Determination of Carbonyls in Natural and other Products

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Summary:Carbonyls, which are present in a large number of foods and natural products, diversify in nature and significantly vary in amounts. They are usually transformed to their 2,4-DNPHs for their analysis. A review of the chromatograpic (paper, TL, GL, HPL), spectroscopic/spectrophotometric (IR, UV, GC-MS), titrimetric and polarographic techniques applied for identifying this important class of compounds has been presented in this manuscript.

Introduction

Carbonyls are important compounds which diversify in nature. Amongst them volatile aldehyde, ketones and dicarbonyl compounds are well known to influence the flavour and quality of a large number of foods and natural products. Various workers have identified a variety of carbonyls from dehydrated pork [1], cheese [2], milk [3], milk fat [4], maple fruit [5], tomatoes [6], apples [7-9], frozen beans [10], etc. Several α-ketoacids have also been found in nature. Fruits like banana, grape-fruit, mountain pawpaw, tangelo, apricot [11-12], papaya, berries and grapes contain a variety of these acids [13,14]. Moreover, presence of free carbonyls in dairy products and vegetable extracts has been indicated [15], while the unsaturated aldehydes have been found to be responsible for card board's flavour in skim milk [16].

Since these compounds are vital part of the flavour constituents, considerable activity has been directed to the isolation, separation and identification of these compounds. Both qualitative and quantitative determination of these compounds as 2,4-dinitrophenylhydrazones (2,4-DNPH) in complex mixtures has been the subject of many techniques. These odoriferous constituents are usually present in limited quantities. Such a situation demands an efficient method of determining the nature and amount of these compounds. Unfortunately, in the wake of unlimited possibilities, no general procedure can be applied for their determination. Generally, every situation calls for a particular approach to identify and quantify such compounds. In a restricted sense one may say that these carbonyls are usually converted into their 2,4-DNPHs, which are later on subjected to analysis. In this connection, chromatographic and spectroscopic techniques are generally applied for identifying these compounds. The chromatographic technique encompasses paper chromatography (including spot-tests), TLC, column chromatography, GLC and HPLC. Apart from these techniques various spectroscopic/spectrometric (colorimetry, IR, UV, GC-MS), titrimetric and polarographic methods have been applied as well. In a few cases techniques other than afore-mentioned have been rather critically reviewed and discussed in this manuscript.

Paper chromatography

Whatman paper

In order to identify the carbonyls Sykora and Prochazka (1953) subjected their 2,4-DNPHs to paper chromatography with petroleum as the stationary phase and ethanol (80%) or propanol (65%) as the mobile phase [17]. They reported R_f values for the DNPHs of various carbonyl compounds. Burton (1954) developed a method, which was useful for the rapid identification of carbonyl compounds. He achieved good separation of 2,4-DNPHs of carbonyl compounds on simple Whatman no. 1 and acetylated Whatman no. 1 papers [18], using an aqueous solution of ethyl acetoacetate as the stationary phase and developing the chromatogram with light petroleum ether reinforced with carbon tetrachloride. In this study acetylated paper appeared to give more compact zones than the untreated paper. The method was

adaptable for the chromatography of DNP-derivatives of amino acids [19] as well.

By using *N,N'*-dimethylformamide as the stationary phase on Whatman no. 7 paper, and a mixture of cyclohexane/cyclohexene (5:3, v/v) as the mobile phase, very satisfactory results were obtained by Sundt and Winter (1958) in separating the 2,4-DNPH of aromatic carbonyl compounds containing one benzene ring. The solvent atmosphere in the jar proved to be very important in obtaining good results. This system is equally suitable for the separation of the 2,4-DNPHs of lower aliphatic carbonyl compounds. For these derivatives, however, decalin is, in general, better suited as the mobile phase [20].

In another investigation satisfactory separation of 2,4-DNPHs of alkyl/aryl, cycloalkyl/aryl and diaryl ketones was easily affected on Whatman paper with water/dimethylformamide as the stationary phase and cyclohexane/carbon tetrachloride/dimethylformamide (20:4:1, v/v) as the mobile phase. In this connection R_f values of 2,4-DNPH of typical aldehydes and ketones are given by Brener *et al.* [21]. The appreciation of the importance of alk-1-en-3-ones in oxidative flavour defects of food [22] and their anomalous chemical behaviour, in relation to other unsaturated carbonyl compounds, prompted a study of some of their properties. Consequently R_f data for the 2,4-DNPHs of C_{4-10} alk-1-en-3-ones was collected and compared with those for C_{1-14} *n*-alka-2,4-dienals, C_{9-10} *n*-alk-2-enals, $C_{5-12,14,16,18}$ *n*-alka-2,4-dienals, C_{9-10} *n*-alka-2,6-dienals, C_{3-13} alkan-2-ones etc. Baykut and Ozeris (1958) used specially treated Schleider Schull 2043 paper, which was impregnated with naphtha (boiling range of 190-220°) and its strips were arranged in a descending chromatographic apparatus. The sample components were separated by the dropwise addition of the naphtha saturated with aqueous acetic acid (97.5%) over a period of 10 hours. It was observed that no colour development was needed for 72.5 γ quantities of 2,4-DNPHs of symmetrical dialkyl ketones investigated [23]. Hulein [24] and Meigh [25] also described a method for the paper chromatographic separation of the 2,4-DNPHs of lower saturated aldehydes, ketones, furfural, acrolein and crotonaldehyde. Later on, Forss *et al.* (1963) extended this method to the separation of 2,4-DNPHs of alk-2-enals, alk-2,4-dienals and alk-2-enals of higher molecular weight [26].

Reversed phase technique

Seligman and Edmonds (1955) used reversed phase technique on cellulose sheet to separate 2,4-DNPHs [27]. Hattori (1956) also utilized inversed phase chromatographic technique, with tetraline as the stationary phase, for the successful separation of various DNPHs. He applied the 2,4-DNPH derivatives to Toyo no. 50 paper, which was then impregnated with tetraline, and the chromatogram was finally developed in a mixture of methanol (90%)/acetic acid/tetraline (10: 1.5:1 v/v). This procedure satisfactorily resolved the 2,4-DNPHs of normal aliphatic aldehydes (formaldehyde to crotonaldehyde) and methyl ketones (acetone to methyl hexyl ketones) (28).

Acetylated paper

In order to adapt the methods of paper chromatography to non-polar hydrophobic substances, the chemical treatment proposed by Kostir was used by Nieman *et al.* (1951), in which the polar OH groups of cellulose were replaced by Ac-groups. This increased the hydrophobic character of paper. They discussed optimum conditions for acetylation of paper. The treated paper was successfully used for the separation of the DNPHs of formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde and acrolein [29]. Kuntzel and Nungesser (1956) also conducted chromatographic separation of 2,4-DNPHs by the method of Nieman *et al.* with the exception that the paper was only 30-5% acetylated since fully acetylated paper was too fragile. Spots of hydrazones of formaldehyde, acetaldehyde, and propionaldehyde were well separated, but those of acetaldehyde, acrolein and crotonaldehyde could not be distinguished. Oxidation product of cod, herring and stark oil were also examined by this procedure, indicating the presence of formaldehyde, acetaldehyde, propionaldehyde, acrolein, diacetal and glyoxal. Respective spots of 2,4-DNPHs of dialdehydes, when sprayed with KOH turned blue. None of the spots turned carmine red with KOH, which indicated the absence of ketones. Moreover, there were no essential difference between the chromatograms for products

from an oil and those from the free fatty acids of the oil [30].

Prey and Kabil converted carbonyl compounds to their 2,4-DNPHs, which were applied to the acetylated Schleicher and Schull paper from their methanolic solution. They used butanol/methanol/formic acid (83:15:2, v/v) and petroleum ether/methanol/ethyl acetate (65:15:20, v/v) solvent combinations, and the latter afforded best separation. They also investigated higher aliphatic hydrocarbons in place of petroleum ether and observed that the differentiation was better in case of hydrocarbons of higher molecular weight, while cyclic hydrocarbons and higher alcohols were not suitable. Carbonyls investigated in this study included HCHO, MeCHO, EtCHO, PrCHO, CH_3-CH=CHCHO, BuCHO, AmCHO, *n*-C_6H_{11}CHO, Me_2CO, Et_2CO, MeCOPr, $(Me_2CH)_2CO$, *iso*-BuCOMe, CH_2Ac_2 and cyclopentanone. Their 2,4-DNPHs were visible in UV light [31]. It has been reported that the only method that is applicable to both mono and dicarbonyl compounds requires acetylated filter paper. However, a solvent system has also been developed that separates their nitrophenyl hydrazones on ordinary filter paper. This system is the only one of 78 systems tried that gave favourable results in separating both types of compounds [32].

Compounds which are insoluble in water but soluble in organic solvents cannot be separated by partition chromatography. Separation is, however, possible by partition between two organic solvents on a specially acetylated filter paper acting as the stationary phase, the preparation of which has been described by Kostir and Slavik (1950). They used this technique to separate water insoluble DNPHs of HCHO, CH_3CHO, Me_2CO, $AcCH_2COOH$, AcCOOH, HOOCCO. CH_2COOH, HOOC.CO. $(CH_2)_2COOH$, and osazones of $HOCH_2$.CO.CHO and CH_3CO.CHO by one or two dimensional chromatograms. The spots were detected by spraying with 10% NaOH which forms blue, red, orange and brown spots with 2-5 γ. The R_f values of the compounds in various solvent systems are given [33].

Burnett (1966) also separated 2,4-DNPH derivatives of C_1-C_8 aldehydes and ketones by two dimensional development in paper chromatography. The first development was with ethyl nitrite:acetonitrile:methanol:water (1:2:3:3, v/v) and the second with light mineral oil:*iso*-octane (1:2, v/v) [34]. Two isomeric compounds, cyclohexylidene acetone and *iso*-propylene cyclohexanone, were separated by Erwin (1958) by circular chromatography of their DNPHs. Similarly, α,β-mesityl oxide was separated from β,γ-mesityl oxide. Thus, the yellow DNPH of pure β,α-mestityl oxide proved to be a mixture of syn and anti components. In this connection acetylated filter paper was used for their separation with 65% aqueous propanol as the eluent using 20-30 γ of the DNPH in ethyl acetate. The chromatographed areas appeared yellow to red in ordinary light and dark in the UV [35].

Volatile constituents

The filter paper partition chromatography of 2,4-DNPH of keto-acids and subsequent investigations have demonstrated that it can be used to affect the separation and identification of a number of other 2,4-DNPHs. This method is simple and rapid and requires only microgram quantities of the 2,4-DNPH, and has been used to separate and identify volatile constituents of orange juice containing aldehydes and ketones [36]. The same methods has also been used to resolve a mixture of the alcohols, which were identified through their 3,5-dinitrobenzoates. It was observed that separation of many compounds could be affected by using appropriate paper/solvent combinations enabling a large enough difference in their R_f values. Moreover, this study revealed that a paper impregnated with silicic acid gave exceptionally clear and sharp spots compared to results in other papers. It was also noticed that a difference of about 0.08 in R_f values was essential to differentiate well between compounds. Meigh (1952) developed a method which gave improved separation of the DNPHs of a number of aldehydes and ketones, and proved useful in the detection of volatile aldehydes and ketones dissolved in petroleum ether in quantities greater than 10^{-7} gram mole. In this connection, the non-aqueous two-phase solvents, methanol-heptane, were used, where the heptane phase was the mobile one. The DNPHs of the keto-acids and dicarbonyl compounds failed to resolve by this procedure [25].

During a study of the less volatile compounds evolved by apples it was necessary to separate a mixture of difficultly soluble DNPHs. In

this connection when a paper made water repellent with a solution of chloropolysiloxane was used, it gave desired resolution [37]. On the other hand, most of the published methods rely on preliminary impregnation of paper by dipping with ethyl lactate [17], propylene glycol [38] and 2-phenoxyethanol [39], or by equilibration with *N,N'*-dimethyl-formamide/methanol [40]. This is followed by development with a fraction of light petroleum. These methods give good separation of saturated aliphatic compounds, but are not satisfactory for less soluble derivatives.

Chromatostrip method was applied by Katayama (1955) to the separation of terpenes, who reported the R_f values of pure terpenes in a solvent system of 15% ethylacetate/*n*-hexane (1:8, v/v). Separation of linalool and eugenol was performed in combination with steam distillation. This work was extended to the analysis of fractionally distilled neutral oil, which revealed the presence of α-pinene, *d*-limonene, linalool, carvone and α-terpineol in it [41]. Later on Srepel (1963) examined volatile oils of *Ruta graveolens*, *R. chalepensis* and *R. corsica* along with other volatile oils by paper chromatography and reported the R_f values of the compounds separated. These results were utilized to determine the relation of the Me/*n*-nonyl and the Me/*n*-hexyl ketones in the oils. It was also inferred that the drug plant collected, when it still carried green fruit, gave the maximum amount of high quality oil [42]. Ellis, and his associates (1959) have described rapid chromatography methods of separating mixtures of 2-alkanone, *n*-alkanal, alk-2-enal and alk-2,4-dienal 2,4-DNPHs into classes and each class into individual compounds [43-45]. These methods have been applied to the determination of changes in the proportions of steam volatile mono-carbonyl classes with the anti-oxidation of pork fat and to the identification of such compounds volatilized from a rancid pork fat [46]. In the latter study, tentative quantitative data were reported for the classes and individual compounds. Aside from the inevitable mechanical losses in the manipulations, it was recognised that the most serious obstacle to quantitative application was the variation in stability of the different classes. The alk-2,4-dienal derivatives were examined to be particularly sensitive to light and air [47].

The oxidation of a fat leads to the scission and degradation of the molecules into short chain aldehydes and ketones [48]. Some of the volatile decomposition products that have been identified from the auto oxidation of ethyl linoleate are methyl ethyl ketone, acetaldehyde, propanal, pentanal, haxanal, crotonal, 2-pentanal and 2-heptanal [49-50]. Kawahara and Dutton (1952) have also identified acetaldehyde, propionaldehyde, 2-pentanal and hexanal from the volatile cleavage products of antioxidized soyabean oil by separation of their diphenyl hydrazones on silica gel column [51]. They have identified these aldehydes, with the exception of hexanal, from the volatile products of antioxidized methyl linoleate as well as by following the same technique [52]. In an other study butter fat, corn oil and pure methyl esters were subjected to thermal oxidative polymerization, and small amounts of volatile compounds were collected. A number of methods have been suggested for the separation of their 2,4-DNPHs [8,17, 26, 27, 36, 40, 53]. Most of these methods were found to be unsatisfactory because of poor resolution or streaking, while some were time consuming. The method developed used a modified version of Silberstein's method [38] which enabled the separation of a more complex mixture of 2,4-DNPHs of higher aliphatic aldehyde [54]. Bhalerao and Kumerrow (1959) developed a technique involving the use of filter paper impregnated with ethylene glycol, and the chromatogram was developed by descending system with methanol saturated *n*-hexane. This technique was applied to separate the 2,4-DNPHs of the aliphatic aldehydes from C_4 to C_9 into distinctive spots and could be useful in the separation of corresponding derivatives of the ketones [48].

Keto acids

Several α-keto acids have been found in nature, *e.g.*, pyruvic, glyoxylic, oxalacetic, α-keto glutaric, α-keto *iso*-valeric, α-keto-β-methyl glutaric acid. But only a few studies on the paper chromatographic behaviour of free -keto acids have appeared [35,56]. It may partly be due to the instability of many α-keto acids during isolation procedure. Nonetheless, a number of investigators [57-61] have resorted to the use of corresponding 2,4-DNPH derivatives for the quantitative determination of α-keto acids [62-66]. In this connection several difficulties arise because of

similar behaviour of certain 2,4-DNPH derivatives coupled with the possibility of two spots on one dimensional paper chromatograms and often four spots on two dimensional chromatograms. This may be attributed to the presence of *syn* and *anti*-hydrazones [67-68]. These problems were overcome by two groups of workers, who hydrogenated the hydrazones to afford amino acids which were identified by paper chromatograms. These hydrogenation techniques proved of value in the identification of several α-keto acids present in blood, urine [17] and certain plant tissues [69-71]. Even several synthetically prepared α-keto acids and their hydrogenated analogues (amino acids) have been subjected to paper chromatography by Meister *et al.* (1956). The information presented by them would serve as a useful basis for the identification of a wide variety of α-keto acids [72].

Three groups of workers separated DNPHs of various keto acids along with formaldehyde, acetaldehyde, acetone and dicarbonyl compounds by paper chromatography with different two phase solvents [33,58, 59]. Lulonen (1955) noted R_f values of the aliphatic keto acid hydrazones (upto C_5), which when plotted against the C-atom number afforded a straight line. Moreover, aromatic keto acid hydrazones were resolved with a mixture of petroleum and isopropanol (7:3, v/v) saturated with water. In this connection R_f value was found to depend upon the amount of isopropanol. Aliphatic keto acid hydrazones were observed to migrate very slowly in this solvent system [73].

Steroids

Neher and Weltstein (1952) have developed a system for separating steroidal compounds. This system, using phenoxyethanol impregnated paper as the stationary phase and heptane as the mobile phase, has been found to be well adopted to the separation of homologues of low molecular weight 2,4-DNPHs. Thus as much as 250 γ of a 2,4-DNPH in a spot can be chromatographed with no streaking. The spots obtained with such amounts of material were readily visible without spraying or use of UV light. Also, as all solvents used got easily evaporated, the separated 2,4-DNPHs were readily eluted and recovered in pure state from the paper [74].

Sugars, etc.

Natarajan and Bains (1955) chromatographed mannose phenylhydrazone (1% in pyridine) with butanol/dimethyl ketone/water (2:7:1, v/v) which gave a well defined band. However, the hydrazone when chromatographed after againg for 24 hours, gave a new band having an R_f value close to that of mannose, arabinose and fructose, beside the original hydrazone band. The new band gave a positive test with aniline hydrogen phthalate and negative with resorcinol, indicating the presence of a pentose group and the lack of a ketose group respectively [75]. Ulbrich and Makes (1964) impregnated Whatman no. 4 filter paper with dimethylformamide and paraffin oil and used it for separating and identifying crystalline 2,4-DNPH formed from glycidyl ether by a sequence of reactions. The R_f values were linearly related to the number of C-atoms in these compounds [76].

Spots tests

Miller and Kirchner (1953) gave procedures for such chromatostrip spot reactions as oxidation, reduction, dehydration, hydrolysis and the formation of such derivatives as 3,5-dinitrobenzoate, phenylcarbamate, phenylhydrazones and semicarbazones [77].In the case of formation of derivatives such as 3,5-dinitrobenzoates and phenylhydrazones, reagents were usually strongly absorbed at the origin.

In the chromatographic system, the R_f value of a compound generally appeared to be related to the size of the molecule and the number and kind of functional groups, which the molecule contained. An increase in molecular weight tended to increase the R_f value, as did a decrease in the number of functional groups. Changing the nature of the functional groups by means of some reaction such as reducing a ketone to an alcohol, always resulted in a change of R_f value, so that the separation by chromatography, was easily affected. The same authors have found these reactions and their subsequent analysis on chromatographic strips to be useful in a number of ways. Thus, the compound may be positively identified by means of the reactions; the information could be obtained with a small amount, and the time could be saved. Moreover, if a derivative is desired from a particular compound, its formation may be readily

checked on a chromatostrip without the necessity of extensive purification and crystallisation required to perform main reactions. In this way, the condition and the progress of the reaction could be checked rapidly.

The ability of 'unifunctional high capacity synthetic resins' to exchange hydrogen quantitatively for metallic or organic cation has been explored for the determination of organic salts on micro scale. This resulted in the analysis of 46 salts of organic acids or bases. The method offers the advantage of speed, simplicity and accuracy over conventional micro methods for determining organic salts. Various workers have utilised these procedures for a variety of spot reactions. Flood (1940) has used impregnated papers for inorganic ion analysis, in which the paper was first treated with aluminium hydroxide or a synthetic zeolite, and then with a suitable reagent for developing the spot [78]. Hopf has used Flood's method as a spot test for ketones and aldehydes by treating the filter paper with alumina, and 2,4-DNP-hydrazines. In this instance 2,4-DNPHs were applied directly to the paper as explained by Kirchner and Keller [53].

Verma Krishna (1979) oxidized arylhydrazines with *N*-bromosuccinimide [80]. The oxidized products were coupled with resorcinol to form azo dyes, which gave intense colour in alkaline media. Hydrazones and osazones were hydrolysed to form the arylhydrazines, which were then detected as above. This procedure was applied to 4-nitro and 2,4-DNP-hydrazines, which were detected by forming their hydrazones with salicylaldehyde and adding alkali to produce a violet colour. Aldoximes and ketoximes formed hydroxylamide on acid hydrolysis. This was detected with ICI in the presence of sulphanilic acid followed by coupling with 8-hydroxyquinoline, which formed a red dye in alkali. Intense colours were immediately produced when primary, secondary, and tertiary aromatic amines were mixed with diacetoxy iodobenzene. All the tests were specific and sensitive. 1,3-Dibromo-5,5-dialkylhydrantoins have been used by Khan (1980) for the spot test detection of arylhydrazines, arylhydrazones and osazones. He oxidized arylhydrazones to their corresponding diazonium salts, which were then coupled with resorcinol to give azo dyes as pointed out earlier [81]. In this study the arylhydrazones were also hydrolysed with hydrochloric acid to give the corresponding arylhydrazines, which were converted to azo dyes with required reagents. Anger and Offri (1964) detected micro amounts of aldehydephenyl-hydrazones or carboxylic acid hydrazide. Their method is based on the formation of colours with 2,6-dichloro-*p*-benzoquinone-4-chloroimine on a spot plate [82]. In other report the same authors detected micro amount of a phenol or aldehyde-phenylhydrazone. This method is based on reaction in the *para* position of the compound with *p*-nitrosophenol in concentrated H_2SO_4 yielding a blue or green indophenol which turns red with H_2O or aqueous HCl [83].

Feigl (1937) identified arylhydrazines, arylhydrazones and osazones by oxidizing with H_2SeO_3 to diazonium salt and their coupling with α-naphthylamine [84]. Feigl and Bendor (1963) have also described spot tests for the detection of micro amounts (upto 0.5 γ) of 2-, 3-, or 4-pyridine carboxyladehydes, which form coloured product with pehnylhydrazine hydrochloride solution (0.1 %). They also used hydroxylamine hydrochloride and Fe $(NH_4)_2(SO_4)_2$ which form characteristic red or pink colour with pyridine carboxylaldehyde (upto 0.5 γ) on addition of a drop of pyridine. In case of phenylhydrazones and osazones their aqueous/alcoholic test solution with addition of pyridine carboxylaldehyde followed by pyridine produces yellow colour [85].

Thin layer chromatography

Thin layer chromatography offers a simple, rapid and interesting method of separation and identification of aromatic 2,4-DNPHs. A variety of carbonyl compounds have been analysed by this teachnique employing different variations of adsorbents and using solvents and other factors. This is quite evident from numerous investigations that have been cited in literature. One could note that Urbach (1963) described the separation of 2,4-DNPHs of the homologous series ($C_{1\text{-}14}$ alkanals, $C_{3\text{-}13}$ 2-alkanones, $C_{4\text{-}10}$ alk-1-en-3-ones (vinyl ketones), $C_{3\text{-}11,16}$ 2-alkenals, $C_{5\text{-}12,14,16,18}$ 2,4-alkadienals and $C_{6,7,10}$ 3-alken-2-ones using various systems. The TLC plates coated with alumina were found to separate the 2,4-DNPHs of aldehydes, saturated methyl ketones and vinyl ketones with a solvent mixture of 4% ether in light petroleum. By adding specific amount of silver nitrate to alumina a further separation of the derivatives into alkanals,

2-alkenals, 2,4-alkadienals and 2,6-alkadienals and 2,6-nonadienals was obtained with 16% ether in light petroleum as the solvent [86].

A TL chromatographic procedure has been reported by Davidek (1966) for the separation of mixture of lower aliphatic aldehydes (upto C_9) in the form of their 2,4-DNPHs. He used starch impregnated with 30% dimethylformamide in ether as the stationary phase and discussed various precautions, which were necessary for their successful resolution [87]. In another study by the same author the same TLC method has been used to resolve 2,4-DNPHs of aliphatic methyl ketones in a homologous series, which has given R_f values for dimethyl, methyl ethyl-methyl propyl- and methyl pentyl ketones respectively[89]. In the same year Fedeli separated 2,4-DNPHs of aldehydes and ketones by TLC on plates coated with a mixture of silica gel and carbowax of molecular weight 1500. He observed that separation was dependent upon the molecular weight of carbowax. He further noted that carbowax having higher molecular weight usually favoured nice separation [89]. Bruemmer and Mueller (1967) have given R_f values for 250 2,4-DNPH derivatives of carbonyl compounds. In this connection they used silica gel-G and water slurry to prepare the TLC plates [90]. Instead of resorting to 2,4-DNPHs Jong *et al.* (1963) described the preparation and separation of carbonyl compounds on TLC plates and columns using different adsorbents [91].

Carl and Thomas (1972) were able to separate 2,4-DNPHs of aldehydes and ketones into three categories by R_f values and colour by TLC on zinc carbonate using pyridine as a developing agent. The hydrazones of the first category, which were yellow-brown in colour, had R_f values (0.63 - 0.95) and were usually obtained from aliphatic monocarbonyls (dimethyl ketone, acetaldehyde, proprionaldehyde, methyl ethyl ketone, diethyl ketone, diphenyl ketone, cyclopentanone and cyclohexanone). The second category of the hydrazones, which were pink-purple in colour, had R_f values (0.07-0.68) and were usually obtained from aromatic monocarbonyls and others (furfural, benzaldehyde, methyl phenyl ketone, benzoin, formaldehyde and glutaraldehyde). The dihydrazones of dicarbonyls formed the third category [92]. Gaba and Jain (1972) also separated 2,4-DNPH derivatives of monocarbonyl compounds from bis-2,4-DNPHs of dicarbonyl compounds by TLC on 2:1 magnesium oxide celite 545 plates with chloroform - methylnitrite as developing solvents. The compounds were visible as yellow to brown and blue to violet spots respectively. Also, the 2,4-DNPH derivatives of 2-alkanones, alkanals, alk-2-enals and alk-2,4-dienals were separated by using (100:1.25 v/v) hexane-methanol as developing solvents [93].

When Schwartz *et al.* (1968) extended the TL chromatographic method of analysis of carbonyls to natural products, they illustrated the separation of various classes of carbonyl and dicarbonyl compounds as their 2,4-DNPHs on different adsorbents [94]. Pradel (1975) has reviewed the determination of carbonyl compounds in milk and other diary products [15]. Keen *et al.* (1976) adopted the procedure of Schwartz for the quantitative isolation monocarbonyl cases (2-alkanones, *n*-alkanals, 2-alkenals and 2,4-alkadienals) from whole milk powder [95]. The saturated aldehydes constituted the major class (40-70%) of monocarbonyl isolated from each powder [10]. Vashist and Handa (1965) coated fine-layer plates with a slurry of silica gel and plaster of Paris to separate the 2,4-DNPHs of oxo-terpenes present in small quantities in essential oils. Different colour spots formed the basis of individual carbonyl compounds [96]. Almost concurrently Kore *et al.* (1965) separated and identified a series of carbonyl compounds as their 2,4-DNPHs by ascending TLC on alumina plates. It was observed that mixture of 2,4-DNPHs of vanilin, hydroxycitronellal, heliotropin, benzaldehyde, jasminealdehyde, decylaldehydes etc were well separated from the mixture of corresponding 2,4-DNPHs of anisaldehyde, phenyl acetaldehyde, cyclomenaldehyde, methylnonyl acetaldehyde and ionone [97]. Later on mixtures of aliphatic and terpene type 2,4-DNPHs were separated by Davidek (1966) by TLC on silica gel layers [98] and Galanos and Kapoulas (1964) put forward R_f values correlation regarding plate variations [99].

Mahadevan *et al.* (1966) described a simple procedure for the separation of mixture of fatty aldehydes, aldehyde dimethylacetals and methyl esters of fatty acids into different classes by 2-dimensional TLC. Separation of individual members of long chain even numbered saturated fatty aldehydes, their dimethyl acetals, and 2,4-DNPH along with the C-18 unsaturated aldehyde

(Oleyl, linoleyl and linolenyl) and their corresponding derivatives was achieved by reversed phase TLC. The pairs having identical R_f values in the reversed-phase system were separated by adsorption chromatography using silver nitrate impregnated silica gel-G plates. With this techniques *cis-trans* isomers could also be separated, which were verified through their 2,4-DNPH derivatives [100]. In the same year Edward Jr. (1966) separated 2,4-DNPHs of stereoisomeric aliphatic aldehydes and unsymmetrical ketones by several reversed-phase thin layer procedures . The separated 2,4-DNPHs resulting from both the *syn* and *anti* forms were subjected to repeated crystallization from ethanol to afford pure *syn* form. It was also noted that recemization of the *syn* form was stimulated by dissolving in chloroform or by the addition of HCl to acetone or methanol solution. Moreover, dimethyl-formamide-heptane was found signifi-cantly effective in separating the isomers, whereas methanol - water-undecane mixture was suitable for separating the members of homologous series [101].

Rasmus and Deyl (1961) described two improved methods for the separation of 2,4-DNPHs of carbonyl compounds. In one method separation of carbonyl compounds was done on chromatoplates, while in the other separation was carried out using centrifugal chromatography. It was found that the quality of separation was much better in the former case than that obtained with modification of centrifugal chromatography [102].

The behaviour of 2,4-DNPHs C_1-C_5 normal aldehydes was examined by Amos *et al.* (1974) on Kiese-gel-G and alumina-G layers with duozonal development system containing three polar components. When the liquid-liquid interaction dominated, it facilitated the separation in the homologous series. In this study, the chromatographic behaviour of related compounds was also compared to that of 3,5-dinitrobenzoic acid esters of C_1-C_5-normal alcohols [103].

Lyle *et al.* (1976) devised a visible reflectance method for quantifying spots in TLC, which gave reliable results similar to those taken in conventional elution procedures. This method was tested by determining hydrazones of formaldehyde and propionaldehyde and three red water soluble dyes [104]. When carrying out a study on radiolysis of cyclohexanones, the need for separating and identifying some specific carbonyl compounds was recognised by Denti and Luboz (1965) [105]. The problem was solved by TLC using silica gel and alumina according to the technique described by Barret *et al.* [106].

The chromatographic behaviour of a number of aliphatic, cyclic, fatty aromatic ketones on a thin layer of silica gel, and of isomeric methyl cyclohexanone 2,4-DNPHs on a layer of silica gel impregnated with a polar solvent, was studied by Buzlanova and Stepanovskaya in 1965 [107] who observed in air characteristic yellow spots for each compound on sulphuric acid sprayed plates. The limits of sensiuvity for various compounds were also determined.

Irena (1969) achieved the separation by running the chromatograms with chloroform-methanol (1:1,v/v) for 2,4-DNPHs of *p*-benzoquinone and chloro-,2,5-, 2,6-dichloro- and trichloro-*p*-benzoquinones. A different solvent combi-nation consisting of chloroform-cyclohexane (1:1, v/v) was used for the separation of benzaldehyde, *o*- and *p*-chlorobenzaldehyde, *o*-and *p*-hydroxybenzaldehyde and benzophenone. The colour of the spots was intensified with ammonia or propylamine vapours. The method was extended for the analysis of the benzhydrol oxidative chlorination products [108]. Dhout *et al.* (1970) used polyvinyl acetate as the stationary phase in TLC for separating 2,4-DNPHs, 2-diphenylacetyl-1,3-indandione hydrazone, 3,5-dinitrobenzoate and 2,4-dinitroanilines by using a combination of four solvents. Separation were achieved in a short time and R_f values were highly reproducible [109]. Srivastava *et al.* (1977) separated twelve cyclo-hexanetrione arylhydrazones by TLC on silica gel plates [110]. Auvinen and Favorskaya (1963) used a special TLC apparatus to separate a series of 2,4-DNPHs of three steroidal ketones (testosterone, testosterone propionate and progestrone) labelled with C^{14} [111]. It has been observed that incubated brain homogenates from normal and ethanol intoxicated rats produce keto acids. These were determined by TLC as their 2,4-DNPHs [112].

Column chromatography

Various workers have described the separation of 2,4-DNPHs of carbonyls by column

chromatography (CC) on a variety of adsorbents. As early as 1935 Strain described the separation of β-ionone and camphor 2,4-DNPH derivatives as well. He used talc as the adsorbent and suggested alumina, aluminium phosphate, magnesium sulphate and fuller's earth as other adsorbents [113]. Lucas *et al.* (1935) separated acetaldehyde DNPH from propionaldehyde DNPH on alumina [114]. Later on Buchman *et al.* (1942) used chromatographic separation also on alumina to separate two cyclobutyl and cyclobutanone derivatives [115]. Robert and Green (1946) reported chromatographic separation on silicic acid -supergel of any mixtures of the 2,4-DNPHs of acetaldehyde, acetone, propionaldehyde and methyl ethyl ketone, except acetone-propionaldehyde combination [116]. Johnston (1947) has also described the separation of the DNPH of certain androgens on an alumina column [117].

Chromatographic adsorption on bentonite has been used by Jonathan (1948) to separate many aliphatic DNPH mixtures using ether and/or hexane as eluents Twenty two pairs of derivatives representing twelve aliphatic aldehydes and ketones were examined. In this connection use of correct solvent mixture for development resulted in clear, definite zones with sharp lower boundaries, which permitted observations of faint zones near the more prominent bands. Occasionally, however, a single derivative had a tendency to rise to two adjacent bands resulting in poor separation by continued washing. In all such cases, the upper of the two bands was of an orange colour and the lower was yellow in colour. To avoid confusion, it was essential to separate the bands clearly [118].

The use of strips of a mixture of silica gel and poly (vinyl alcohol) deposited on glass has been reported by Schmitt *et al.* (1956) for the separation of the 2,4-DNPHs of aliphatic aldehydes. This has been extended to include the insoluble dicarbonyl derivatives as well [119]. Pippen *et al.* (1957) explored possibility of separating known mixtures, particularly of some higher aldehydes and ketones, and certain other combination of 2,4-DNPHs on silicic acid-celite columns. Columns packed to a height of 75 cm permitted separation of hydrazone mixtures of adjacent members of homologous series of saturated aldehydes upto C_{11}. In addition, the feasibility of separating various combinations by derivatives of 34 aliphatic aldehydes and ketones was also investigated [120]. Lukovnikov and Komissii (1953) devised a method of separating butyraldehyde, propionaldehyde, acetaldehyde, formaldehyde, methanol, ethanol, propanol, *iso*-propanol and butanol from a mixture resulting from oxidation of hydrocarbons [121].

In connection with identification of flavour constituents of dairy products Pradel (1978) investigated the effect of heat on the activity of magnesia for use as an adsorbent on column [122]. The results were compared with those of Schwartz *et al.* (1964) [123] and refuted the results of Synder (1967) regarding the effect of heat on the activity of magnesia. The activated magnesia, Sea Sorb-43, has often been used as an absorbent for detecting food flavour [122].

Change of 2,4-DNPHs on alumina and silica gel chromatographic columns was examined by Forss and Dunstone (1957). They observed that the unsaturated aldehydes were responsible for cardboard flavour in skim milk. The compounds like C_{5-11} 2-enals and C_{6-11} 2,4-dienals were separated by passing their 2,4-DNPHs through chromatographic columns. Most of these derivatives passed unchanged through alumina and silica gel columns, while some of the 2,4-DNPHs of the aliphatic saturated aldehydes and ketones, which were also present, were observed to change. Acetaldehyde 2,4-DNPH was the main product of these changes, but formaldehyde and other 2,4-DNPHs were also obtained. The proportion changed was greater when relatively small amounts of the 2,4-DNPHs were applied to the columns, and the effect was much more marked on silica gel than on alumina. Therefore, silica gel adsorption columns were not recommended for fraction of mixtures of the 2,4-DNPHs of saturated aliphatic aldehydes and ketones, while alumina column posed much less rick. It was further observed that partition columns eluted with methanol and light petroleum as solvents gave good separation [16].

The principle of chromatographic adsorption was also applied earlier by Montes (1952) to the analysis of essential oils with the aid of columns of silicic acid and bentonite. He separated binary and tertiary mixtures of 2,4-DNPHs of 23 aldehydes and ketones of essential oils and determined their physical constants [125]. In 1970 Vorontsov *et al.* studied the adsorption of 2,4-

DNPHs of acetone and methyl propyl ketone on graphitized-C black, and also compared the adsorption isotherms of these substances [126].

Mixtures of DNPH derivatives of androsterone, dehydro-*iso*-androsterone and eticholanolone were separated by Dietrich (1957) by column chromatography with aluminium oxide as adsorbent and ethanol/benzene mixture as elutent. It was observed that the amount of water which had to be added to the adsorbent to affect satisfactory separation varied with each of its batch. The concentration of the DNPH derivative in the elute was determined from the absorption at 367.5 nm [127].

Howard and Tatchell (1954) communicated a method for the separation of 2,4-DNPHs, which proved efficient and independent of special adsorbents. It utilized a reversed-phase partition column, which was first used for the separation of long chain aliphatic acids. Binary mixtures of the 2,4-DNPHs of formaldehyde, acetaldehyde, *iso*-butyraldehyde, acetone, 3-methylbutan-2-one and 6-methylheptan-2-one were readily separated by this method [128].

Peyron (1959) devised a method of blocking functional carbonyl groups in vegetable extracts with acylhydrazones, transformed them into coloured products, and detailed procedure for eluting the transformed products on activated alumina column. The eluted yellow and orange fractions were concentrated and radially chromatographed [129].

Gas liquid chromatography

The fractionation of carbonyl compounds and their identification generally involves very complex operations. This refers particularly to the analysis of odoriferous constituents and to the study of ozonolysis fragments of complex organic substances, where carbonyl compounds are present in very limited quantities. The problem is even more complex, where the compounds in the mixtures have different volatilities. In this case separation involves the formation of non volatile derivatives and their resolution. Chromatographic techniques are very useful in the fractionation of 2,4-DNPH mixtures into single components, but the use of GLC for identification purpose is impossible in many cases, unless the carbonyl compounds can be regenerated and subsequently analysed by gas chromatography.

In one of the more promising method described by Rall (1960), the DNPHs of volatile carbonyl compounds were pyrolysed with 1-ketoglutaric acid and the liberated carbonyl compounds were flashed into a gas chromatograph and separated on 30% carbowax 20M or 30% LAC-446 columns [130]. In an other study he suggested that the method could subsequently be made quantitative [131]. Stephens and Teszler (1960) described a modified procedure in which formaldehyde DNPH was added to the mixture of the derivatives in an effort to aid the flashing of the liberated carbonyls into the gas chromatograph, equipped with a thermal conductivity detector [132]. Standard deviations were reported for acetaldehyde and propionaldehyde DNPHs over a range of 30 to 250 μg of derivatives. This procedure was extended to include acetone, *iso*-butyraldehyde, 2-butanone, valeraldehyde and *iso*-valeraldehyde DNPHs and applied for their quantitative determination in the steam distillates of various tobaccos [133]. Further work spelled out various limitations of the quantitative aspects of this procedure. Consequently, rigorous investigation of the various parameters involved in the analysis was initiated by Jone and Manroe (1965), who applied flame ionization detectors [134].

A direct method of GLC analysis for carbonyl compound derivatives would greatly assist in the study of complex mixtures in small amounts. In this connection Harris has examined mixtures of dioximes of volatile aldehydes on celite - di-2-ethylhexalphthalate columns [135], while Lohr and Warren (1962) have examined the oximes of benzaldehyde and salicylaldehyde on celite-silicone column. Columns of SE 30(2%) or chromosorb W were used with a flame ionization detector and nitrogen was used as a carrier gas. The peaks obtained were well shaped and no decomposition products were noted under the reported conditions [136]. Soukup *et al.* [1964] also used direct GLC of the 2,4-DNPH derivatives of a variety of carbonyl compounds using SF-96(100) silicone oil as a liquid phase at appropriate temperatures. The compounds included alkyl aldehydes and ketones upto C_{12}, alkylphenyl ketones, some terpene

aldehydes and ketones and aromatic aldehydes. It proved to be simple and rapid having fewer limitations than any single column or paper chromatographic method [137]. Later on Leonard and Kiefer (1966) described a method for determining formaldehyde and low boiling carbonyls by gas chromatography as their *anti*-2,4-DNPH. They used a column packed with 5% SE-30 silicone on chromosorb-G of a special mesh, and remarked that the derivatives were thermally stable under the conditions used [138]. In 1975 Sasamura *et al.* prepared 2,4-DNPHs of various carbonyl compounds, such as C_{1-12} *n*-alkanals, *iso*-butyral-dehyded, acrolein, crotonaldehyde, acetone, 3-pentanone, 2-octanone and benzoin, and analysed them by gas chromatography. Thus, 100 DNPHs were identified from their retention times. Double peaks of most of the 2,4-DNPHs investigated were attributed to their *syn* and *anti*-isomeric forms [139].

Kevei and Blazovich (1965) pointed out that chemical transformation prior to chromatography was helpful in the identification of components in foods. They discussed transformations such as hydrolysis of esters, separation of carbonyl compounds with 2,4-dinitrophenylhydrazine in the analysis of fruit aroma and smoke solution [140]. Rather recently Jiao *et al.* (1987) used a gas chromatographic method with column packed with chromosorb-103 and chromosorb-101 as the stationary phase for direct determination of *N*-containing low-C organic compounds in waste water. The method is preferred to conventional spectrophotometric or colorimetric methods in propellant manufactured waste water areas [141]. Almost concurrently Tani and Suzuki (1987) discussed the retention behaviour of various 2,4-DNPHs and pointed out that these were affected by variation in polarities of the substituent groups of the carbonyl compounds [142].

High pressure liquid chromatography

High pressure liquid chromatography is becoming a powerful technique for the analysis of 2,4-DNPHs of a number of aliphatic and aromatic carbonyl compounds, especially present as trace components in a variety of products. The HPLC separation of the 2,4-DNPHs of carbonyl compounds offers many advantages over the separation. The reported GC methods capable of GC separating different aldehyde derivatives from ketone derivatives are not capable of separating them when they have the same molecular weight [143].

Honda and Kakehi (1978) used conjugated aldehydes formed in the periodate oxidation of glycosides, and converted them quantitatively into their 2,4-DNPHs in 1,2-dimethoxyethane. The resulting hydrazones were separated by HPLC using specific columns. The best results were obtained when Lichrospher SI-100 was used and the column was developed with a chloroform-*iso*-octane-methanol mixture by gradient elution. The hydrazone separated were detected at 353 nm. All the hydrazones of hydroxy aldehydes gave their absorption around this wave length. The possible use of this procedure may be for the elucidation of the structure of carbohydrates/conjugated aldehydes. However, glyoxal was an exceptional aldehyde which formed *bis*-hydrazone. Its peak was partly superimposed on that of hydrazine reagent and therefore accurate determination of glyoxal was not possible by this technique [144].

The 2,4-DNPHs of a number of aliphatic and aromatic carbonyl compounds have been synthesized and successful separations of C_1-C_5 2,4-DNPHs was obtained on Pellicular Le Column [145]. However, the retention times of the 2,4-DNPHs of carbonyl compounds with six or more carbons tend to merge, because the solubilities in the hydrocarbon mobile phase are nearly the same.

A method for the quantitative conversion of traces of aldehydes and ketones to their DNPH at room temperature has been mentioned by Selim (1977), who prepared 2,4-DNPH of a number of aliphatic and aromatic carbonyl compounds. The compounds were separated on a reversed phase Bondapak column. It was observed that derivatives of identical molecular weight could be easily separated by HPLC but not by GC. In this connection separation of DNPHs of propionaldehyde and acetone was achieved on a reversed phase HPLC using acetonitrile/water (3:2,v/v) as a mobile phase. Similarly derivatives of aldehydes and ketones having upto ten carbons were also separated. Even 2,4-DNPHs of aromatic aldehydes like α-tolualdehyde and salicylaldehyde were easily separated on the same column using acetonitrile/water (13:7, v/v) as the mobile phase. Thus, in case of aldehydes/ketones having even higher molecular weight, higher concentration of acetonitrile was

recommended. Thus, reversed phase LC offers some advantage over adsorption chromatography for the separation of non-polar derivatives [146].

Nanogram quantities of certain groups of compounds such as alcohols, aldehydes, ketones, acids etc cannot be selectively detected by GC ro LC. One approach to overcome the detection problem is to tag the compound by adding a group that renders it detectable. This approach has been used extensively in GC and is gaining importance in HPLC. For this purpose derivatives that absorb strongly in uv light have been prepared for HPLC. Hydroxy steroids have been benzoylated and 3,5-dinitrobenzoates of glycols have been formed [147]. The HPLC response of fatty acids has been improved by the formation of benzyl [148], *p*-nitrobenzyl [149], 2-naphthyl [150] and phenacyl [151,152] esters. The detectability of 17 keto steroids has been enhanced by the formation of their 2,4-DNPHs and as little as 10 ng of a steroid was detected [153,154].

Shimada *et al.* (1980) prepared selective derivatives of keto-steroids for use in HPLC with electrochemical (EC) detection. Thus, 17 keto steroids in human blood were quantitatively derivatized into *p*-nitrophenylhydrazones and efficiently separated by HPLC/EC on a special column [155]. Concurrently, Vigh and Puchony (1980) determined the capacity factors of C_{6-16} *n*-alkanols, C_{6-12} *n*-alkanal-DNPHs and C_{6-11} 2-*n*-alkanone-DNPHs at various temperatures on a special reversed-phase packings with aqueous methanol as eluent. The results revealed that the capacity factors were functions of both temperature and carbon number [156]. Later on Tani *et al.* (1983) examined temperature effects on the difference of the elution order of 2,4-DNPHs and 1-dimethylamino naphthalene-5-sulphonyl hydrazones of aliphatic saturated aldehydes and their isomeric ketones on a reversed-phase column [157].

Spectroscopic methods

Infrared

Jone *et al.* (1956) initiated a spectrophotometric study of forty 2,4-DNPHs to determine if sufficient information was available from the infrared spectra to classify the parent carbonyl compounds [158]. The infrared derivatives as potassium bromide disks revealed that position of the N-H stretching band indicated whether the parent carbonyl was an aldehyde or ketone. The aliphatic or aromatic character could be determined with reasonable certainty by examination of their C-H stretching region. Even olefinic derivatives were found to have characteristic bands which facilitated their identification [159,160].

During the investigation of 2,4-DNPH derivatives of the carbonyls by infrared spectroscopy the location and evaluation of the relative intensities of such bands as the N-H stretching at 3.0 to 3.15 microns (3333 to 3279 cm^{-1}), the phenyl C-H stretching at 325 microns (3076 cm^{-1}) and the aliphatic C-H stretching at 3.45 to 3.55 microns (2899 to 2817 cm^{-1}) regions were also readily apparent [161,162].

Takeuchi and Suzuki (1963) determined IR absorption spectra of the 2,4-DNPHs of aliphatic saturated and unsaturated monoene aldehydes. Total concentration of the 2,4-DNPH was determined by the absorption intensity of 1330 cm^{-1} band, the molar absorptivity of which was constant (1470 ± 20) for the hydrazone of any aldehyde examined. Molecular weight of the aldehyde was determined by the intensity of 2870 cm^{-1} band. Content of unsaturated aldehyde was determined by the intensity of the 980 cm^{-1} band. This method was successfully applied to the determination of the aldehydes formed by the oxidation of methylerucate in air [163]. In an other work by the same authors dimethyl, methyl-ethyl, diethyl, ethyl-propyl, methyl-propyl, dipropyl, ethyl-butyl and *iso*-butyl-methyl ketones were examined by IR spectroscopy after reaction with 2,4-dinitrophenylhydrazine. Two isomers, 2-pentanone and 3-pentanone, were also analysed in mixtures. Total amount of 2,4-DNPH was determined by the intensity of the 1330 cm^{-1} band, and isomers were determined by solving simultaneous equations using the absorbances at 1330, 1130 and 1065 cm^{-1} bands. Amounts of ketones in mixture with ethanol and butanol were determined after conversion into hydrazones followed by separation on alumina. The IR spectra of the eluted fractions were then used for their estimation [164].

Ultraviolet

Gian (1963) carried out separation of various 2,4-DNPHs on alumina using combination of solvents and thus achieved quantitative recoveries of formaldehyde and acetaldehyde derivatives. The eluents were subjected to their uv absorbance for their analysis [165]. Tunmann (1956) used four solvent systems to separate 2,4-DNPHs. The separated streaks were developed and eluted with ethanol. The concentration of each component was calculated from the UV absorption of its respective solution [166]. A systematic spectrophotometric study of a series of aliphatic, olefinic, aromatic and heterocyclic aldehyde and ketone 2,4-DNPHs had also been carried out. It was found that the information afforded by their UV and visible spectra in neutral as well as basic solution presented a means of differentiating the type of parent carbonyl compound. A time study of the deterioration of the colour of the 2,4-DNPH in alcoholic base provided further information as to the structure of the parent carbonyl compound [167]. This approach was utilized by Suzuki and Marnta (1963), who also observed that saturated aliphatic aldehydes indicated maxima at 358 nm, and the ketones at 363 nm in ethanolic solution, but both showed similar absorbance curves. Thus, it was possible to determine total carbonyl compounds in the experiments, but the determination of an aldehyde in the presence of a ketone was impossible. However, when they were made alkaline, the maxima in the UV region disappeared and the new maxima appeared at 440 nm. Moreover, the transmittance of the DNPH of the aldehyde decreased in a rate proportional to the molar concentration, while that of DNPH of the ketone remained stable. Thus, the determination of aldehyde and ketone in mixture was possible by measuring UV transmittance in a neutral solution and the rate of loss of transmittance in the visible region in alkaline solution [168].

Furest and Feustel (1962) chromatographed, 2,4-DNPHs of aldehydes and ketones on specially treated papers and exposed their spots in a UV spectrometer, along with an untreated filter paper which was used as a reference material. The carbonyl compounds were identified and quantitatively determined from their UV spectra by matrix methods. Depending on the carbonyl contents of various compounds, the maximum absorbance error was upto 6% [169]. Suzuki *et al.* (1964) also separated 2,4-DNPHs of aliphatic aldehydes by ascending paper chromatographic method. The UV spectra of the separated spots indicated maximum absorbances of DNPH of saturated aldehnydes, unsaturated monoeneal-dehydes and unsaturated dienealdehydes at 370, 385 and 405 nm, respectively [170].

Colorimetric analysis

Different aspects of colorimetric analysis have been studied. Cavallini (1950) determined absorption maxima and extinction coefficients of 2,4-DNPHs of a series of keto acids such as pyruvic (368, 708), mesoxalic (374, 766), oxalacetic (381, 805), α-ketoglutaric (380, 762), α-ketoadipic (378, 710),β-ketoadipic (374, 1155), oxalsuccinic (383, 786), phenylpyruvic (379, 691) *p*-hydroxyphenyl pyruvic (379, 590), glyoxalic (365, 958) and succinic semialdehyde (368, -) acids respectively. It was noted that all showed change in absorption curves in alkaline solution after six hours, except the DNPH of glyoxalic acid, which indicated change in one hour [171]. Later on Kawano *et al.* (1962) obtained DNPH of α-keto acids (glyoxalic, pyruvic, α-ketobutyric, α-ketovaleric, α-ketocaproic, α-keto*iso*caproic, α-*iso*-valeric, α-keto-β-methylvaleric, α-keto-β-di-methylbutyric acid and α-ketoglutaric), extracted them with benzene and 0.1 N sodium bicarbonate. In this case the photometric measurements were made at the wave length, where the absorption spectra of *cis*- and *trans*- isomers of DNPH intersected (1972).

During the evaluation of the published methods Toren and Heinrich (1955) noticed that the 2,4-DNPH butadiene furfural was much more soluble in *iso*-octane (2,2,4-trimethyl pentane) than is the 2,4-dinitrophenyl hydrazine reagent. This difference in solubility was utilized to develop an extraction procedure, which permitted the direct spectrophotometric measurement of 2,4-DNPH concentration, without the addition of alkali. Thus, when the prepared 2,4-DNPH of this carbonyl compound was added to a two phase system composed of *iso*-octane and an alcohol-water-phosphoric acid mixture, the hydrazone was selectively extracted into the *iso*-octane and its concentration was determined at 340 nm. The excess reagent remained in the aqueous mixture and did not interfere [173].

Vecera and Jurecek (1951) described the colorimetric determination of phenylhydrazine, which is based on Postorosky reaction involving oxidation of phenylhydrazine with selenium dioxide to the diazonium halide and its coupling with α-napthylamine. The resulting red dye had its absorption maxima at 530-5 nm and is practically stable. This procedure was used for the estimation of phenylhydrazine and phenyl-hydrazone in antipyrine, and the results were read by means of calibrating curve made for Ph_2 C=NNH-Ph [174].

Saclicyloyl hydrazide has been proposed as a useful fluorimetric reagent for carbonyl compounds [175]. Philip (1959) used it to form salicyloyl hydrazones of *o*-hydroxybenzaldehyde, *p*-hydroxybenzaldehyde and *p*-dimethylamino benzaldehyde, which possessed specific fluorescence characteristics. Consequently, spectrophotofluorimeter was used to evaluate their fluorescence properties [176].

The modified method of Marshall and Rogers (1945) was employed by Hoffman and Wollert (1968) for the quantitative determination of guanyl hydrazone, which is based on the complex formation of organic-*N*-bases with an acid indicator (bromo thymol blue) in an organic medium. The coloured complex formed was extracted with an organic solvent (benzene) and determined photometrically. In case of blood plasma or aqueous organic extracts, it was essential to treat with chloroform and hydrochloric acid. Consequently, the hydrochloric acid extract was determined quantitatively by the indicator method at pH 7.8. This method was found to be sensitive to detect 0.2 ug guanyl hydrazone in plasma [177].

Titrimetric analysis

It was demonstrated by Fritz *et al.* (1958) that 2,4-diphenylhydrazine (which contains a bsic functional group) could be titrated as a base in glacial acetic acid, and as an acid with tetrabutyl ammonium hydroxide. This suggested that 2,4-DNPHs should titrate as weak acids and thus afford another simple method for the characterization of this important group of derivatives [178]. By the proposed procedure small amounts of the samples were analysed with a considerable accuracy (± 2%) by the use of 0.01 to 0.02 N titrants. It was found that phenyl hydrazone, *p*-nitrophenyl hydrazone and phenyl osazone derivatives as well as 2,5-dinitrophenylhydrazine were titratable by this procedure. In addition, 5,5-dimethyl-1,3-cyclohexanedione (dimedone), a specific reagent for aldehydes, as well as derivatives of dimedone were titratable [179-184].

These investigations were followed by Bolm and Cario (1959), who described a method for the determination of carbonyl compound in complex mixtures. The 2,4-DNPHs of the carbonyls were separated on paper. The bands were cut and placed into small titration vessel containing oxygen-free acetic acid. Sufficient sodium acetate was added, followed by excess 0.003N titanium dichloride and the mixture was stirred for 5-10 minutes. After acidification, the excess was back titrated with ferric chloride with rhodamine as an indicator. In principle this method may be applied to all compounds containing groups reducible by titanium dioxide [185].

Zellner (1942) prepared *o*-carboxyphenyl hydrazones of *iso*-butyraldehyde, butyraldehyde, glyceraldehyde and acetone, and observed that the parent aldehyde could be regenerated by heating the hydrazone with a large excess of benzaldehyde. It was further pointed out that the hydrazone could be titrated to confirm accurate composition [186]. Later on Pifer *et al.* (1953) also demonstrated the usefulness of very weak titrants (0.001 N) in some non-aqueous solvent systems [187].

Polarographic analysis

According to Petrova and Novikora (155) aldehydes can be determined by indirect polarography by conversion to the 2,4-DNPH in ethanol, followed by polarographic determination of unreacted 2,4-dinitrophenylhydrazine, which begins to reduce at 0.05V. The results were said to be accurate within less than 2-3% for aldehyde concentration between 0.6-105 millimoles/litre [188].

The chromatopolarographic method has been applied by Kemula *et al.* (1963) to the analysis of aldehydes/ketones after their quantitative reaction with 2,4-dinitrophenyl-hydrazine. Because of the low solubility of 2,4-DNPH in water, it was necessary to use aqueous solution containing a high percentage (80%) of dimethylformamide for the partition chromatography on

rubber columns. This medium does not disturb the polarographic measurements. It was also observed that the first members of the homologous series of saturated aliphatic aldehydes could also be separated without transformation into various hydrazone, by an absorption column prepared of cellulose acetate [189]. Bobarevic *et al.* (1965) transformed hydrazides into various hydrazones and used polarographic method to estimate them [190]. Neish (1953) deproteinized rat-tissue homogenates, and treated them with 2,4-dinitrophenylhydrazine. The α-keto acid 2,4-DNPHs, thus formed were separated and estimated polarographically [191].

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