



Review

Derivatization of carbonyl compounds with 2,4-dinitrophenylhydrazine and their subsequent determination by high-performance liquid chromatography[☆]Shigehisa Uchiyama^{*}, Yohei Inaba, Naoki Kunugita

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ABSTRACT

Derivatization of carbonyl compounds with 2,4-dinitrophenylhydrazine (DNPH) is one of the most widely used analytical methods. In this article, we highlight recent advances using DNPH provided by our studies over past seven years. DNPH reacts with carbonyls to form corresponding stable 2,4-DNPhydrazone derivatives (DNPhyrazones). This method may result in analytical error because DNPhyrazones have both *E*- and *Z*-stereoisomers caused by the C=N double bond. Purified aldehyde-2,4-DNPhydrazone demonstrated only the *E*-isomer, but under UV irradiation and the addition of acid, both *E*- and *Z*-isomers were seen. In order to resolve the isometric problem, a method for transforming the C=N double bond of carbonyl-2,4-DNPhydrazone into a C–N single bond, by reductive amination using 2-picoline borane, has been developed. The amination reactions of C1–C10 aldehyde DNPhyrazones are completely converted into the reduced forms and can be analyzed with high-performance liquid chromatography. As a new application using DNPH derivatization, the simultaneous measurement of carbonyls with carboxylic acids or ozone is described in this review.

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1. Introduction

Carbonyl compounds such as aldehydes and ketones have received much attention as hazardous substances in studies of environmental and biological chemistry. Long-term exposure to relatively high levels of formaldehyde is known to increase the risk to human [1–4]. In 2004, the International Agency for Research on Cancer (IARC) reclassified formaldehyde as a human carcinogen that causes nasopharyngeal cancer and also concluded that there is a “strong but not sufficient evidence for

a causal association between leukemia and occupational exposure to formaldehyde” [5]. It is a great public health problem whether there is an association between formaldehyde exposure and leukemia. IARC classification of formaldehyde has started controversial discussions. Recently, some new epidemiological reports including meta-analysis were published [1,3,6–10]. The two studies [7,8] found an elevated mortality rate from myeloid leukemia in individuals occupationally exposed to formaldehyde. In addition, formaldehyde affects indoor air quality, and is known to trigger acute adverse health effects such as skin, eye, nose, and throat irritation. It has been reported that formaldehyde is significantly associated with a higher risk of the “Sick Building Syndrome (SBS)” [11–13]. Acetaldehyde, an analogue of formaldehyde, is listed as possibly carcinogenic to humans (Group 2B) by IARC. Ethanol in alcoholic drinks is mainly oxidized in the liver by alcohol dehydro-

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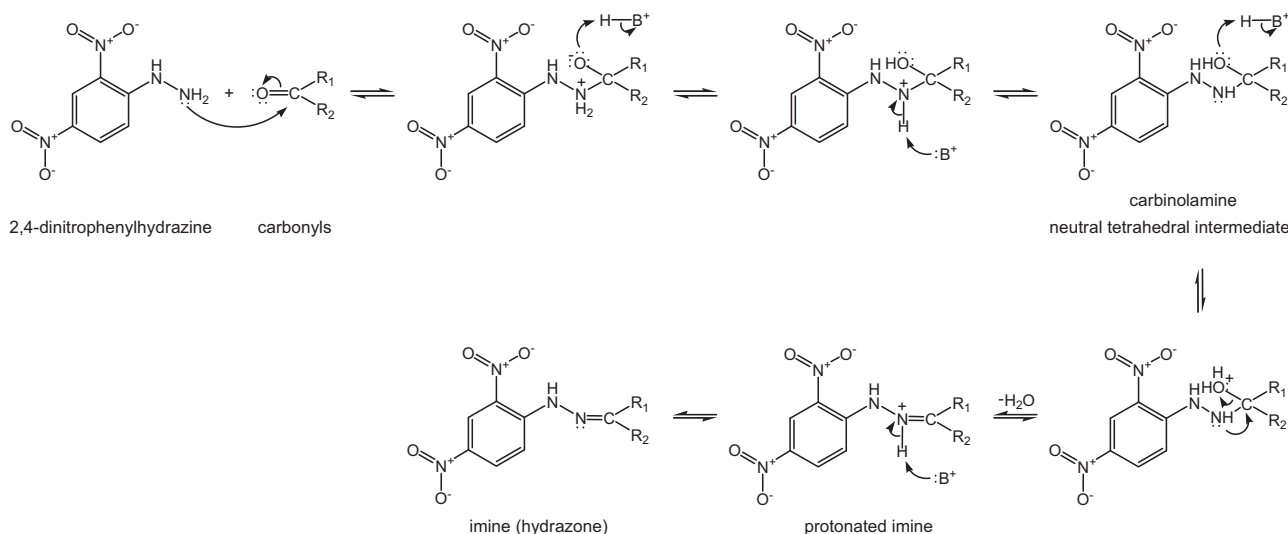


Fig. 1. Scheme of the derivatization reaction of DNPH with carbonyls.

genes to acetaldehyde. Acetaldehyde is subsequently oxidized to acetic acid by aldehyde dehydrogenase 2 (ALDH2). Approximately 40% of Japanese have single nucleotide polymorphisms (SNPs) of the ALDH2 gene. The ALDH2 *2 allele produces an inactive protein subunit, which is unable to metabolize acetaldehyde. Exposure to high levels of acetaldehyde may be responsible to increase the risk of head and neck cancer and esophageal cancer [14–16]. Estimation of aldehydes is also the most common approach for the study of lipid peroxidation [17]. Malonaldehyde can be very useful as a presumptive marker for the development of oxidative stress in tissues and plasmas [18]. Determination of hexanal as an indicator of the lipidic oxidation state in some food samples has been presented [17].

The specific reaction of carbonyl compounds with 2,4-dinitrophenylhydrazine (DNPH) forming the corresponding 2,4-DNPhyrazones is one of the most important qualitative and quantitative methods in organic analysis. This method has been used to measure aldehydes and ketones in plasma [18–20], urine [21–23] and other biological samples [24–26], as well as environmental air [27] and water samples [28]. It was first published by Allen [29] and Brady [30]. Carbonyl compounds react with DNPH to form hydrazones as Fig. 1 [31]. In the first step of the mechanism for hydrazone formation, the amine attacks the carbonyl carbon. Gain of a proton by the alkoxide ion and loss of a proton by the ammonium ion form a neutral tetrahedral intermediate. The neutral tetrahedral intermediate, called a carbinolamine, is in equilibrium with two protonated forms. Protonation can take place on either the nitrogen or the oxygen atom. Elimination of water from the oxygen-protonated intermediate forms a protonated hydrazone that loses a proton to yield the hydrazone. Hydrazone formation is reversible. In acidic aqueous solutions, the hydrazone derivatives are hydrolyzed back to the carbonyl compound and DNPH, and then the reaction attains equilibrium. The main advantage of the DNPH derivatization method is the ability to analyze various aldehydes and ketones simultaneously in a complex mixture. Sampling can be performed using acidic solutions of DNPH in impingers [32] or with acidic solid sorbents coated with DNPH in a cartridge. A number of cartridge devices containing solid sorbents coated with DNPH have recently been provided for sampling aldehydes. The solid sorbents include XAD-2 [33,34], silica gel [35,36], glass beads [37], octadecylsilane bonded silica gel [38], Florisil [39], and glass fiber filters [40]. In aqueous samples, an acidic solution of DNPH is used to form the corresponding hydrazones followed by high per-

formance liquid chromatography (HPLC) separation and ultraviolet (UV) detection at 360 nm (depending on the absorption maximum wavelength of the hydrazones) [28,41–43] or mass spectrometry (MS) [44–46]. Due to the importance of the method, it has been introduced as a standard procedure by several national standardization bodies. Recent research has resulted in the identification of chemical interferences caused by the presence of ozone [47–49] or nitrogen dioxide [50].

This review looks at the fundamental principles of and new applications for the derivatization of carbonyl compounds with DNPH; mainly through our studies over past seven years.

2. Isomerization of carbonyl 2,4-DNPhyrazones

Usually, the DNPH derivatizations are performed under acidic conditions and 2,4-DNPhyrazones derivatives are separated by means of HPLC followed by detection using UV spectrophotometry at 360 nm (depending on the absorption maximum wavelength of the hydrazones). However, this DNPH derivatization method may cause an analytical problem as 2,4-DNPhyrazones have both *E*- and *Z*-stereoisomers due to the C=N double bond. Fig. 2 shows the structure of *E*- and *Z*-stereoisomers of acetaldehyde DNPhyrazone. The formation of isomeric 2,4-DNPhyrazones from unsymmetrical carbonyl compounds in the liquid phase has long been known [51–53]. Behforouz et al. [54] and Tayyari et al. [55] reported that a trace of acid catalyzed the *E*-*Z* isomerization, which was detected via melting point anomalies. However, until recently, this evidence seemed irrelevant with regard to possible analytical problems it would cause in the determination of aldehydes and ketones. Purified alkanal-2,4-DNPhyrazones demonstrated only the *E*-isomer. However under UV irradiation and the addition of acid, both *E*- and *Z*-isomers were seen [56]. The spectral patterns of *Z*-isomers were different from those of *E*-isomers and the absorption maximum wavelengths were shifted towards shorter wavelengths by 5–8 nm. Fig. 3 shows the variation of *Z*- to *E*-isomer ratios of acetaldehyde and propanal DNPhyrazones derivatives with various phosphoric acid concentrations. An equilibrium *Z/E* isomer ratio was observed in 0.02–1% (v/v) phosphoric acid solutions. Propanal- and other aldehyde-2,4-DNPhyrazones derivatives showed similar behavior. The isomer ratios of alkanal-2,4-DNPhyrazones are listed in Table 1. In the case of acetaldehyde- and propanal-2,4-DNPhyrazones, the equilibrium *Z/E* isomer ratios were 0.32 and 0.14, respectively. However, when irradiated with ultraviolet light

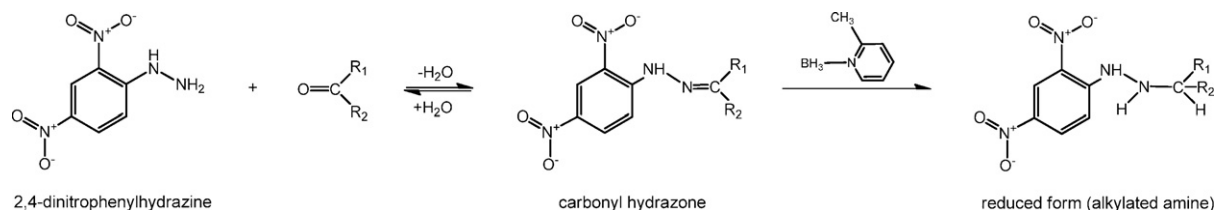


Fig. 4. Scheme of the reductive amination of carbonyl 2,4-DNPhydrazones with 2-picoline borane.

2-hexanone and 4-methyl-2-pentanone showed lower equilibrium constants than corresponding 3-ketones. Consequently, only a minimum concentration of catalytic acid must be added. The better method for the determination of ketone-2,4-DNPhydrazones by HPLC or GC is to add phosphoric acid to both the standard reference solution and samples, forming a 0.001 mol/L acid solution, and analyzing after 27 h.

3. Reductive amination of aldehyde 2,4-DNPhydrazones

As mentioned above, the traditional method for the measurement of carbonyl compounds, using DNPH to form the corresponding 2,4-DNPhydrazones derivatives, is subject to analytical errors because DNPhydrazones form both *E*- and *Z*-stereoisomers as a result of the C=N double bond. In order to resolve the isometric problem, it is necessary to transform the C=N double bond to a C-N single bond through use of a reducing agent. Various kinds of reducing agents, such as sodium cyanohydrinborate ($NaBH_3CN$) [59,60], sodium triacetoxyborohydride ($Na(OAc)_3BH$) [61–65], pyridine-borane ($pyr-BH_3$) [66–68], titanium(IV) isopropoxide/sodium borohydride ($Ti(Oi-Pr)_4/NaBH_4$) [69–72], borohydride exchange resin [73], zinc borohydride/silica gel ($Zn(BH_4)_2/SiO_2$) [74], and phenylsilane/dibutyltin dichloride ($PhSiH_4/Bu_2SnCl_2$) [75] have been developed for this conversion. The choice of the reducing agent is very critical to the success of the reaction, since the reducing agent must reduce imines selectively. Pyridine-borane has been widely used as a reductive amination reagent for aldehydes and ketones [68]. However, this reagent is quite unstable to heat and attempted distillation of the liquid residue at reduced pressures sometimes results in violent decompositions [76–78]. Thus, extreme care must be used if this reagent is handled in large quantities. Sato et al. [79] have developed an expeditious, easy-to-handle and environmentally friendly approach to the synthesis of a variety of amines through a three-component one-pot reaction of carbonyl compounds, amines, and 2-picoline borane. The later is a thermally stable transparent solid that be stored on a shelf for months without appreciable loss of the reduction capability. The use of 2-picoline borane eliminates the problems encountered with the use of other less stable reducing agents such as pyridine borane.

Recently, we developed a method for transforming the C=N double bond into a C-N single bond, using reductive amination of DNPhydrazones derivatives with 2-picoline borane [80]. Reductive amination of aldehyde DNPhydrazones is achieved by adding 2-picoline borane to the acetonitrile solution used to elute the DNPH-cartridge. Fig. 4 shows a scheme of the reductive amination of carbonyl 2,4-DNPhydrazones with 2-picoline borane. Aldehyde DNPhydrazones (C1–C10) are completely converted into their reduced forms within 40 min in the presence of 1 mmol/L 2-picoline borane and 20 mmol phosphoric acid. Fig. 5 shows the chromatograms at the state of coexistent aldehyde DNPhydrazones and their reduced forms. Before the addition of 2-picoline borane, only *E*- and *Z*-DNPhydrazones isomers are detected (upper panel). After the addition of 2-picoline borane, peaks of the reduced

forms began to appear between the *Z*- and *E*-isomer peaks of the corresponding DNPhydrazones. Twenty minutes after the addition of 2-picoline borane solution, reductive amination proceeds to 46–50% (middle panel). Sixty minutes later (80 min total), all DNPhydrazones derivatives, including *Z*- and *E*-isomers, are completely converted to their respective reduced forms (lower panel). These reduced forms are very stable and do not change when stored for two weeks at room temperature. The absorption maximum wavelengths of the reduced forms from C1 to C10 aldehyde DNPhydrazones were 351–352 nm, which shifted 6–7 nm towards shorter wavelengths when compared to the corresponding

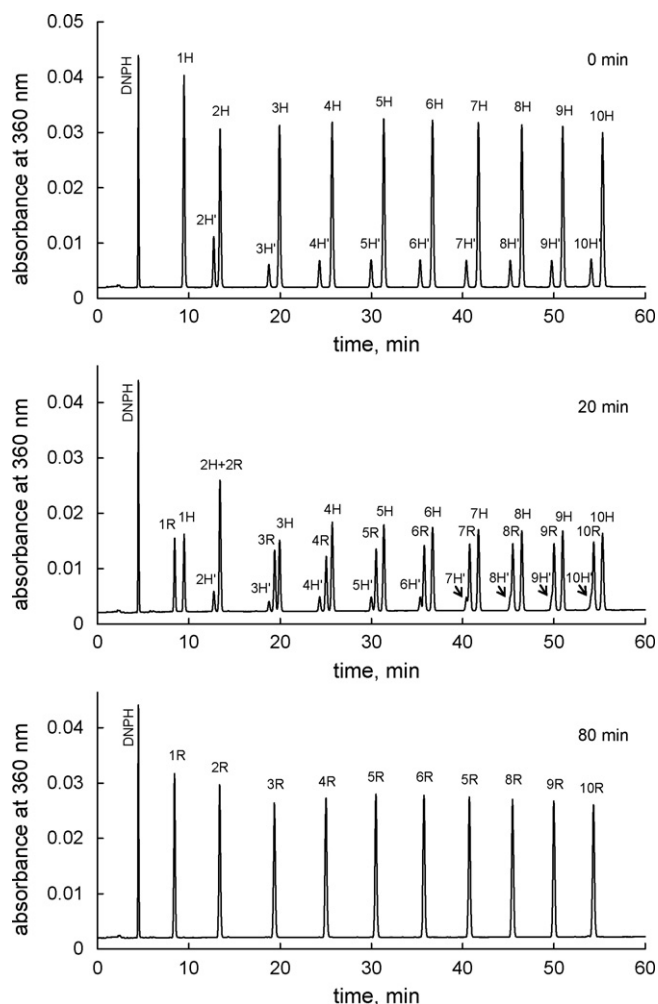


Fig. 5. Chromatographic profiles of DNPhydrazones and their reduced forms changing with reaction time. Number of peak name indicates carbon number of precursor aldehyde (1: formaldehyde, 2: acetaldehyde, 3: propanal, 4: butanal, 5: pentanal, 6: hexanal, 7: heptanal, 8: octanal, 9: nonanal, and 10: decanal). "H" indicates DNPhydrazones derivative and "R" indicates reduced form of DNPhydrazones derivative. Prime sign indicates *Z*-isomer of DNPhydrazones derivative. Reproduced with permission from Fig. 3 in Ref. [80].

DNPhydrazones. The molar absorption coefficients were 1.5×10^4 (C1)– 2.2×10^4 L/mol/cm (C10). Complete separation between C1 and C10 aldehyde DNPhydrazones and the corresponding reduced forms can be achieved by operating the HPLC in gradient mode using an Ascentis RP-Amide column (150 mm \times 4.6 mm i.d.). The RSDs of DNPhydrazone (Z+E) peak areas ranged from 0.40 to 0.66 and those of the corresponding reduced forms ranged from 0.26 to 0.41. This demonstrates that the reductive amination method gave improved HPLC analytical precision because of the absence of stereoisomers.

4. Derivatization of phthalaldehydes

Glutaraldehyde is a powerful biocide that was first introduced in 1963. Until relatively recently it has been the only widely available disinfectant for the reprocessing of flexible endoscopes and other heat-sensitive equipment. Orthophthalaldehyde (OPA) was introduced in 1999 as a safer alternative to glutaraldehyde, even though there was little evidence available to support such claims. OPA is a potential dermal and respiratory sensitizer and irritates the skin and respiratory tract [81]. Various analysis methods for difunctional glutaraldehyde have been developed. For the most part, they are based on solid substrate sampling and involve the use of derivatizing agents [82–86]. When derivatized with DNPH, OPA was collected using a silica gel cartridge impregnated with acidified 2,4-dinitrophenylhydrazine (DNPH-cartridge) and derivatives were analyzed by HPLC. The derivatization was examined by comparing the process with three phthalaldehyde isomers (ortho-, iso- and tere-) [87]. Fig. 6 shows chromatograms of OPA-DNPhydrazone, isophthalaldehyde (IPA) - DNPhydrazone and terephthalaldehyde (TPA) - DNPhydrazone synthesized with a fourfold molar excess of DNPH and with a fourfold molar excess of aldehyde. Chromatograms resulting from the use of excess aldehyde or excess DNPH are designated with the suffix “-A” or “-D” respectively. Only one peak is observed in OPA-DNPhydrazone, and two peaks are observed in IPA-DNPhydrazone and TPA-DNPhydrazone. In the early eluting peaks, peak areas of IPA-A and TPA-A are much larger than those of corresponding IPA-D and TPA-D. In the late eluting peaks, peak areas of IPA-D and TPA-D are much larger than those of corresponding IPA-A and TPA-A. Dialdehydes such as phthalaldehydes may give two types of derivatives, namely mono- and bis-DNPhydrazone derivatives. The early eluting peaks are mono-DNPhydrazone derivatives and late eluting peaks are bis-DNPhydrazone derivatives. In the case of iso- and terephthalaldehyde, derivatives synthesized with excess aldehyde consisted mainly of mono-derivatives and derivatives synthesized with excess DNPH consist mainly of the bis-derivative. In the case of OPA, only the bis-derivative was detected and the mono-derivative was never observed under any conditions. OPA is completely retained by the DNPH-cartridge. The derivatization reaction was incomplete and unreacted OPA was flushed from the cartridge during the subsequent solvent extraction process. Unreacted OPA and DNPH react in the extraction solvent solution. Immediately after solvent extraction, both mono- and bis-DNPhydrazone derivatives of OPA are present in the solution. Over time, the mono-derivative decreased and the bis-derivative increased in concentration until only the bis-derivative remained; allowing accurate determination of the OPA concentration. The transformation of mono-derivative to bis-derivative was faster in polar aprotic solvents such as acetonitrile, dimethyl sulfoxide and ethyl acetate. Transformation is found to occur most quickly in acetonitrile solvent and is completed within 4 h. It is suggested that the reaction of OPA and DNPH proceeded in polar aprotic solvents and mono-derivative was completely transformed to bis-derivative according to the reaction of Fig. 7. It is possible to measure OPA as

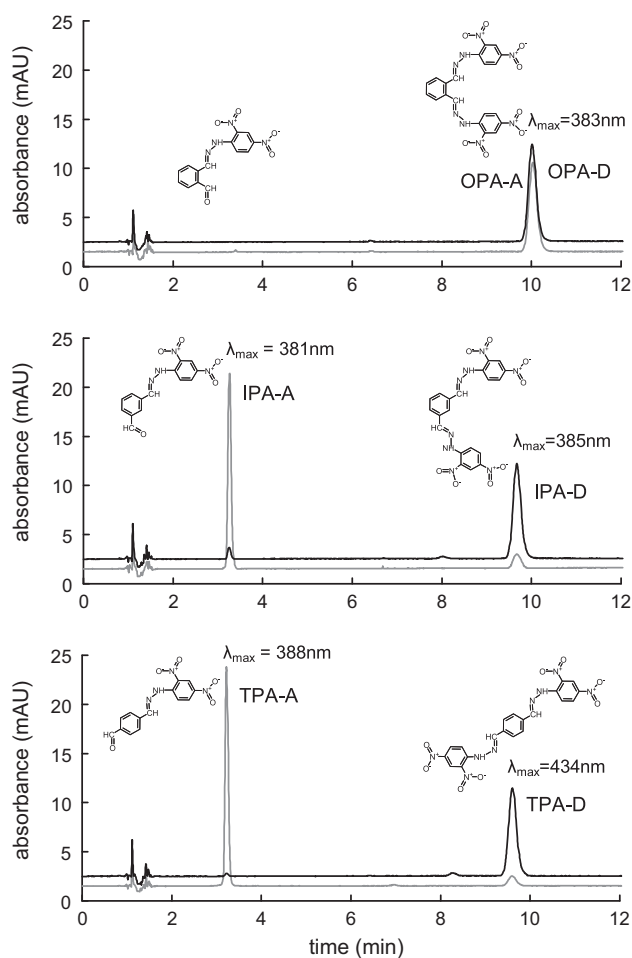


Fig. 6. HPLC chromatograms of OPA-DNPhydrazone (upper), IPA-DNPhydrazone (middle) and TPA-DNPhydrazone (lower) at maximum wavelengths by photo diode array detector. Light-colored chromatograms indicate the derivatives synthesized with excess of aldehyde and dark-colored chromatograms indicate the derivatives synthesized with excess of DNPH. The concentration was 2 mg/L. Reproduced with permission from Fig. 1 in Ref. [87].

the bis-derivative using a DNPH impregnated silica cartridge and HPLC analysis.

5. Application of DNPH derivatization to new analytical methods

5.1. Simultaneous determination of carboxylic acids and carbonyls

It has been recognized that DNPH only reacts with the carbonyl functional groups in aldehydes and ketones and not with those in compounds such as carboxylic acids, esters and amides. However in our experiments, we have found that carboxylic acids such as formic acid and acetic acid react with DNPH to form the corresponding carboxylic-2,4-dinitrophenylhydrazides under specific conditions [88]. A DNPH-cartridge saturated with formic acid vapor becomes gradually discolored and completely changes to light yellow in 6 h at 25 °C. The HPLC chromatogram of the eluant from this DNPH-cartridge indicates complete consumption of DNPH accompanied with formation of formic-2,4-dinitrophenylhydrazide (formic-DNPhydrazone). Fig. 8 shows the peak area changes with time of DNPH and formic-DNPhydrazone at wavelength 360 nm. Acetic acid, propionic acid and butyric acid exhibit similar behavior with longer reaction time in order of

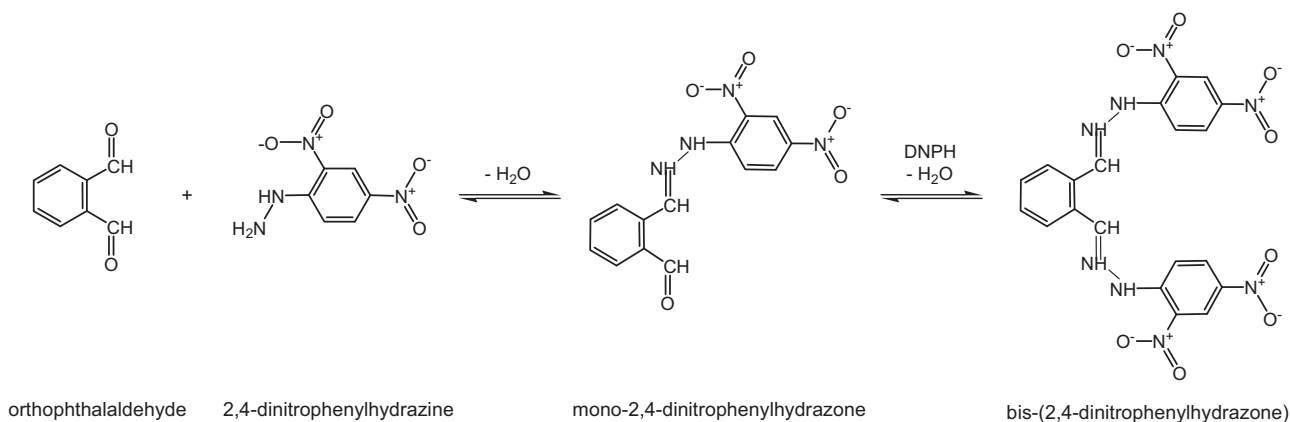


Fig. 7. Scheme of the derivatization reaction of DNPH with orthophthalaldehyde.

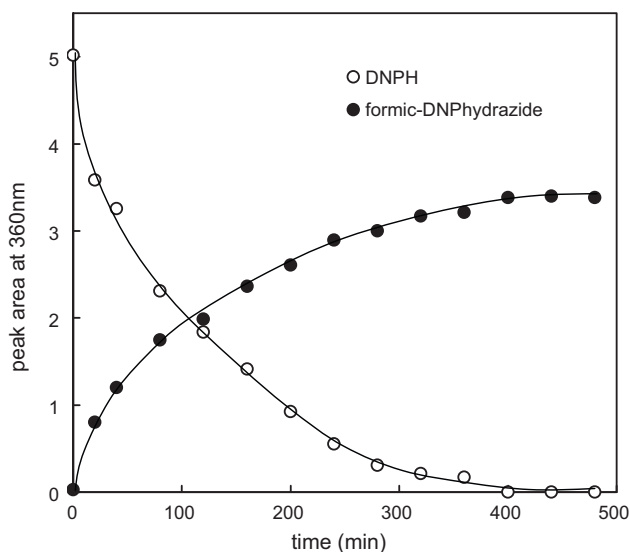


Fig. 8. The reaction of adsorbed formic acid and DNPH with time. ($\lambda = 360\text{ nm}$). Reproduced with permission from Fig. 1 in Ref. [88].

increasing carbon number. Fig. 9 shows the derivatization reaction of DNPH with carboxylic acid. It is suggested that carboxylic acids react with DNPH to initially form corresponding hydrazone derivatives, which then isomerize to hydrazides by keto-enol tautomerization. These hydrazide derivatives have excellent thermal stability with melting points higher than those of the corresponding hydrazones by 32–50 °C. They exhibit maximum absorption wavelengths of 331–334 nm and molar absorption coefficients of $1.4 \times 10^4\text{ L/mol/cm}$. In reversed-phase HPLC analysis, the separations of hydrazide and hydrazones derivatives may be incomplete. The retention times of DNPhydrazone peaks vary with mobile phase

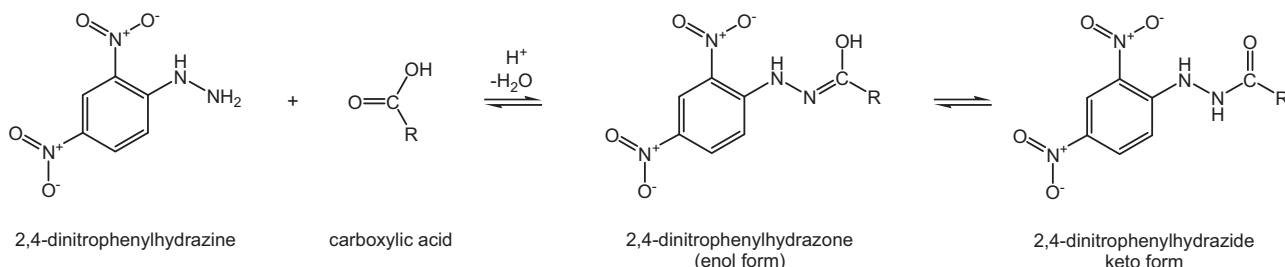


Fig. 9. Scheme of the derivatization reaction of DNPH with carboxylic acid.

pH. The addition of base to the mobile phase shortens the retention times of C1–C6 DNPhydrazone peaks and shifts the UV/vis spectrum profiles to longer wavelengths. Under the conditions of 0, 0.1, and 1.0 mmol/L dibasic potassium phosphate, the spectra of formic DNPhydrazone are unimodal with a maximum wavelength of 339 nm, bimodal with a maximum wavelength of 339 and 423 nm, and unimodal with a maximum wavelength of 423 nm, respectively. The DNPhydrazone derivatives of carboxylic acids exist in equilibrium with their enol tautomer and exhibit an isosbestic point at 370 nm. Complete separation of C1–C6 carboxylic acids and aldehydes was achieved on an RP-Amide column with the use of ACN–H₂O (40:60) containing dibasic potassium phosphate (0.1 mmol/L) as the mobile phase and UV detection at 370 nm. Fig. 10 shows chromatogram of C1–C6 hydrazide and hydrazone derivatives using an RP-Amide C16 column. The derivatization reaction to hydrazide progressed essentially to completion for the DNPH-cartridges containing 0.2–1% (v/w) phosphoric acid. The best condition for the simultaneous measurement of carboxylic acids and aldehydes is 1% (v/w) phosphoric acid because acidic conditions are needed for the measurement of aldehydes. Cartridges packed with DNPH-coated silica particles (DNPH-cartridge) are used for sampling formic acid and aldehydes. Formic acid is physically adsorbed on the silica particles as the first step of the sampling mechanism. A gradual reaction with DNPH follows. Formic acid reacts very slowly with DNPH at room temperature (20 °C), but reacts completely at 80 °C over 4 h.

5.2. Simultaneous determination of ozone and carbonyls

A new method for the simultaneous determination of ozone and carbonyls in air using a two-bed cartridge system has been developed [89,90]. Each bed consists of reagent-impregnated silica particles. The first contains *trans*-1,2-bis-(2-pyridyl)ethylene (2BPE) while the second contains 2,4-dinitrophenylhydrazine (DNPH). Fig. 11 shows the reaction pathways for the simulta-

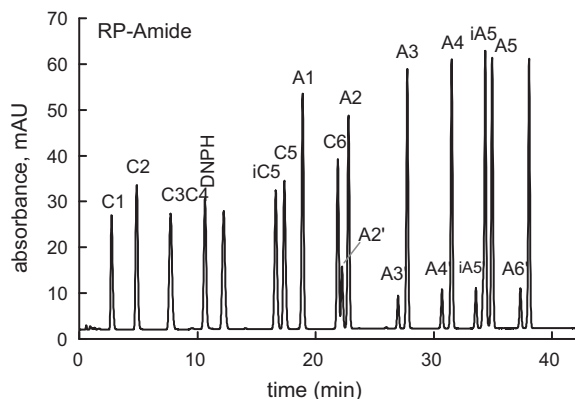


Fig. 10. Chromatographic profiles of C1–C6 carboxylic-DNPhydrazides and aldehyde-2,4-DNPhydrazones on an Ascentis RP-Amide column (100 $\mu\text{mol/L}$) at maximum absorption wavelengths between 300 nm and 500 nm. A prime sign indicates the Z-isomer C1, formic acid DNPhydrazide; C2, acetic acid DNPhydrazide; C3, propionic acid DNPhydrazide; C4, butyric acid DNPhydrazide; i-C5, i-pentanoic acid DNPhydrazide; n-C5, n-pentanoic acid DNPhydrazide; C6, hexanoic acid DNPhydrazide; A1, formaldehyde DNPhydrazide; A2, acetaldehyde DNPhydrazide; A3, propionaldehyde DNPhydrazide; A4, butyraldehyde DNPhydrazide; i-A5, i-pentanal DNPhydrazide; n-A5, n-pentanal, DNPhydrazide; A6, hexanal DNPhydrazide.

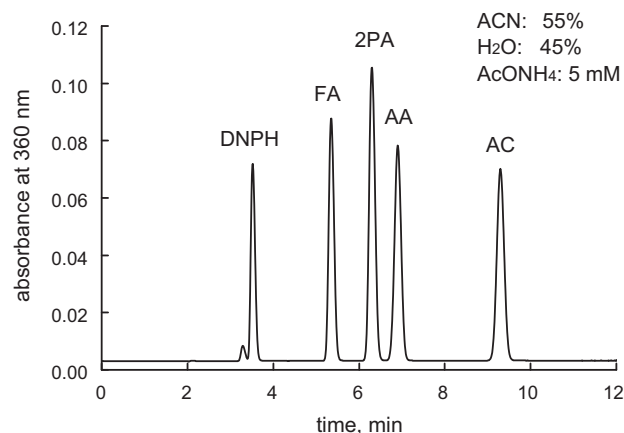


Fig. 12. Chromatogram of pyridine-2-aldehyde and other carbonyl 2,4-DNPhydrazones. Reproduced with permission from Fig. 2 in Ref. [90].

neous determination of ozone and carbonyls. Air samples are drawn through the cartridge first through the 2BPE bed and then through the DNPH. Ozone in the air sample is trapped in the first bed by the 2BPE-coated silica particles to produce pyridine-2-aldehyde. Airborne carbonyls pass unimpeded through the 2BPE and are trapped in the second bed by the DNPH-coated silica particles. They produce carbonyl DNPhydrazones. Fig. 12 shows the chromatographic profiles of 2PA (derived from ozone) and carbonyl DNPhydrazones. DNPH and carbonyl 2,4-DNPhydrazones are not influenced by ozone because of effective trapping by the 2BPE. Extraction is performed in the direction reverse to air sampling. When solvent is eluted through the 2BPE/DNPH-cartridge, excess DNPH is washed into the 2BPE bed where it reacts with pyridine-2-aldehyde and forms the corresponding hydrazone derivative. The use of a 2BPE/DNPH-cartridge has made possible the simultaneous determination of ozone

and carbonyls. A separate ozone scrubber is not necessary with the 2BPE/DNPH cartridge because the 2BPE portion of the sampler serves this function. Initially, *trans*-1,2-bis-(4-pyridyl)ethylene (4BPE) was used for the BPE/DNPH-cartridge [89]. However, the method suffered from long reaction times in the eluate, low solubility of the DNPH derivative and a strong dependence on atmospheric moisture. These problems could be overcome using *trans*-1,2-bis-(2-pyridyl)ethylene (2BPE) in place of 4BPE [90]. The efficiency of the reaction of ozone with 2BPE to form pyridine-2-aldehyde (2PA) is higher than the corresponding reaction with 4-BPE. Under the optimized elution conditions, the reaction times of 2PA and 4PA with DNPH are within 15 min and 120 min, respectively. During elution from the sampling cartridge, 2PA formed from 2-BPE and ozone is easier to dissolve in the elution solvent. A stronger influence of humidity was observed in ozone recovery by the 4-BPE/DNPH method. 2BPE exhibits a maximum reaction efficiency of 84% at 32% R.H., while 49% R.H. is required for 4BPE to attain a maximum reaction efficiency of 82%. Humidity has much less influence on the reaction of 2-BPE with ozone. Above 18% R.H., the reaction efficiency of 2-BPE with ozone is in the range 80–84%. Thus, 2-BPE is the more useful reagent for ozone analysis. The measured concentrations of ozone and carbonyls by the improved 2-BPE/DNPH

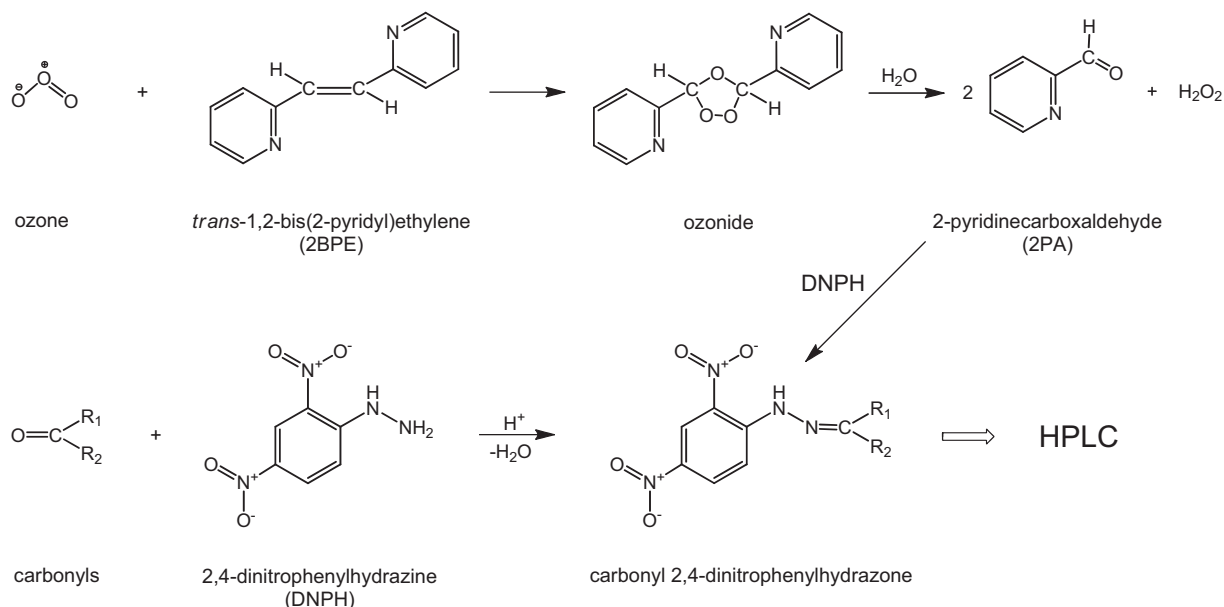


Fig. 11. Scheme of the simultaneous determination of ozone and carbonyls.

method corresponded with the values obtained using an ozone auto analyzer and a DNPH cartridge coupled with a KI-ozone scrubbing cartridge.

6. Conclusions

The specific reaction of carbonyl compounds with DNPH forming the corresponding DNPhhydrazones is one of the most important qualitative and quantitative methods in analytical chemistry. In this review, basic research such as isomerizations of DNPhhydrazones and reductive amination of aldehyde 2,4-DNPhhydrazones were described. Moreover, applications of new analytical methods, such as the analyses of carboxylic acids and ozone, were introduced. We expect that the traditional DNPH derivatization method will be more useful to analyze carbonyls or other compounds.

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