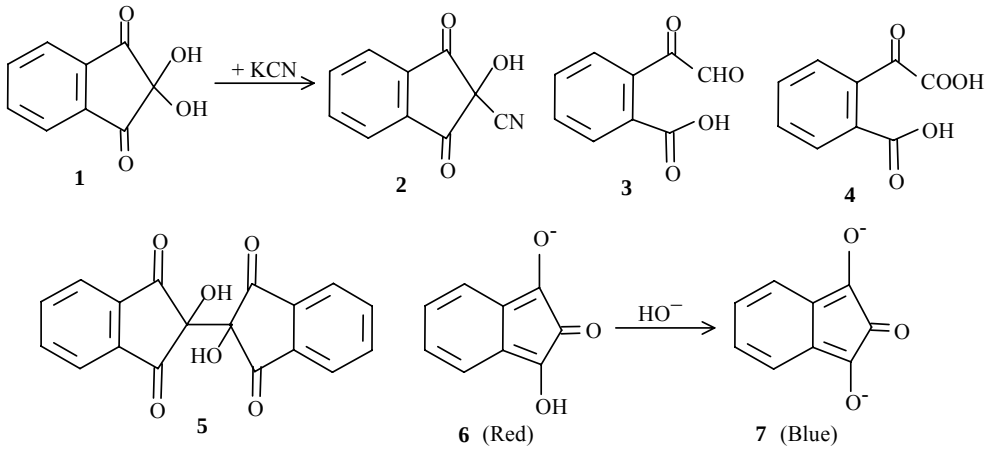

Fig. 1. Ninhydrin reaction with cyanide.

Hydrindantin formation

Cyanide reacts with ninhydrin under alkaline conditions to form a colored compound, which is very stable in the absence of oxygen and the other oxidizers.[11,12,19] On the other hand, small amounts of cyanide are supposed to catalyze the formation of hydrindantin.[10,16] In order to prove the correct mechanism of hydrindantin formation, 1 mmole of ninhydrin was treated with 1 mmole of KCN, the equivalent amount of cyanide, in the presence of sodium carbonate (Drochioiu *et al*., 2004a). The both red colored solutions were diluted accordingly and their absorbance was read in the wave range from 400 nm to 600 nm. In addition, the reaction of ascorbic acid with ninhydrin to afford hydrindantin was also performed.

The three spectra were compared with each other. Both solutions of hydrindantin and of the cyanide-ninhydrin 1:1 adduct showed a maximum absorbance at 485 nm. Nevertheless, the second solution showed a shoulder at 460 nm. Its size was 94 % of the intensity of the 485 nm peak. Although another compound seemed to be also formed when ninhydrin reacted 1:1 with cyanide, we concluded that cyanide reacted stoichiometrically with ninhydrin to form hydrindantin as the major product. The intermediate compounds **8** – **10**, which are shown in the reaction sequence, proved to be not separable from the reaction mixture (Fig. 2).

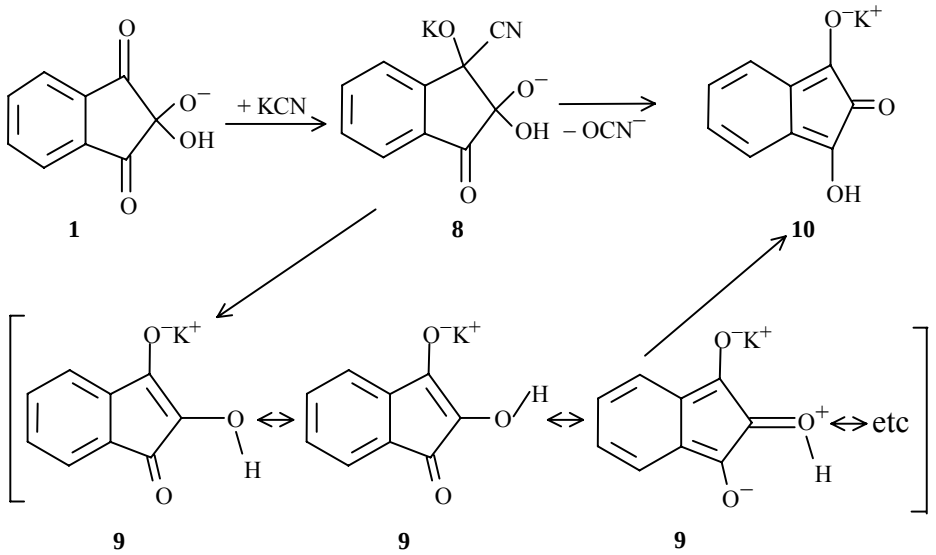

Fig. 2. Possible mechanism of cyanide reaction with ninhydrin under alkaline conditions.

On using the 2:1 molar ratio of cyanide and ninhydrin, the absorbance at 485 nm increased 2.12 times and that at 460 nm 2.98 times as compared to the 1:1 molar ratio. That clearly proved that hydrindantin is not the sole product of the reaction and hydrindantin, once formed, reacts with another molecule of cyanide to afford a novel compound. To identify it, the following reaction was performed: 1.78 g (10 mM) of ninhydrin, **1,** was added to 1.3 g of KCN (20 mM) solved in a 2% solution of sodium carbonate. The reaction took place under nitrogen. The white powder of ninhydrin dissolved immediately to form a deep red colored solution. Upon adding a hydrochloric acid solution, white-pink crystals separated (compound **12**). The suspension was filtered and the precipitate washed out several times with twice distilled water directly on the filter paper and dried at room temperature.

The crystals thus obtained melted at 124 – 126 ^{o}C. The structure of the product **12** as well as that of the other compounds in the reaction sequence was established by elemental and spectral (MS, IR, ^{1}H NMR) analyses. The compound **12** was also dissolved in the solutions of sodium carbonate and sodium hydroxide, respectively, with color changing to red (λ_{max} 485 nm) and blue (λ_{max} 590 nm). Upon evaporating the solvent under vacuum, the colored compounds were obtained in crystalline form. A solution of **11** was bubbled with air when Ruhemann's adduct **2** separated. Upon evaporating a solution of **11** in hot air stream, 2-(2-cyano-2-hydroxy-acetyl)-benzoic acid, **13**, was obtained. A reaction mechanism was proposed according to the experimental data and the Ruhemann's reaction sequence reconsidered (Figure 3).

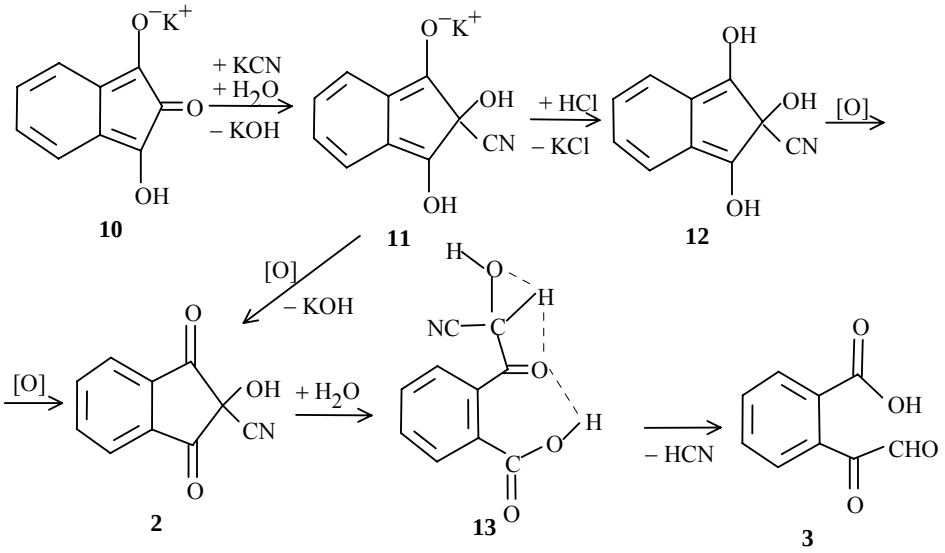

Fig. 3. Decomposition of cyanide adduct with ninhydrin.

Because the compound **12** may be both red- and blue-colored at various pH values, it was considered that the colors are due to the anions of 2-cyano-1,2,3-trihydroxy-2*H* indene. The transformation of hydrindantin, **9**, into its 2*H* indene form, **10**, which is much more stable, would allow for two ionizable groups.

Therefore, the red color is attributable to the monovalent anion, the blue color to the divalent anion.

The ease of oxidation on exposure to air and oxidizers is consistent with the indene structure of **10** – **12**. Also, the rapid formation of acetoxy derivative **14** in the solution of sodium carbonate showed that the ionizable hydroxyl group is very reactive.

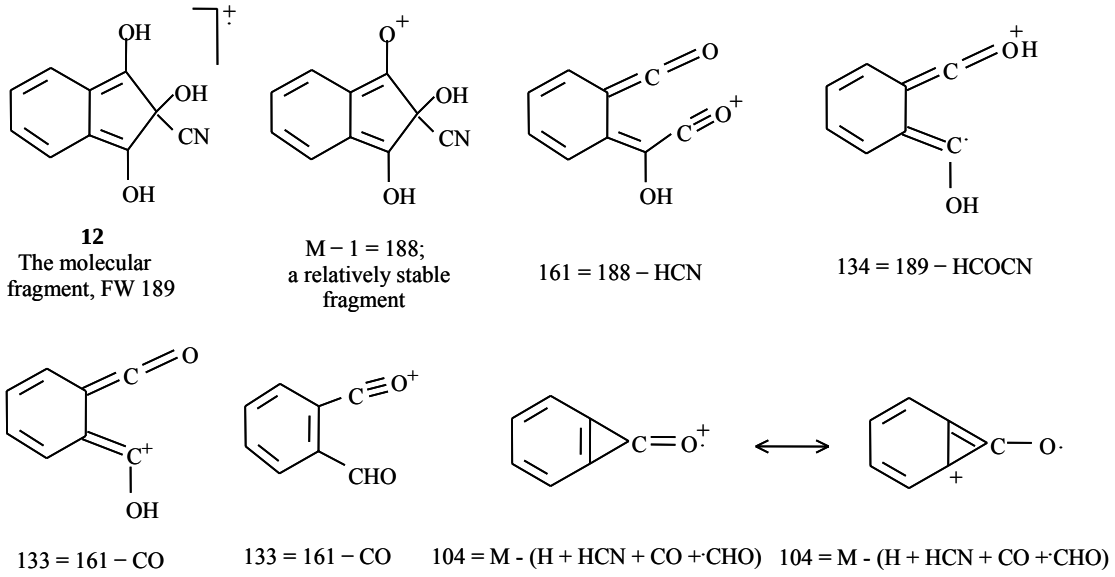

Fig. 4. The fragmentation schema for the resulted compound 2-cyano-1,2,3,-trihydroxy-2*H* indene.

The fragmentation schema was also in the best agreement with the proposed structure for **12**. The molecular weight of **12** of 189 Da was determined by mass spectrometry (Figure 4). The main fragments were found to be at 104, 188, 76, 133, 134, and 106 units, respectively. In addition, all the properties of **12** supported its structure, which was confirmed spectroscopically.

Thus, the red color of **11**, which is an anion form of **12**, vanished on shaking air into its solution, particularly on heating. The compound **12** proved to be a powerful reducer due to its HO groups in the positions 1 and 3, which remind us the similar behavior of the two HO enolic groups in ascorbic acid. Moreover, the compound **12** was quite stable in solid state or at low pH value. The solution changed color from red to blue with increasing pH. The color turned again to yellow if the pH decreased. Under anaerobic reaction conditions, the red color was stable indefinitely even at 100^{o}C. Also, under more alkaline conditions, the reaction gave a blue solution, which was stable for weeks in the absence of air. Upon adding hydrochloric acid, the blue color turned to red and then to white-pink. Bromine, chlorine and other oxidizers destroyed the colored compound to form HCl and HBr. Silver, mercury, and copper salts hindered the investigated reaction. Under alkaline conditions, ninhydrin reacted with these ions to give precipitates or colored solutions. Hydroxylamine may be used to mask the mercury and silver ions. Copper formed a greenish 1:2 molar complex with ninhydrin, which was unable to react with cyanide. Nevertheless, when the ninhydrin was present in large excess the reaction with cyanide was again possible. The stability of the coloration was much enhanced in the presence of the reducers such as ascorbic acid. Nevertheless, large amounts of ascorbic acid reacted with ninhydrin to form hydrindantin, which masked the formation of red colored salts of **12**. Therefore, cyanide reduced ninhydrin, **1**, to form hydrindantin, **10**, which reacted with another cyanide molecule to afford a stabilized 2*H* indene, **11**. This one is stable under anaerobic conditions, but can be easily oxidized to Ruhemann's compound **1** and give **13** with increasing pH.

The mechanism of reaction

The experimental data demonstrated that cyanide ion attacks nucleophylically the C=O group at position 1 of ninhydrin, **1**. Then, the negative charged oxygen at position 2 (due to alkaline medium) attacks the carbon atom of the cyano group to release cyanate ion OCN, and to afford 1,2-dihydroxy-3-keto-3H indene. This one turns into the 2*H* indene isomer, which is more stable. The process whereby ninhydrin is rapid dissolved in a solution of KCN with the formation of a purple solution was thus explained by the 2*H* indene structure with the two ionizable HO groups in the positions 1 and 3. Following that, cyanide ion reacts again with the C=O group at position 2 to form 2-cyano-1,2,3-trihydroxy-2*H* indene or its anions according to the pH value.

If one of the HO groups in the position 2 of ninhydrin, **1**, is blocked with urea or alcohol, or changed by another group, the reaction between cyanide and ninhydrin is hindered. Thus, Ruhemann's compound **2** cannot form 2-cyano-1,2,3-trihydroxy-2*H* indene, and the reaction pathway was completely different. Also, a solution of ninhydrin containing sodium carbonate cannot afford 2-cyano-1,2,3-trihydroxy-2*H* indene if previously treated with an alcohol or urea that block one of the HO groups at position 2. To prove the fact that cyanate ion is really released as a by-product, we identified it in the solution as ammonia after hydrolysis with sulfuric acid and distillation with 32% sodium hydroxide as recommended by Deepa's group. Thus, the possibility of other reaction mechanisms has been excluded.

Cyanide detection and determination with ninhydrin

Cyanide also reacts with ninhydrin in alkaline solution to form a red- or blue-colored product with a λ_{max} of 485 or 590 nm, depending on the solution pH value (Drochioiu, 2002a; Drochioiu, 2002b). This reaction can be used to measure low levels of cyanide in industrial effluents (Nagaraja *et al*., 2002). Nevertheless, the process whereby the colored compounds are formed from ninhydrin remained obscure.

Therefore, all the compounds involved in cyanide reaction with ninhydrin were synthesized, isolated and characterized and a new mechanism of reaction was advanced (Drochioiu *et al.*, 2004a; 2005). The reaction was proposed also for cyanide determination in blood and other body fluids (Drochioiu & Mangalagiu, 2002). A review on cyanide determination refers to the forensic usage of ninhydrin (Drochioiu *et al.,* 2007). However, there is no review on the application of ninhydrin to cyanide detection.

Micelli (2005) improved cyanide determination with ninhydrin to analyze waste water in oil indusrey by flow injection during a Ph.D. investigation. By adopting an on-line standard addition protocol, the sensitivity of the proposed method was enhanced drastically, without affecting the determination range (Themelis *et al*., 2009). The assay was validated in terms of linearity (up to 200 $\mu g\ L^{-1}$), limit of detection (c_L = 2.5 $\mu g\ L^{-1}$), limit of quantitation (c_Q = 7.5 $\mu g\ L^{-1}$), precision (s_r < 2.5% at 100 $\mu g\ L^{-1}$) and selectivity. High tolerance against critical species such as sulfides and thiocyanates was achieved. The applicability of the method was demonstrated by analyzing tap and mineral water samples at levels below the limits established by international E.U. and U.S. organizations.

Chueachot *et al*. (2007) determined trace amounts of cyanide in wine sample.

Headspace single-drop microextraction and cuvetteless microspectrophotometry for the selective determination of free and total cyanide involving reaction with ninhydrin was investigated (Jain *et al*., 2010).

We used the ninhydrin-based cyanide assay in blood samples (Drochioiu & Mangalagiu, 2002). The method needs further improvements due to its sensitivity toward oxygen and other oxidizers.

It is also of great interest the following poison transformation within the body and searching for its metabolites (Zbancioc *et al*., 2011).

According to Santelli et al. (2006), the ninhydrin-based cyanide assay takes advantages of the reaction of cyanide ions with ninhydrin in basic medium in a flow injection system.

These authors reported a linear range of 0.01 to 0.04 μg/mL with a detection limit of 1.5 ng/mL by using 500 μl sample injection. Regarding to interferences, cyanide could be determined in the

presence of 100 mg/L of thyocianate and sulfide, both species normally found in industrial effluents. For total cyanide determination strong acid distillation was recommended due to the presence of cyano-metallic complexes in the refinery effluents. The more significant advantage of the proposed method is the lack of use of carcinogenic reagent such as pyridine and psychotropic compound such as barbituric acid, both used in the recommended methods. Therefore, the ninhydrin-based method is really a friendly analytical procedure.

Conclusions

Although ninhydrin is most useful in fingerprint process, it could be a valuable forensic tool for detecting and determining cyanide poisons. This work revealed several novel properties of the newly proposed ninhydrin containing reagents. Ninhydrin reagent can be used to detect cyanide either as separated and purified compound or as a component in a mixture. Some properties of ninhydrin could be of interest in selective identification of cyanide ions.

Evidence for a an indene structure of the compound 2-cyano-1,2,3-trihydroxy-2*H* indene, which occurs in the reaction sequence of ninhydrin with cyanide has been brought. Forensic applications of the above-mentioned reaction consist of cyanide ion identification in air, water and biological fluids, cyanide determination in blood samples of fired or killed people, cyanide analysis of smokers, etc.

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