

**FACTORS AFFECTING THE 2,4-DINITROPHENYL HYDRAZINE REACTION  
WITH LIPID CARBONYLS**

by

**MORGAN KANDRAC**

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Professor Karen M. Schaich

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## **ABSTRACT OF THE THESIS**

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By MORGAN KANDRAC

Thesis Director:

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The basic reactions of lipid oxidation were first reported more than 50 years ago, yet measurement of lipid oxidation remains a challenge for both industry and academia. Particularly missing are sensitive, accurate methods for quantitating and identifying secondary non-volatile oxidation products such as monomer carbonyls, both saturated and unsaturated, and core aldehydes remaining on triacylglycerols. Methods for quantitating carbonyls by reaction with 2,4-dinitrophenylhydrazine (DNPH) have been plagued with inconsistencies and problems that have limited applications to analyses of lipid carbonyls. This thesis re-evaluated the chemistry underlying reaction of 2,4-dinitrophenylhydrazine with lipid carbonyls to develop a robust method that is chemically accurate and quantitative, yet simple enough for both research and industrial quality control analyses.

DNPH reaction conditions and characteristics were tested using pure saturated, monounsaturated, and di-unsaturated aldehydes that are typical lipid oxidation. DNPH dissolved in N,N-dimethylformamide (DMF) and acidified with varying concentrations and types of acids to form the base reagent was reacted for a range of times with aldehydes diluted in acetonitrile, and reagent mixtures were applied to HPLC columns for

separation of product hydrazones, unreacted DNPH, and unreacted aldehyde (if any); this procedure also detected side products. Peaks were detected by diode array detection and quantitated by comparison peak areas to standard curves generated from each aldehyde.

Conditions under which saturated and unsaturated aldehydes reacted to completion within 20 minutes with minimal generation of hydrazone isomers and no carbonyl condensation products were identified as pH 2.52 with a molar ratio of 2.5:1 2,4-DNPH:Carbonyl. Reactions were incomplete at higher pH and less efficient at lower pH. Reaction slopes for the various aldehydes varied by <10% in contrast to previous observations of large differences with aldehyde structure. Reaction variability was <2%, and lower limits of detection and quantification were <50 µg/L. Formic acid was comparable to HCl as acidifying reagent. 3,5-Diaminobenzoic acid (DABA) was unable to provide an alternate proton source without lowering pH. No HPLC column tested - Ultra C18 with 2.1 mm internal diameter, pentafluorophenyl, and aqueous C18 - was able to completely resolve all critical pairs of hydrazones.

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## 1. INTRODUCTION

For decades, the 2,4-dinitrophenyl hydrazine (DNPH) reaction has been used qualitatively to identify the presence and structures of aldehydes and ketones in reagents. The reaction has been applied to detect and quantitate carbonyls in proteins [1, 2], in polluted atmospheres [3, 4], and in oxidizing lipids [5-7]. Each of these quantitative assays, however, has encountered problems with direct optical detection because product hydrazones have absorbance maxima too close to that of DNPH, and the technique suffered from poor reproducibility.

To overcome identical absorption maxima in DNPH and its hydrazones, oxidized proteins reacted with DNPH have been separated by polyacrylamide gel electrophoresis then identified by reaction with antibodies raised against proteins containing hydrazone adducts [1, 2, 8]. For atmospheric carbonyls, DNPH is loaded onto solid phase cartridges, air is drawn through, and hydrazones produced by reactive carbonyls are then eluted from the cartridges (U.S. EPA, 1999). Solution reactions of oxidizing lipids with DNPH were largely abandoned until proposals for separating hydrazones by high pressure liquid chromatography (HPLC) were developed [9]. However, routine and accurate quantitative analysis of lipid carbonyls has still not been achieved, probably because the method has been used blindly for the most part, without adequate elucidation of the mechanisms involved and testing of analytical conditions. Consequently, difficulties with reproducibility and accuracy of carbonyl detection are widely recognized.

Issues with identical absorbance maxima, acidity, and instability of hydrazones were encountered when this laboratory first began assessing the DNPH assay for tracking secondary products of lipid oxidation. To overcome these limitations, a solid phase

cartridge method was tested and found inappropriate for reacting liquid phase (rather than gaseous) carbonyls [10]. DNPH was not stable on the cartridges, separation of unreacted DNPH from hydrazones of multiple carbonyls with a wide variation in chain length was unpredictable and poorly reproducible, and the method was time-consuming and expensive since cartridges were not reusable. However, the HPLC separations were effective, so the approach shifted to direct reaction of DNPH with oxidized lipids followed by separation of product hydrazones by HPLC.

An HPLC method was originally developed by Xie using aldehyde standards and applied to tracking oxidation of methyl linoleate [11]. Yao [12] then extended the method to oxidizing oils and extracts, separating monomer carbonyls from core carbonyls with an HPLC gradient. In the process, Yao observed that reaction response curves (rate and concentration dependence) varied markedly with carbonyl chain length and unsaturation, that prolonged use of sulfuric acid led to degradation of the HPLC column, that carbonyl condensation products were detectable under standard conditions, and that a large excess of DNPH appeared to be required to ensure complete reaction with carbonyls. It was clear that fundamental aspects of the DNPH reaction required detailed investigation if the assay was to provide accurate quantitation of carbonyl products in oxidizing lipids.

Izzo [13] further tested the DNPH assay conditions to identify factors interfering with quantitation and reaction conditions that would allow full reaction of all carbonyls without degradation of early-forming hydrazones or formation of carbonyl condensation products. He demonstrated that standard conditions added excess acid, thereby protonating DNPH to an unreactive form. He suggested that a reaction at pH 3 would facilitate reaction of carbonyls with DNPH while preventing carbonyl condensation. He

also hypothesized slow formation of the intermediate carbinolamine as the rate-limiting step in reaction of long chain and unsaturated carbonyls. However, questions about optimum reaction time and effects of amount and type of acid remained.

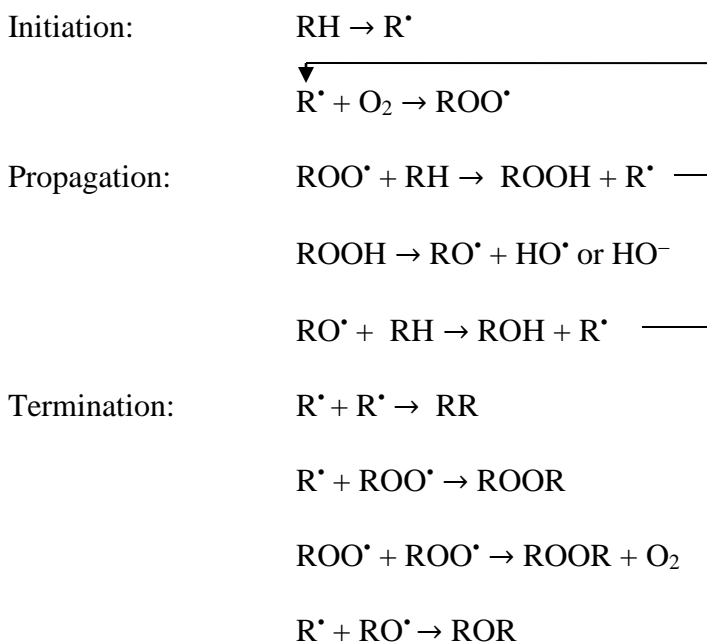
Continuing these investigations, this project assessed factors affecting full reaction and quantitation in the DNPH reaction with lipid monomer carbonyls. Focus points were effects of acid concentration and type on hydrazone formation and reaction time, solvent effects, potential reaction catalysts, and approaches for improving reproducibility. Modifications of HPLC separation of hydrazones were tested to improve the resolution and quantitation of products. This project also addressed the differences in reactivity between saturated and unsaturated aldehydes.

Even though the DNPH reaction has been used for analysis of carbonyls for decades, important details of the chemistry still need systematic investigation. For application to studying lipid oxidation mechanisms, the assay must be accurate, reproducible, quantitative, dependable, and sensitive. This research focuses on developing the DNPH reaction into a rugged assay for identifying and quantitating non-volatile carbonyl oxidation products at micromole levels through optimization of reaction, detection, and quantitation.

## 2. LITERATURE REVIEW

### 2.1 Lipid Oxidation

Lipids oxidize by a free radical chain reaction, in which the driving force is peroxy radical abstraction of hydrogen atoms from adjacent molecules, producing hydroperoxides that decompose to form new radicals that continue the chain reaction. An overly simplified, traditional version of this is shown below [14, 15].



However, questions about the completeness of this reaction scheme have been raised, based on identification of multiple alternate reactions for peroxy and alkoxy radicals that compete with hydrogen abstraction even early in lipid oxidation [16-18]. Integration of these alternate pathways with the traditional free radical chain (denoted in red) is shown in Figure 1.

# INTEGRATED SCHEME FOR LIPID OXIDATION

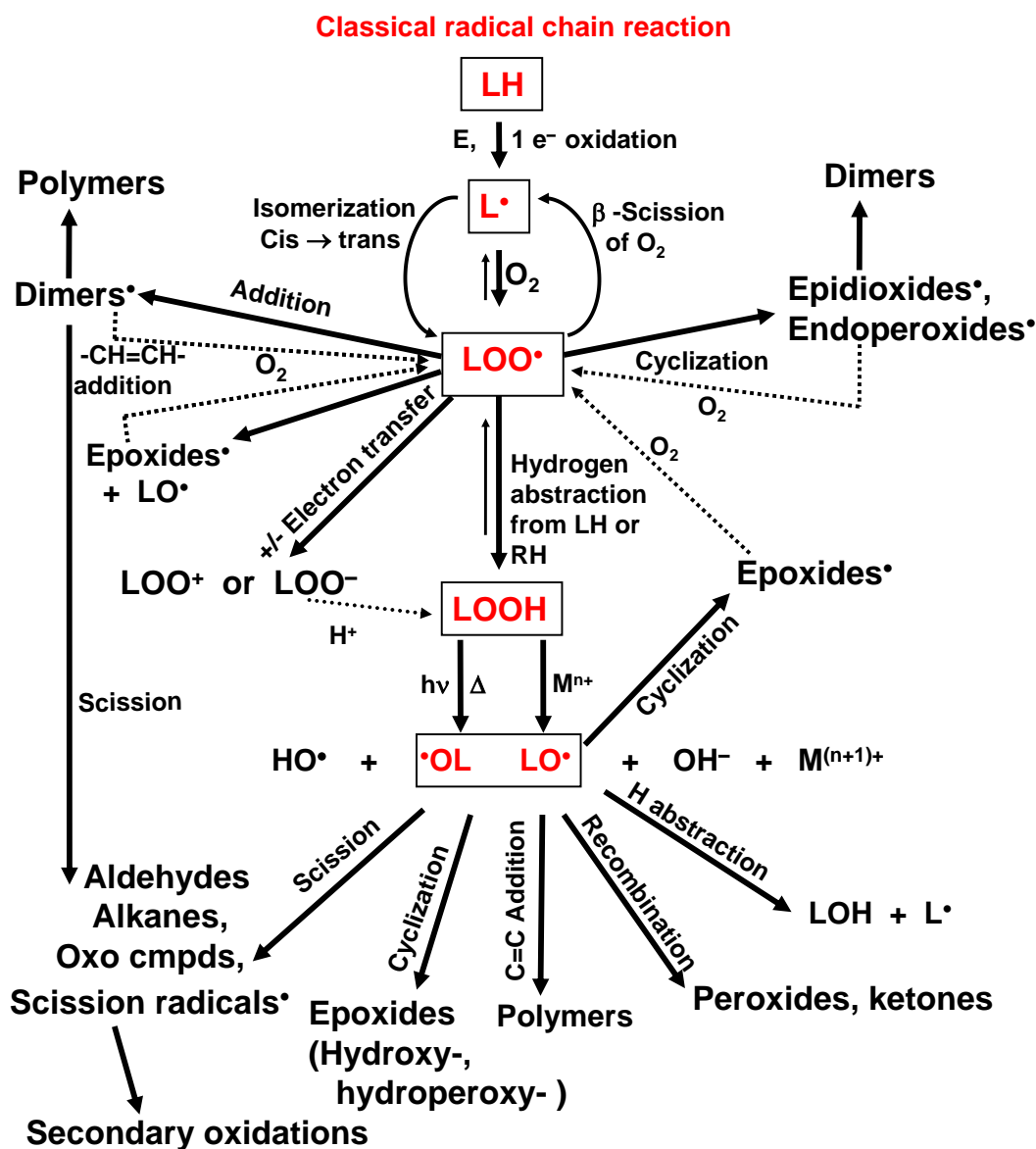


Figure 1. Proposed scheme integrating alternate reactions with traditional hydrogen abstraction in lipid oxidation [18]. Used with permission.



An important consequence of these alternate reactions is that lipid oxidation cannot be described adequately by measuring only conjugated dienes formed with  $L^\bullet$  in polyunsaturated fatty acids, peroxide value, and secondary products such as volatile hexanal [16, 17]. Monitoring both non-volatile and volatile products in parallel in oxidizing methyl linoleate, Xie [11] and Bogusz [19] identified epoxides as a major product formed parallel to and at higher levels than hydroperoxides, pentane as by far the most prevalent volatile product, and heptanal and octenal as significant aldehydes. Integration of products strongly supported  $LOO^\bullet$  addition to double bonds as the dominant oxidation pathway in pure neat oils, yielding epoxides and  $LO^\bullet$ , the latter of which can then internally rearrange to additional epoxides or undergo scission to aldehydes. Integration of multiple products also indicated that products from C-9 and C-13 were not equivalent as held by traditional theory [14, 15]. The mechanism for formation of C-9 and C-13 hydroperoxides in linoleate autoxidation is shown in Figure 2. Whether formation of hydroperoxides is not equal as previously claimed or is equal at both positions but subsequent reactions vary (scissions dominate on the terminal end of the fatty acid while internal rearrangements are more facile near the acid group) is currently being investigated. All of these observations show the importance of measuring products from multiple pathways when evaluating the extent and especially mechanisms of lipid oxidation.

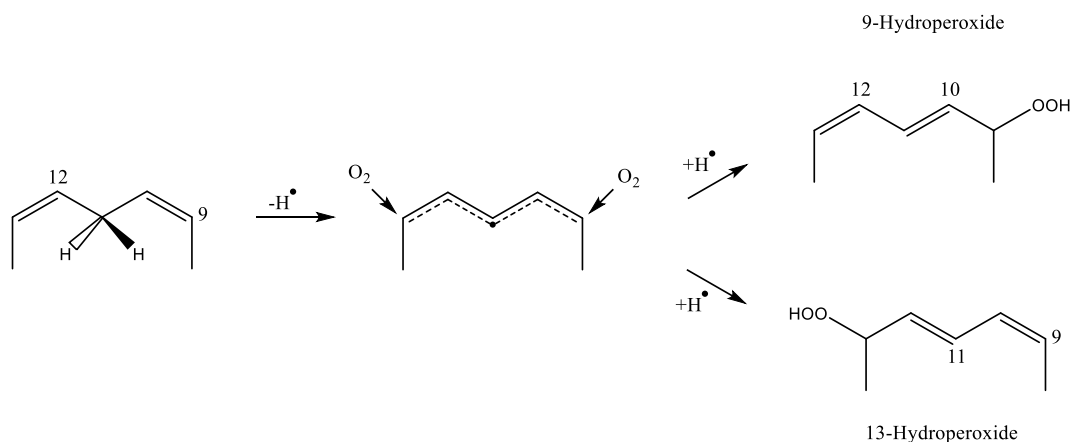


Figure 2. Traditional theory holds that hydroperoxide formation occurs preferentially at the external double bond positions, e.g. C9 and C13 in methyl linoleate [20] .

Tracking alternate reactions in elucidating lipid oxidation mechanisms requires assays of multiple products that are both sensitive and accurate so that a mass balance between pathways can be calculated. Assays detecting micromolar levels of products as classes are available for hydroperoxides in the xylenol orange assay [21] and for epoxides by complexation with diethyldithiocarbamate [22], but comparable assays for carbonyls and lipid alcohols are still not readily available. Elucidation of mechanisms also requires information about formation of specific products. Individual hydroperoxides [23] and epoxides [22] can be identified by HPLC and volatile scission products can be identified by gas chromatography [19]. Yao [12] began the process of gleaning structural information about triacylglycerol core aldehydes and soluble carbonyls from the DNPH assay. The task of this thesis is to make this assay quantitatively accurate as well.

The production of lipid carbonyls from different pathways illustrates why quantitative reaction with identification is so important. Identifying and quantifying

individual lipid carbonyls can elucidate the mechanism by which oxidation or degradation has occurred. In free radical autoxidation of linoleate, C-9 and C-13 hydroperoxides form, and scission of those hydroperoxides generates characteristic aldehydes: 3-nonenal, 2,4-decadienal, pentanal, and hexanal [18]. The typical scission products formed from the 9 hydroperoxide linoleate are shown in Figure 3. Quantitation of these products in equivalent amounts would suggest the equal formation of 9 and 13 hydroperoxides, while larger amounts of 3-nonenal or *t,t*-2,4-decadienal could indicate greater formation of the 9 hydroperoxide. Perhaps more importantly, identification of heptenal and octenal was a key factor revealing the active addition of lipid peroxy radicals to linoleate double bonds to generate high levels of epoxides in parallel with hydroperoxides [11].

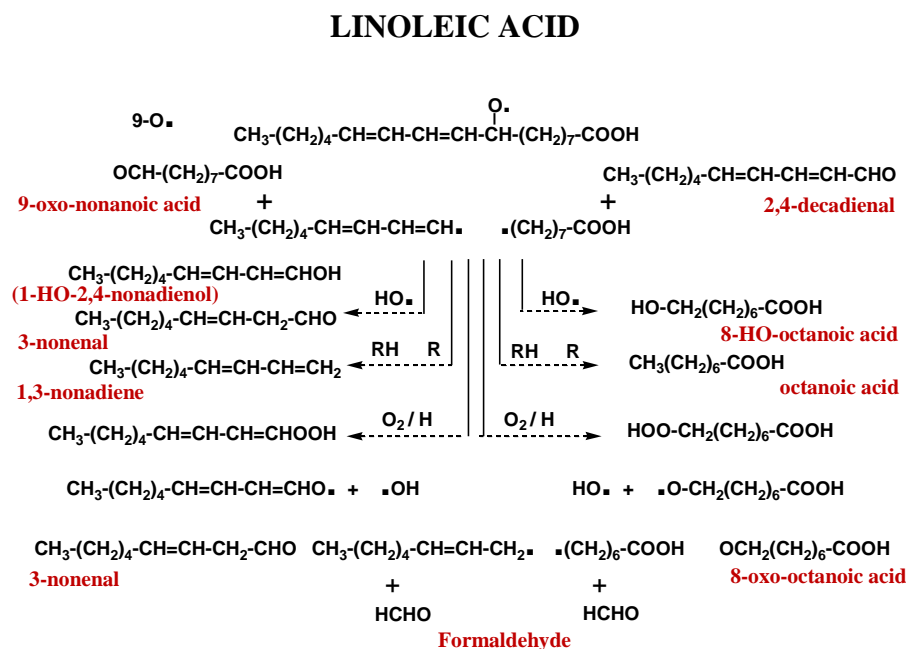


Figure 3. Typical scission products of oxidizing linoleic acid [18]. Used with permission.

Identifying and quantifying carbonyl products can also distinguish whether a lipid

has undergone sensitized photooxidation or free radical autoxidation. Sensitized photooxidation of linoleate generates a mixture of 9, 10, 12, and 13 hydroperoxides [24-26], so scission products include aldehydes characteristic of all four hydroperoxides, doubling the number of aldehydes produced from free-radical autoxidation, as shown in Table 1. Identifying the different aldehydes generated under different conditions contributes significantly to distinguishing pathways, while quantifying aldehydes is required to confirm the primary mechanism of oxidation, predominant hydroperoxide and scission pathways, as well as to calculate overall mass balance of pathways in the oxidation of a lipid.

Table 1. Characteristic aldehydes produced during free radical oxidation and sensitized photooxidation of linoleate. Modified from [18].

<b>Oxidation Mechanism</b>	<b>Free Radical Autoxidation</b>		<b>Sensitized Photooxidation</b>	
Linoleate Hydroperoxide	Beta Scission	Alpha Scission	Beta Scission	Alpha Scission
9-OOH	3-nonenal	2,4-decadienal	3-nonenal	2,4-decadienal
13-OOH	Pentanal Butanal	Hexanal	Pentanal Butanal	Hexanal
10-OOH	n/a	n/a	2-octenal	3-nonenal
12-OOH	n/a	n/a	2-heptenal	heptanal 2-hexenal

If we further extend analysis of carbonyls to the oxidation of vegetable oils, we must consider the fact that vegetable oils contain a mixture of saturated and unsaturated

fatty acids, the unsaturated of which are a mixture of oleic, linoleic, and linolenic acid, depending on the oil [27]. This mixture of unsaturated fatty acids increases the range of carbonyls that can be produced during oxidation. Without accurate fatty acid determination followed by accurate quantitation of oxidation products, one cannot discern the primary oxidation pathway or fatty acid contributing to oxidation.

## **2.2 Identification and Quantitation of Soluble Carbonyls**

### **2.2.1 *p*-Anisidine Value**

Unsaturated aldehydes can be determined by reaction with *p*-anisidine (Figure 4) to form a condensation product that absorbs at 350 nm [16]. The *p*-anisidine value is defined as “100 times the optical density measured at 350 nm in a 1 cm cuvette of a solution containing 1.00 g of the oil in 100 mL of a mixture of solvent and reagent” [28]. The *p*-anisidine value is commonly combined with the peroxide value of an oxidized oil or fat to give the TOTOX (total oxidation) value. The TOTOX value is considered a measure of both early and secondary oxidation as it combines values for early stage hydroperoxides and secondary stage aldehydes.

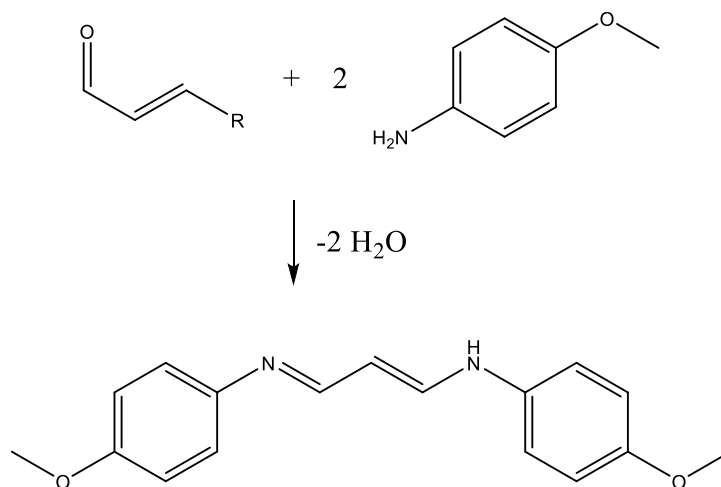


Figure 4. Reaction scheme of an unsaturated aldehyde with *p*-anisidine [16].

Although a standardized procedure exists and the procedure is widely used in the food industry, there are disadvantages to the *p*-anisidine assay. *p*-Anisidine is highly toxic [29] and its reaction with aldehydes is neither quantitative nor specific. Although both saturated and unsaturated aldehydes react with *p*-anisidine, the reagent has a marked preference for 2,3-unsaturated aldehydes so underestimates total carbonyls [30]. Anisidine also reacts slowly with hydroperoxides, so it is not a specific indicator of secondary oxidation. Additionally, the method cannot be used with highly colored oils, particularly those containing carotenoids that also absorb in the 350-nm wavelength range [16]. Thus, while this assay has been used routinely in monitoring thermal degradation of oils, it really is not accurate and cannot be used for studies of mechanism.

### 2.2.2 Thiobarbituric Acid (TBA) Value

When heated in an acidic solution, 2-thiobarbituric acid (TBA) reacts with malonaldehyde to form a Schiff base that absorbs at about 530 nm (Figure 5).

Malonaldehyde is a secondary oxidation product produced by fatty acids containing three or more double bonds, such as alpha-linolenic acid or arachidonic acid [16]. TBA value is defined as “the increase of absorbance measured at 530 nm due to the reaction of the equivalent of 1 mg of test sample per 1 mL volume with 2-thiobarbituric acid” [31].

The TBA reaction has been used extensively in analyses of lipid oxidation in foods, particularly muscle foods where arachidonic acid is a major fatty acid component. In general, TBA results have correlated well with sensory analyses [16]. However, TBA has been found to react with many compounds other than malonaldehyde, including saturated and unsaturated aldehydes, acids, esters, imides and amides, amino acids, and oxidized proteins. Therefore, the TBA reaction is unsuitable for complex food materials and biological systems containing nonlipid materials that can contribute to the color reaction [32].

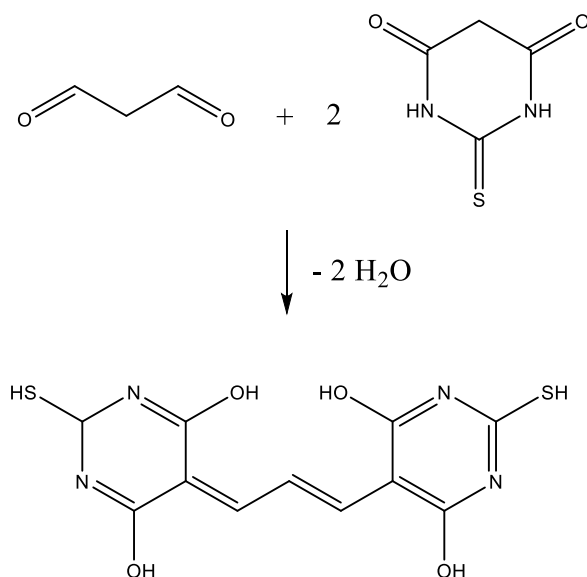


Figure 5. Reaction scheme of malonaldehyde with 2-thiobarbituric acid [33].

The non-specificity of TBA's reaction is only one of many problems with this assay. Variations in sample preparations also affect the accuracy and reproducibility of the method [34]. Lack of molecular specificity, low detection sensitivity, high sensitivity of results to reaction conditions, and the many different colored products observed in the TBA reaction also attest to its chemical complexity and inability to provide detailed information about secondary oxidation products [32]. While this assay can provide a general indicator of lipid oxidation in systems with highly unsaturated fatty acids, the oxidation products detected are insufficient for use in studies of lipid oxidation mechanism.

## **2.3 Analysis of Soluble Carbonyls by Reaction with 2,4-Dinitrophenyl Hydrazine**

### **2.3.1 History of 2,4-DNPH Reaction**

Purgotti [35] and Curtius & Dedichen [36] are credited with the initial investigation of 2,4-dinitrophenylhydrazine (DNPH) as a reagent to identify aldehydes and ketones. However, the reagent was made popular in 1925 by Brady and Elsmie's [37] review, after which DNPH became known as 'Brady's reagent'. DNPH was considered an advantageous reagent for identifying the presence of aldehydes and ketones because the reagent was reliable, rapid, easily prepared, and the hydrazone products are stable, easily crystallized, and exhibit sharp melting points [38].

Initially, DNPH complexation was used widely as a reagent for melting-point identification of aldehydes and ketones [38]. The resulting hydrazones were crystalline and readily purified by recrystallization. Soon, however, there were conflicting results reported from many sources regarding melting points of pure standard compounds. The



differences were too large and varied to be explained by insufficient purification. It was speculated that the differences were due to the acid chosen as catalyst in preparation of the derivatives. Allen [39] noted that sulfuric acid was more difficult than hydrochloric acid to remove from the crystals, persisting through several recrystallizations. Another explanation suggested geometrical isomerism of the hydrazones catalyzed by the presence of acid [39].

The variability of hydrazone melting points was resolved in the 1980s when NMR and X-ray crystallography studies of derivatives were performed, confirming that acid was indeed still trapped within the crystals, causing syn-anti isomerization of the hydrazones [40]. Acid-catalyzed isomerization led to discrepancies in melting points since each stereoisomer has a characteristic melting point. To eliminate this problem, Behforouz [41] suggested a new method for purifying hydrazones by washing crystals with sodium bicarbonate.

Spectroscopic studies of hydrazone derivatives began in the 1940s and 1950s. Initially, the main goal was to identify a relationship between parent carbonyl compound and characteristic color by UV/Vis or IR absorbance [42-44]. However, the issue remained that individual hydrazones could not be identified in mixtures. Spectroscopy could only be applied for determining total carbonyl content until adequate separation methods were developed.

Through the 1960s and 1970s, strong acid was still regularly used in the preparation of hydrazones, and when applications of the reaction switched from synthetic to quantitative, no attention was given to the role of either the acid or the pH in quantitation. This period saw initial investigation of stereoisomeric forms of hydrazones

as chromatography advanced and thin layer chromatography (TLC) became increasingly popular. Edwards [45] noted multiple occurrences of stereoisomers reported in the literature, and demonstrated their formation by synthesizing hydrazones from aliphatic aldehydes C5-C9 and separating the isomers by TLC. During this period, many researchers began to use the DNPH reaction coupled with TLC to separate or isolate individual hydrazones and identify carbonyls from a variety of foods and food products [46, 47].

With continued development of chromatographic techniques such as gas, liquid, and column chromatography, it became possible to easily separate hydrazones from one another and from unreacted DNPH [48-52]. Gas and liquid chromatography separation techniques allowed for the separation of carbonyls from a variety of sample sources, including olive oil [5], alcoholic spirits [53], and beer [54]. Rather than just reporting total carbonyl content, researchers began to accurately identify individual aldehydes and ketones present in samples and to quantify them. Despite this attention, however, an assay that can accurately identify and quantify a comprehensive panel of saturated, unsaturated, and core carbonyls produced by lipid oxidation has not yet been developed.

### **2.3.2 2,4-DNPH Reaction Mechanism**

The reaction of DNPH with an aldehyde or ketone is an acid-catalyzed addition-elimination reaction (Figure 6). The key requirement is initial activation of the carbonyl bond by the addition of a proton from acid. This forms a carbocation that polarizes the carbonyl bond and attracts the nucleophilic amine group of DNPH more strongly. Activation of the carbonyl is especially important for carbonyls that are unsaturated

and/or long in carbon chain length because increased carbon length imposes steric hinderance around the carbonyl, slowing nucleophilic attack. Unsaturation from neighboring double bonds decreases electrophilicity of the carbonyl carbon through contributions of pi electrons, overall decreasing reactivity. In these instances, acid activation overcomes the steric hinderance and electronic contributions that slow reaction and decrease the reactivity of long carbon chain length and unsaturated carbonyls. The amine then adds to the activated carbonyl by nucleophilic addition, forming a tetrahedral carbinolamine intermediate. This is followed by a 1,2 elimination of water and the formation of the 2,4-dinitrophenylhydrazone [55]. It is important to note that this reaction is reversible at all stages, a behavior that can complicate quantitation.

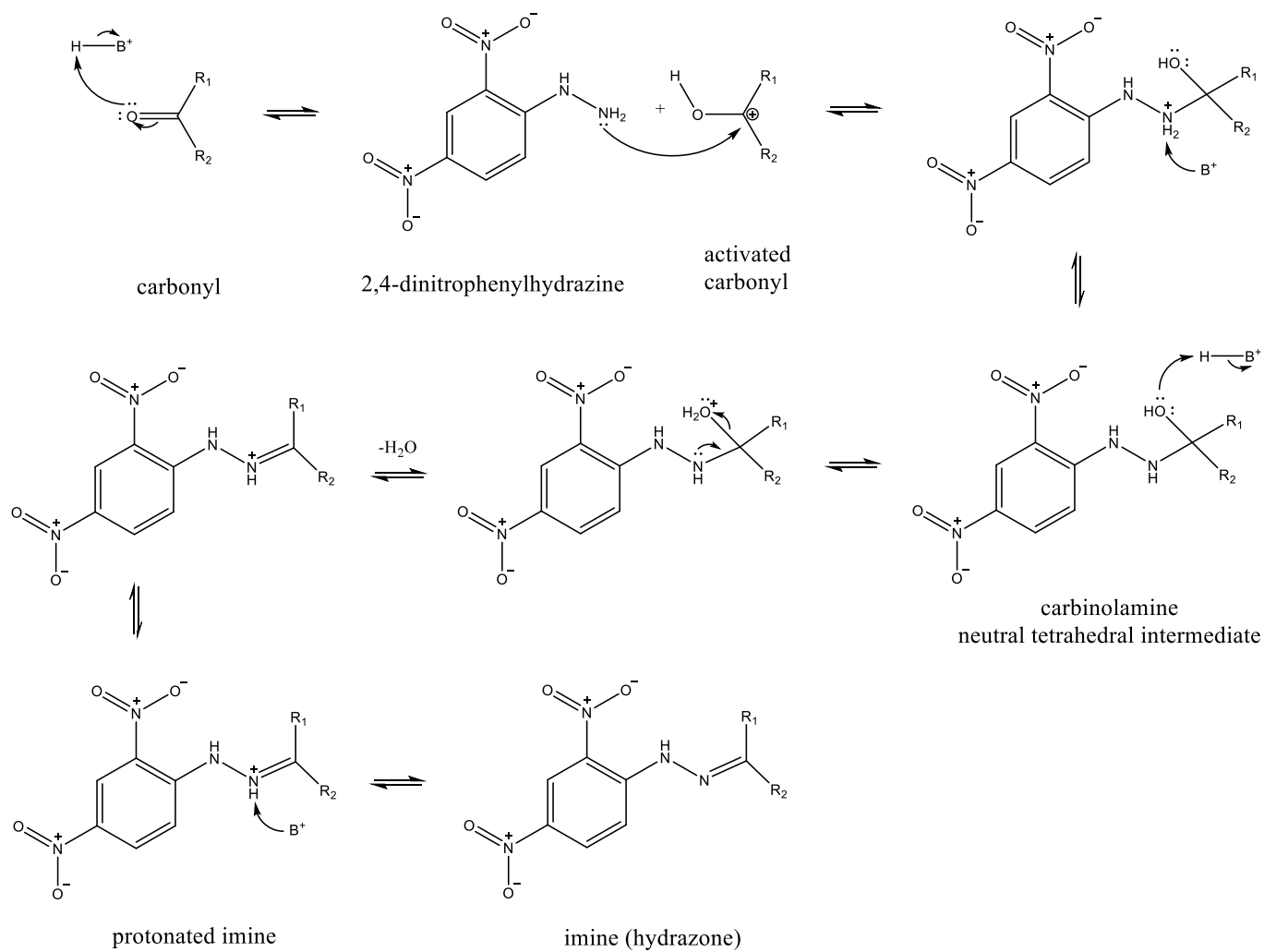


Figure 6. Scheme for derivatization of carbonyls with DNPH [55].

While acid is a necessary requirement for reaction, it also induces multiple complications. The first complication is the protonation of DNPH (Figure 7). In an aqueous environment, DNPH exhibits a pKa of 1.5 [56]. From the Henderson-Hasselbalch equation, if the pH of a solution is 1.5, half of the dissolved DNPH exists protonated, while half is not [57]. Protonated DNPH is not able to react with carbonyls. Comparable to browning reactions, a protonated amine cannot react with a carbonyl, as it is no longer nucleophilic [58]. Thus, the acidity conditions must be monitored closely to ensure that enough unprotonated DNPH is available in solution to react with carbonyls.

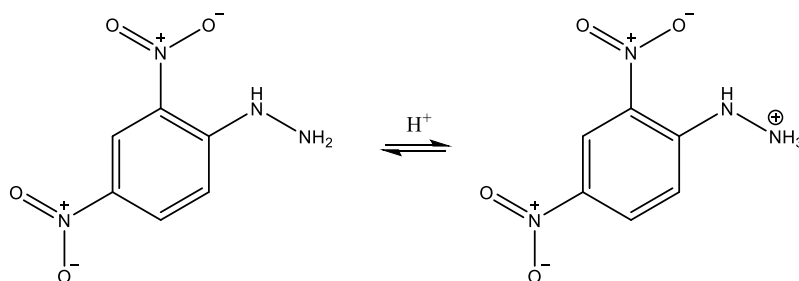


Figure 7. Scheme of protonation of 2,4-DNPH [56].

DNPH concentrations are especially important considering the very limited solubility of DNPH in water (0.2 mM) relative to carbonyls [56]. Solubility increases tenfold in isopropanol (2 mM) [59], and another tenfold in acetonitrile (15 mM [60] – 30 mM [61]). The greatest solubility of DNPH is in N,N-dimethylformamide (0.5 M) and dimethyl sulfoxide (1 M) [62]. In samples where the carbonyl concentrations are unknown, ensuring excess DNPH is critical for accurate quantitation. Thus, with the potential for protonated, unreactive DNPH, the acid level needs to be minimized so that

the majority of solubilized DNPH is available for reaction and easily in excess of the carbonyl content.

The second complication with acid is potential for side reactions such as carbonyl condensation. Under strongly acidic conditions, aldehydes can condense with one another via aldol condensation to form dimers [63] (Figure 8). Accurate quantitation is not possible if side reactions such as carbonyl condensations also occur in the system.

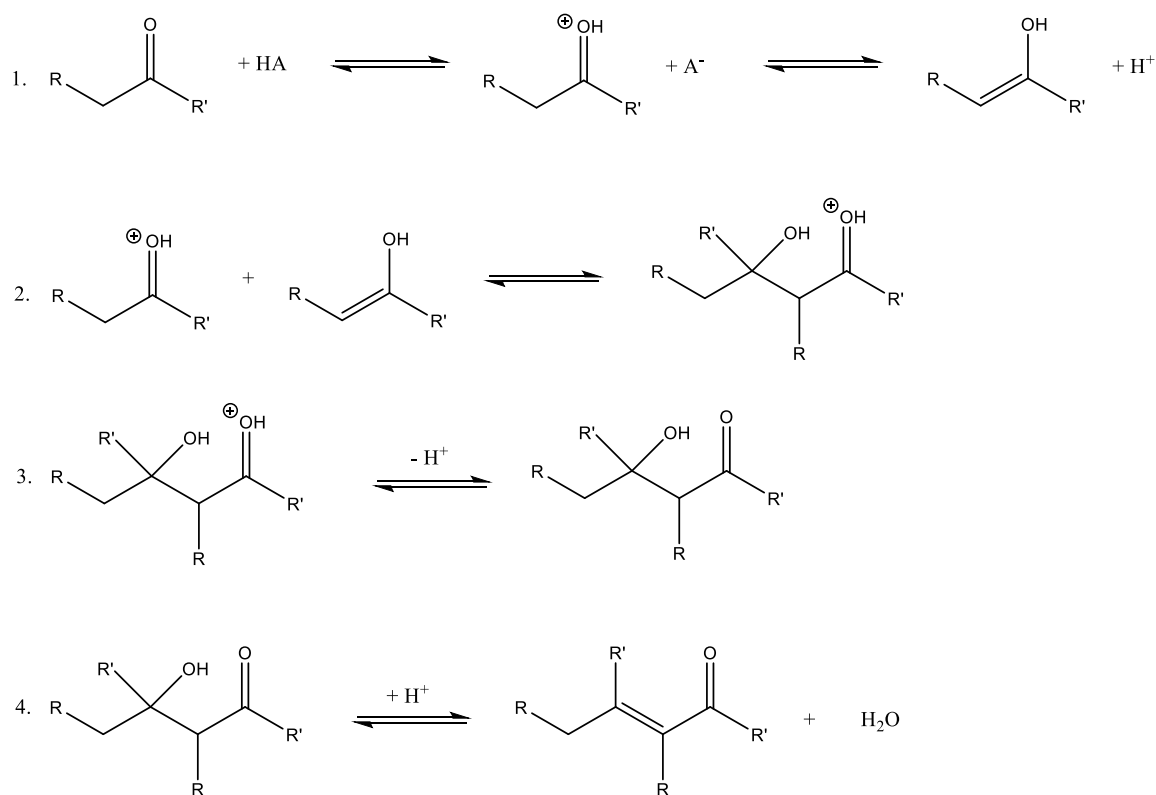


Figure 8. Acid catalyzed mechanism for aldol condensation of carbonyls. 1. Enolization, 2. Nucleophilic Addition, 3. Proton Transfer, 4. Dehydration. [64]

A third complication added by acid is enhancement of hydrazone isomerization [65]. Free rotation about the C-N bond (between aldehyde and DNPH) allows for structural rearrangement of carbinolamine tetrahedral intermediates into either syn or anti stereostructures in the final hydrazone (Figure 9). When the hydrazone is an aldehyde-DNPH adduct, the E-isomer structure designates  $R_1$  as a hydrogen and  $R_2$  as the remaining carbon backbone of the aldehyde. Hydrazones can be easily purified by recrystallization and removal of acid. However, often some acid remains entrained in the crystals and upon redissolution for analysis, acid on the order of 0.1-1% phosphoric acid causes isomerization of 15-30% of E to the Z isomer (final ratio 0.15-0.3 Z/E). Reducing the concentration of acid reduces the extent of isomerization [66].

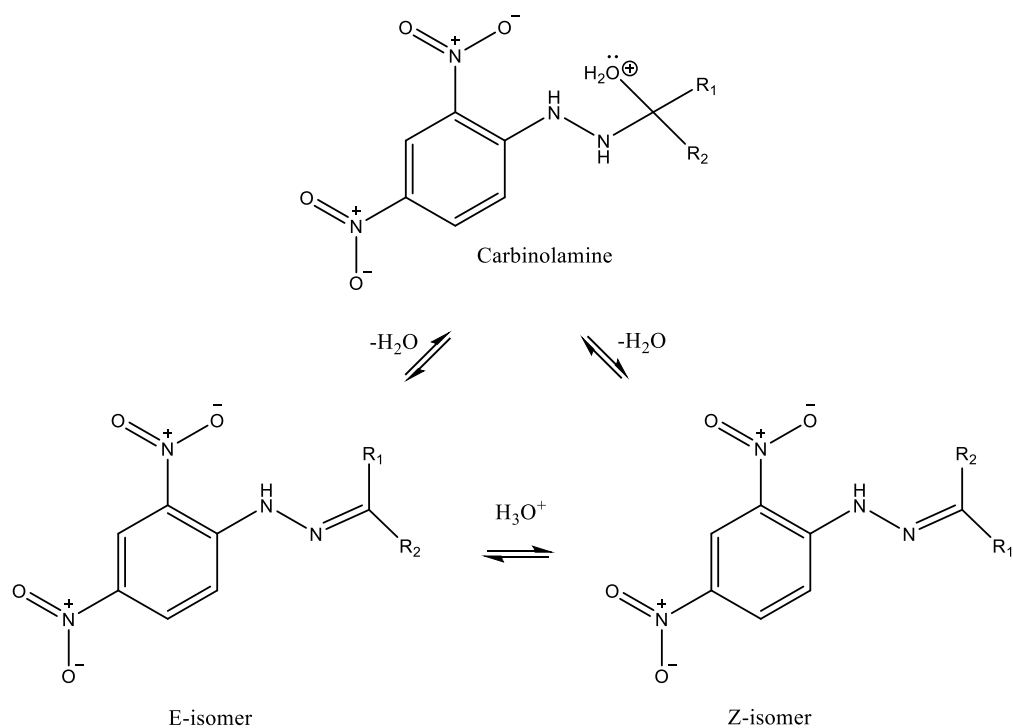


Figure 9. Isomerization of hydrazones [66].

### 2.3.3 Synthesis of 2,4-Dinitrophenyl Hydrazones

Hydrazones are synthesized with an acidified 2,4-DNPH reagent that is some iteration of 2,4-DNPH dissolved in either acid or acidified alcoholic (ethanol or isopropanol) solution, and the reagent is then reacted directly with a carbonyl or added to a solution of the carbonyl [67]. In some cases, the reaction mixture is also heated to ensure complete reaction [38].

The original reagent used by Brady had a characteristically low concentration of 2,4-DNPH and required a large volume of the reagent for reaction. In preparation of the reagent, 4 grams of 2,4-DNPH were suspended in 1.245 liters of 2 N hydrochloric acid, giving a final concentration of 3.21 g 2,4-DNPH/L (16 mM). This entire DNPH solution was then reacted with approximately 1.5 g of aldehyde. After the dinitrophenylhydrazone had formed and precipitated, the product was filtered and recrystallized from ethanol [37]. Low solubility in acid limits the use of this reagent when dealing with a sample of unknown carbonyl content.

To overcome solubility limitations, Allen [35] dissolved DNPH in ethanol instead of acid. A saturated solution of dinitrophenylhydrazine was prepared by refluxing 1 gram of 2,4-DNPH with 100 mL of ethanol, resulting in a concentration of 2,4-DNPH is 10 g/L. For reaction, 5 mL of 2,4-DNPH, 5 mL of ethanol, and a few drops of the carbonyl compound were mixed in a test tube and carefully heated to boiling. Concentrated hydrochloric acid (1-2 drops) were slowly added, the mixture was boiled for an additional two minutes, and water was added dropwise to incipient cloudiness or crystallization and products were then filtered [38]. In this approach, solubilizing DNPH in ethanol rather than 2 N HCl did increase DNPH reagent concentrations, but with the reduction in acid



significant heating was required for facilitate synthesis of hydrazones. Such heating is not suitable for analyzing oxidized triglycerides as it will induce additional lipid oxidation, as well as transformation and polymerization of breakdown products.

The method described in *The Systematic Identification of Organic Compounds* by Shriner [67] combined methods of Brady and Allen for qualitative identification of carbonyl compounds. Here, 3 g of 2,4-DNPH were dissolved in 15 mL of concentrated sulfuric acid, which was added to 20 mL of water and 70 mL of 95% ethanol to give a final concentration of 2,4-DNPH is 28.6 g/L. This 2,4-DNPH reagent must be mixed thoroughly and filtered. For the identification of carbonyls, one or two drops of a test solution (about 50 mg of carbonyl compound in 2 mL of 95% ethanol) are mixed with 3 mL of the 2,4-DNPH reagent, and the formation of a precipitate confirms the presence of carbonyls in the test solution [67].

Use of Shriner's preparation of 2,4-DNPH dissolved in ethanol solution with concentrated sulfuric acid has been reported extensively in the literature. As an alternative, some researchers reduced the acid strength or switched to hydrochloric acid to reduce causticity or increase compatibility with mass spectrometry detection [7, 61]. Here, moderate solubility of 2,4-DNPH in ethanol is the most limiting factor. DNPH has much higher solubility in dimethylformamide (100g/L), which ensures that excess reagent can be easily added to a lipid sample of unknown carbonyl content. Dimethylformamide also offers the additional advantage of dissolving triacylglycerols where alcohols do not. For both reasons, therefore, dimethylformamide was selected as the 2,4-DNPH solvent in this study. HCl was selected as the main test acid.

#### 2.3.4 Solution Chemistry of 2,4-DNPH Reaction with UV-Vis Optical Detection

2,4-DNPH derivatization of carbonyls has been used primarily for qualitative identification of aldehydes and ketones; DNPH does not react with other carbonyl-containing functional groups such as carboxylic acids, amides, and esters [38]. As discussed in the previous section, acidified 2,4-DNPH reagent is typically prepared by dissolving 2,4-DNPH in an acidic ethanol solution, an aldehyde or ketone is added, and the hydrazone product is precipitated out of solution, filtered, and analyzed [37, 38, 67]. The precipitate can be isolated for mass spectrometric determination of structure or the amount present can be determined in solution by optical analysis if it is a pure hydrazone of an individual aldehyde or ketone.

The hydrazine chromophore gives 2,4-DNPH high optical absorbance with a  $\lambda_{\text{max}}$  at 360 nm in aqueous or alcoholic solutions [68]. A low concentration of  $10^{-5}$  M is sufficient for detection by UV/Visible spectroscopy. Unfortunately, the majority of 2,4-DNPH hydrazone derivatives exhibit  $\lambda_{\text{max}}$  values that are too close to the parent compound, so the reagent and products cannot be distinguished from one another in solution and change in absorbance does not accurately track the reaction. To use the 2,4-DNPH reaction as a test tube assay, all 2,4-DNPH must be sufficiently reacted or removed prior to analysis. In a sample of unknown carbonyl content, it is impossible to determine if all 2,4-DNPH has reacted and allowing excess 2,4-DNPH to ensure full reaction exacerbates the problem. Hence, UV/Visible spectroscopy is an unsuitable method for routine quantification of DNPH hydrazones, especially if many samples are involved.

A modification of the solution assay adds strong base to the reaction to convert the hydrazones into quinoidal ions that exhibit an absorbance at 480 nm [68]. The marked difference in UV-Vis lambda max allowed this new approach to be used for quantitation of total carbonyl content. However, the limitation that individual carbonyls could not be identified from a mixture of hydrazone products remained because quinoidal ions of varying hydrazones all exhibited similar lambda max values, around 480 nm. Thus, the test tube assay remains as a method that is only suitable for determining total carbonyl content.

### **2.3.5 HPLC Separation of 2,4-Dinitrophenyl Hydrazones**

One way to overcome limitations of optical detection is to separate hydrazones from unreacted DNPH by HPLC. This approach offers the additional advantage of isolating individual hydrazones and identifying them by comparison to standards and by mass spectrometry. Separation of DNPH hydrazones by HPLC typically utilizes RP-C18 columns with a combination of water, methanol/isopropanol, and/or acetonitrile as the solvent system along with gradient elution to improve speed and resolution of eluting hydrazones [69]. Column temperatures range from 20 to 50 °C [70, 71], with higher temperatures used to reduce retention time of analytes [72]. Peaks are detected by Ultraviolet or Diode Array detectors and quantitated by comparison to calibration curves established for each hydrazone. Peaks are tentatively identified by comparison to retention times of standards [12], with confirmation by mass spectrometry (MS) when available. MS detection is particularly useful for distinguishing aldehyde from ketone hydrazones [4, 12] and identifying isomers. Key limitations in separation are with critical

pairs, combinations of hydrazones from saturated and unsaturated carbonyls, e.g.  $C_{\text{sat}}$  and  $C_{n+2 \text{ unsat}}$  or  $C_{\text{sat}}$  and  $C_{\text{diunsat}}$ . Co-elution of critical pairs such as nonanal and *t,t*-2,4-nonadienal prevents quantitation of either compound and limits identification if a mass spectrometer detector is not available.

Various columns and elution solvents were compared for separation of hydrazones from 15 volatile aldehydes by HPLC and RRLC (rapid resolution liquid chromatography) [69]. HPLC separation on Zorbax Eclipse XBD C18 (150 mm x 2.1 mm x 5  $\mu\text{m}$ ) and Supelcosil C18 (250 mm x 4.6 mm x 5  $\mu\text{m}$ ) columns with an ACN:H<sub>2</sub>O gradient provided satisfactory separation. Minor resolution issues existed for some compounds, such as propanone and acrolein, and isomeric tolualdehydes. RRLC separation on a Zorbax Eclipse Plus C18 (50 mm x 2.1 mm x 1.8  $\mu\text{m}$ ) column with mobile phase of IPA:MeOH:THF:H<sub>2</sub>O (30% methanol, 52.5% water, 10.5% IPA, 10% THF with a linear gradient to 80% methanol, 15% water, 3% IPA, 2% THF in 5 minute) provided the best separation of all 15 hydrazones. Propanone, acrolein, and propionaldehyde hydrazones were resolved, but not isomeric tolualdehydes. Final optimization resulted in 6 minutes of analysis time and consumption of 2 mL solvent per run. This research demonstrates the necessity and importance of optimizing separation conditions depending on the panel of hydrazones analyzed.

Conditions affecting precise quantitation of hydrazones has been well investigated [55, 66, 73, 74]. Uchiyama identified the analytical inaccuracy of quantitation of hydrazones due to formation of stereoisomers that occurs under acidic environments and in the presence of UV irradiation. The isomers readily separate from one another under normal HPLC separation conditions and experience slightly different lambda max values

[66, 73]. A proposed solution for dealing with the stereoisomers was reductive amination of hydrazones using 2-picoline borane prior to HPLC analysis [74]. The reduced hydrazones exhibited increased stability (2 weeks at room temperature) compared to traditional hydrazones (24 hours at room temperature). The molar absorption coefficients still varied with chain length, but there was marked improvement in precision of quantitating peak area of reduced hydrazones due to the absence of isomers.

### **2.3.6 Food Science Application – Lipid Oxidation**

The 2,4-DNPH assay has seen limited use in food science applications, primarily to identify and quantify on a relative basis the presence of carbonyls produced during lipid oxidation as a replacement for or adjunct to gas chromatographic detection of volatile carbonyl compounds. Standardized assays have not been developed due to multiple problems leading to irreproducibility and inaccuracy in results. However, now there is a compelling need to identify full carbonyl profiles for oxidized lipids or foods, including non-volatile carbonyl products, to better understand oxidation mechanisms and track reaction pathways so that the shelf life and stability of lipids and lipid-containing foods can be improved.

HPLC is the best method for separating hydrazones prior to quantitative and qualitative analysis. Separation and analysis of single classes of aldehydes such as only saturated aldehydes or aromatic aldehydes is generally straightforward. However, mixtures of aldehydes from food lipids are never this simple. Edible oxidized edible oils generate a range of aldehydes from four to about twelve carbons with varying degrees of (un)saturation. Lipids extracted from grains, produce, and meats are even more complex,

again producing a combination of aldehydes that vary in volatility, solubility, saturation, and chain length. The robustness of HPLC separation must be increased to resolve these complex mixtures of carbonyls in these systems.

Mathew et al. [75] identified 36 carbonyl compounds found in sea buckthorn berry and oil samples by enzymatic hydrolysis followed by DNPH-LC-UV and ESI-MS/MS. Several classes of carbonyl compounds were separated; of these nine carbonyl compounds were quantifiable using calibration curves. Predominant aldehydes in berry samples included formaldehyde, acetaldehyde, and acetone. In pulp oil, longer aldehydes and carboxy aldehydes dominated, demonstrating benefits of enzymatic hydrolysis when analyzing oxidation products originating from TAGs.

Work by Dannenberger et al. [76] is interesting as they analyzed how diet affects the concentration of long chain aldehydes, C10-C18, and 12-methyltridecanal released from plasmalogens in the phospholipids of *longissimus* muscle of bulls. Muscle lipids were extracted and separated into classes by TLC, derivatized by 2,4-DNPH, then the hydrazones were purified by TLC and analyzed by HPLC. Pasture feeding was found to significantly increase the 12-methyltridecanal and octadecanal concentrations and decrease the octadec-9-enal concentration in the muscle phospholipids.

Cao et al. [7] analyzed nonpolar lipophilic aldehydes and ketones in six oxidized edible oils (sunflower, peanut, camellia, rapeseed, perilla, soybean) by DNPH-HPLC-ESI-QqQ-MS. Close relationships among the amounts of aldehyde carbonyls and the initial contents of oleic, linoleic and  $\alpha$ -linolenic acids were revealed by principal component analysis. The results provided some information about secondary oxidation products in edible oils as well as possible sources of parent fatty acids. Major limitations

in quantitation of carbonyls were that a hexanal-DNPH calibration curve was used to quantify all carbonyls and a similar molar extinction coefficient was assumed for all DNPH derivatives, which is not the case. Concentrations of carbonyls were expressed as peak area per gram of oil, which is only a relative basis of quantitation.

Damanik and Murkovic [77, 78] used LC-MS/MS to analyze the kinetic formation of aliphatic aldehydes produced in Rancimat-oxidized triolein and palm oil in the presence and absence of antioxidants, beta carotene and vitamin E. Traditional reaction conditions were applied and again a hexanal-DNPH calibration curve was used to quantitate individual carbonyl concentrations as well as total carbonyl content.

Bastos et al. [79] determined carbonyl compounds in soybean oil during continuous heating at 180 °C. Carbonyls were extracted from the oxidized oil with various solvents, agitation, and sonication time, derivatized with 2,4-DNPH, and analyzed by UFLC-DAD-ESI-MS. The extraction parameters were optimized for reproducibility. Ten of the identified carbonyl compounds were quantified using calibration curves of standards.

Da Silva et al [80] determined carbonyl compounds in ten species of marine algae by collecting volatiles by headspace purge and trap method, reacting these with DNPH, and separating the resulting hydrazones by HPLC. Eight carbonyl compounds were identified and quantitated while three additional carbonyls were only identified. Under optimized conditions, all carbonyl compounds were separated in 32 minutes.

Da Silva et al. [81] determined rates of production of selected volatile carbonyl compounds released from palm and soybean oils heated at 180 °C in the presence of air, through different time intervals and at different surface-to-volume ratios (S/V), in

continuous and intermittent processes. Carbonyls in headspace over the oils were collected and derivatized on silica C18 cartridges impregnated with an acid 2,4-DNPH solution, followed by elution with acetonitrile and analysis by HPLC-UV and, in some cases, HPLC-MS with electrospray ionization. Among the carbonyl compounds quantified, acrolein was the main product released from both oils at all surface:volume ratios, followed by hexanal and 2-heptenal. Soybean oil released higher levels of acrolein than palm oil. Carbonyl production was directly related to surface:volume ratios during heating. During intermittent heating, there was a trend toward increasing generation of saturated aldehydes and decreasing unsaturated aldehydes.

Seppanen and Csallany [82] used traditional reactions with DNPH then separation of hydrazones by HPLC to determine aldehydes in thermally oxidized soybean oil. Thirteen nonpolar carbonyl compounds (butanal, 2-butanone, pentanal, 2-pentanone, hexenal, hexanal, 2,4-heptadienal, 2-heptenal, octanal, 2-nonenal, 2,4-decadienal, decanal, and undecanal) and three polar carbonyls (4-hydroxy-2-hexenal, 4-hydroxy-2-octenal, and 4-hydroxy-2-nonenal) were identified, but two different gradients had to be used to separate and identify the polar carbonyls from the nonpolar carbonyls. Quantitation was estimated by normalizing to hexanal (1 ng hexanal hydrazone = peak area of 2000) and an assumption of a similar molecular extinction coefficient for all compounds.

Zhu et al. [5] used dynamic headspace sampling and 2,4-DNPH derivatization to analyze volatile carbonyl compounds in virgin olive oil. Olive oils were heated to 45 °C, and through nitrogen purging, volatilized carbonyl compounds were collected in a 2,4-DNPH cartridge at a flow rate of 1 L/min for 60 minutes. Derivatized carbonyls were



then eluted and separated by UHPLC. Nine characteristic carbonyls were quantitated using cyclopentanal as an internal standard.

Suh et al. [83] developed a targeted lipidomic method for the simultaneous determination of thirty-five aldehydes and ketones derived from fish oil by using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Fish oil was decomposed in Tris buffer in the presence of Fe(II) at physiological pH and temperature. Effects of incubation time (up to 22 hours) on formation of the main toxic reactive carbonyl species (acrolein, crotonaldehyde, HHE, HNE, ONE, glyoxal and methylglyoxal) and related the formation patterns to parent omega-3 or omega-6 fatty acid were investigated. Aldehydes were quantitated using calibration curves for each compound.

Wang and Cui [6] compared generation and derivation of reactive carbonyl species from omega-3 and omega-6 fatty acids using DNPH-LC-MS/MS analyses. Autoxidation of  $\alpha$ -linolenic acid and linoleic acid occurred with iron(II) as catalyst. The only products quantified were acrolein and crotonaldehyde. The results showed that  $\alpha$ -linolenic acid, an omega-3 fatty acid, generated more acrolein and crotonaldehyde than linoleic acid.

Sjovall et al. [84] determined TAG core aldehydes during rapid oxidation of corn and sunflower oils with *t*-Bu-OOH/Fe(II). TAG samples were directly derivatized, hydrazones were isolated by normal phase TLC then dissolved in IPA at 80 °C prior to injection and separation by RP-HPLC. Quantities were estimated from abundance of major molecular ions.

This brief review shows the intense current interest in quantifying carbonyl compounds from oxidized oils and foods. However, while each study reports

quantitation, at this point the quantitation must be considered only relative and not accurate on an absolute basis since traditional high acid DNPH reaction conditions were used, full reactivity of the carbonyls was never verified, and equal reaction of all aldehydes was erroneously assumed. In addition, HPLC separation conditions in general were not optimized and analysis times were typically long (e.g. 60 minutes). Thus, there is a compelling need to develop more accurate methodologies for DNPH reaction with and quantitation of carbonyls.

DNPH reaction conditions for quantifying carbonyls were derived almost directly from protocols for qualitative detection of carbonyls. To date there have been no systematic investigations of the DNPH reaction with carbonyls designed to identify exact conditions required for full reaction of different classes of carbonyls and derive procedures for accurate absolute as opposed to relative quantification of carbonyls. Accurate information about total carbonyls is necessary to assess extent of lipid oxidation in foods, and information about individual as well as total carbonyls is critical for elucidating complex mechanisms of lipid oxidation. Only with this information can we fully elucidate lipid oxidation in more complex lipid systems and foods.

### 3. RESEARCH GAPS

Even though the DNPH reaction has been used for analysis of carbonyls for decades, important details of the chemistry still need systematic investigation to develop accurate, reproducible quantitative assays.

**Acid – how much?** How much acid is sufficient to form the carbocations from any carbonyls present without protonating and inactivating the DNPH or catalyzing carbonyl condensation? This question has not been addressed in other studies of DNPH reactions. 2,4-DNPH has a reported pK of 1.55 in aqueous systems [56]. This suggests that the final reaction pH must be no lower than about 2.5 to avoid loss of DNPH by protonation and incomplete reaction since protonated DNPH cannot react with an activated carbonyl. Conversely, when there is not enough acid present in the reaction system, carbonyl species, especially longer chain length or unsaturated aldehydes, cannot be effectively activated, and reaction either does not occur or does not go to completion. The balance of acid in this reaction system is perhaps the most key element that must be addressed to achieve an effective and reproducible assay.

**Acid – what kind?** It has long been recognized that acid is required to make the carbonyl carbocation necessary for condensation with DNPH. To meet this requirement, a strong acid such as sulfuric acid has been added typically in excess to drive DNPH reactions with carbonyls. However, the type of acid has never been optimized. Sulfuric acid is not suitable for mass spectrometry (MS) applications because, as a mineral acid, it causes significant background in a mass spectrum. MS will be the ultimate identification method when samples of unknown carbonyl composition are tested in the future. Also, sulfuric acid causes HPLC column degradation over time so this acid should be avoided

when possible. Can other acids be substituted for sulfuric acid? Formic acid may be the most suitable choice for MS applications since it is an organic, non-mineral acid and will produce less background in a mass spectrum. However, since it is a weak acid, higher concentrations will be likely necessary to achieve the same reactivity as a strong acid. For reaction conditions, hydrochloric acid may be the most suitable choice: it is a strong acid, available in high purity, easily added and calculation of concentrations is straightforward. Alternate acids that provide required reaction conditions while at the same time are less damaging to HPLC columns and are compatible with MS detection need to be identified.

**How to quantitate mixed carbonyls when reaction kinetics vary with chain lengths.** Reaction kinetics of individual carbonyls play a particularly important role in accuracy of quantitation when the test sample contains mixed carbonyls with different chain lengths and degrees of unsaturation. Previous research in this lab indicates that under traditional conditions, aldehydes with increasing unsaturation and chain length take longer to react than short chain aldehydes under the same reaction conditions. Reaction times need to be long enough to detect long chain and unsaturated aldehydes but not so long that short-chain hydrazones begin to degrade. Can conditions be identified to facilitate reaction of longer chain aldehydes so that hydrazones of mixed chain lengths can be analyzed within the same time window? If not, for accurate quantitation it may be necessary to test and quantitate mixed carbonyls in two stages -- after a short reaction time for short chain aldehydes, and after a longer reaction time for long chain and unsaturated aldehydes.

**How to quantitate mixed carbonyls when optical properties of hydrazones vary with carbonyl structure.** Quantitating a mixture of carbonyls requires accurate

detection in addition to accurate reaction and resolved chromatographic separation. Since optical properties of hydrazones vary with carbonyl structure, the calibration curve of one carbonyl cannot be used to quantitate the remaining carbonyls in a mixture. For greatest accuracy, a calibration curve must be determined for each hydrazone when detecting peaks optically, but this is indeed tedious and time-consuming. Can this problem be overcome by considering different methods of detection?

**Optimum solvents for both carbonyls and 2,4-DNPH.** As identified from earlier laboratory experiments, dimethylformamide (DMF) improves solubility of both DNPH and lipids but also may be altering the level of protonated DNPH. It still is not clear if DMF influences the availability of DNPH to react. Eliminating DMF and switching to other solvents such as isopropanol does not seem feasible, so the minimum amount of DMF required for solubility of reagents and maximum amount tolerable without interference with DNPH must be determined.

**Optimum HPLC column choice.** Hydrophobic C18 columns have been most commonly used to separate hydrazones according to their carbonyl backbones, e.g. saturated aldehydes elute by chain length, with shortest eluting first. When unsaturation comes into play, compounds with increasing numbers of double bonds elute faster than saturated compounds with the same chain length so some key hydrazones co-elute as critical pairs. Can this problem be overcome by simply modifying solvent and elution conditions, or will alternate columns or other approaches be necessary?

#### **4. RESEARCH OBJECTIVES**

Our laboratory is engaged in broad-based research on lipid oxidation mechanisms, one goal of which is to develop dependable, accurate and sensitive methods to analyze all

products and stages of lipid oxidation. This dissertation research focuses on developing the DNPH reaction into an accurate rugged assay for identifying and quantitating non-volatile carbonyl oxidation products at micromole levels.

The specific objectives of this research are:

### **Reaction optimization**

- Determine highest pH that will support DNPH reaction (carbocation formation) without protonating DNPH to inactive form or catalyzing carbonyl condensation
- Test the effects of acids other than H<sub>2</sub>SO<sub>4</sub> (e.g. HCl and formic) on kinetics and characteristics of reaction to reduce column degradation and increase MS compatibility
- Test reaction effects of DMF used to solubilize DNPH and lipids
- Evaluate potential of 3,5-diaminobenzoic acid (DABA) to provide a proton source without lowering pH

### **Detection and quantification optimization**

- Compare separation of a panel of carbonyl-DNPH hydrazones on Reverse Phase-C18 and Pentafluorophenyl columns
- Improve the resolution of critical pairs and isomers of carbonyl-DNPH hydrazones by adjusting the elution conditions such as solvents, or gradient and column
- Determine the limit of detection, limit of quantification and RSD% for DNPH-HPLC analysis of carbonyl-DNPH derivatives

## 5. METHODS AND MATERIALS

### 5.1 Experimental Design

A flow diagram for optimizing the 2,4-DNPH reaction for detection and quantification of lipid carbonyls is presented in Figure 10. A series of pure aldehydes, saturated and unsaturated, that are known to be generated in oxidation of oleic, linoleic, and linolenic acids provided starting carbonyls to test reaction conditions and optimize HPLC separations.

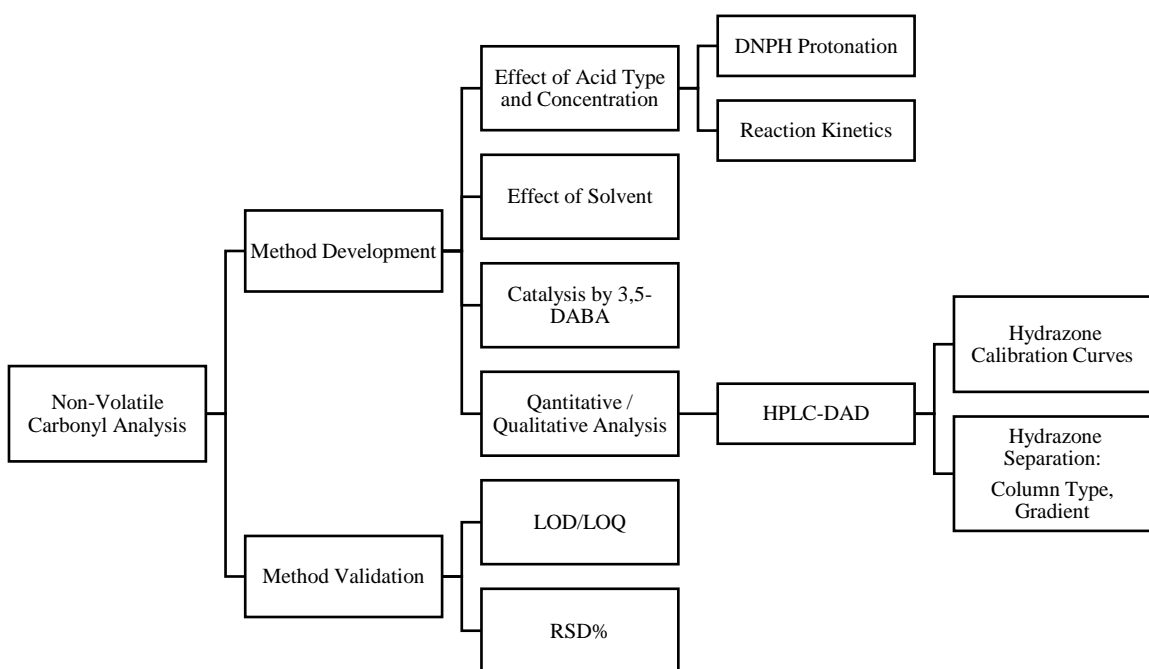


Figure 10. Experimental design for testing factor effects and optimizing conditions for reaction of DNPH with lipid carbonyls (aldehydes).

Development of an improved 2,4-DNPH reaction with lipid carbonyls focused on determining the effects of acid concentration and type on DNPH protonation and reaction kinetics with aldehydes, as well as solvent effects and possible reaction catalysts. Changes in HPLC separation parameters were studied to improve the separation and quantitation of products. Improvements in reaction parameters in conjunction with improved separation should facilitate qualitative identification and increase quantitation accuracy for individual carbonyl products produced from lipid oxidation.

## 5.2 Materials

2,4-Dinitrophenylhydrazine (2,4-DNPH) was obtained from Sigma Aldrich (St. Louis, MO) and recrystallized three times from acetonitrile. 3,5-Diaminobenzoic Acid (3,5-DABA) was purchased from Acros Organics (Waltham, MA). 37% Hydrochloric acid, 98% formic acid, dimethylformamide, and the following carbonyl standards were purchased from Sigma-Aldrich (St. Louis, MO): butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, undecanal, *trans*-2-pentenal, *trans*-2-hexenal, *trans*-2-heptenal, *trans*-2-octenal, *trans*-2-nonenal, *trans*-2-decenal, *trans,trans*-2,4-nonadienal, and *trans,trans*-2,4-decadienal. HPLC grade acetonitrile and isopropanol were purchased from multiple suppliers. Water for all reagents and reactions was purified to 18 M $\Omega$  resistivity in a four-cartridge Milli-Q<sup>TM</sup> water purification system (Millipore, Billerica, MA).



### **5.3 Testing and Optimization of HPLC-DNPH Analyses of Carbonyls**

#### **5.3.1 Preparation of Acidified 2,4-DNPH Reagent**

DNPH reagent acidified with HCl was prepared by dissolving 40 mg recrystallized 2,4-DNPH in 990  $\mu\text{L}$  N,N-dimethyl formamide (DMF); 10  $\mu\text{L}$  hydrochloric acid of varying concentrations (0.01 - 12.1 M) was added and the solution was vortexed for 30 seconds.

DNPH reagent acidified with formic acid was prepared by dissolving 40 mg recrystallized 2,4-DNPH in 850  $\mu\text{L}$  N,N-dimethylformamide; 150  $\mu\text{L}$  of 98% (21 M) formic acid (final volume 1 mL) was added and the solution was vortexed for 30 seconds. Taking differences in dissociation between the two acids into account, this amount and concentration of formic acid was equivalent to the HCl (op cit) in  $[\text{H}^+]$  concentration released during reaction.

Acidified 2,4-DNPH reagents were prepared daily and used immediately.

#### **5.3.2 Effect of Acid and Solvent on UV-Vis Absorbance of 2,4-DNPH**

To prepare the working reagent, 40 mg recrystallized 2,4-DNPH was dissolved in 990  $\mu\text{L}$  of test solvent, then 10  $\mu\text{L}$  HCl of varying concentrations were added to give final concentrations of  $3.0 \times 10^{-5}$  M 2,4-DNPH with 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 or 0.1 M acid. Optical spectra were recorded with a Cary 50 UV-Vis spectrophotometer and plotted in overlay mode to view changes in absorption spectra with increased acid. Protonated DNPH was distinguished from the native form by the characteristic shift in lambda max from 360 to 320 nm.

### 5.3.3 Preparation of Hydrazones from Carbonyl Standards

For reaction, 2 mg carbonyl standard were added to 150  $\mu\text{L}$  acidified 2,4-DNPH reagent plus 100  $\mu\text{L}$  acetonitrile in a disposable 12x75 mm tube and vortexed for 30 seconds. After reaction for 0-60 minutes at room temperature (20-25 °C), the samples were diluted with acetonitrile (ACN), immediately filtered by syringe filter (0.22  $\mu\text{m}$ ), and analyzed by HPLC.

### 5.3.4 Effect of Acid on Rate of 2,4-DNPH Reaction with Aldehydes of Varying Saturation

To determine the acid concentration needed for maximum DNPH reaction with saturated, monounsaturated, and diunsaturated aldehydes, nonanal, *t*-2-nonenal, and *t,t*-2,4-nonadienal were reacted individually with 2,4-DNPH acidified with varying HCl concentrations (12.1, 1, 0.75, 0.5, 0.25, 0.1, or 0.01 M) to give a range of final reaction pH.

For reaction, 2 mg of aldehyde, 150  $\mu\text{L}$  of acidified 2,4-DNPH, and 100  $\mu\text{L}$  ACN were vortexed for 30 seconds. The reaction mixture was incubated for 0, 5, 10, 15, or 20 min at room temperature (20-25 °C), then diluted with 2 mL ACN, filtered through a 2-micron filter, and analyzed by HPLC. Time 0 was effective time zero rather than no reaction: immediately after vortexing, the sample was diluted, filtered, transferred to an HPLC vial, and injected onto the column. It is possible that some small amount of reaction occurred in that handling time. Nevertheless, this provides the baseline for comparison with other reaction conditions.

### 5.3.5 Tests of Reduced HCl Acidity with Complete Aldehyde Panel

Aldehydes were reacted and analyzed individually as well as in a full mixed panel to establish reproducibility in synthesizing hydrazones and to ensure that unsaturated aldehydes of all chain lengths and degrees of unsaturation react fully under conditions of reduced acid.

Aldehyde panel: butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, undecanal, *t*-2-pentenal, *t*-2-hexenal, *t*-2-heptenal, *t*-2-octenal, *t*-2-nonenal, *t*-2-decenal, *t,t*-2,4-nonadienal, *t,t*-2,4-decadienal.

Acidified 2,4-DNPH reagent: 40 mg of DNPH was dissolved in 990 microliters of DMF and 10 microliters of 0.5 M HCl.

Reaction mixture: 2 mg of aldehyde, 100 microliters of ACN, and 150 microliters of 2,4-DNPH reagent acidified with 0.5 M HCl were mixed and vortexed for 30 seconds, allowed to react for 20 minutes at room temperature (20-25 °C), diluted with 1 mL of ACN and analyzed by the standard HPLC method outlined in 5.3.3. The hydrazones were prepared and analyzed in triplicate.

### 5.3.6 Stability of Hydrazones at Reduced HCl Acidity

*t,t*-2,4-Decadienal hydrazone was prepared according to Section 5.3.1 utilizing 0.5 M HCl to prepare the 2,4-DNPH reagent, resulting in a reaction pH of 2.52. Samples were analyzed by HPLC 0 minutes, 20 minutes, 1, 6, 12, and 24 hours after reaction to determine stability of hydrazones.

### 5.3.7 Reaction Stoichiometry of DNPH:H<sup>+</sup>:Carbonyl

After determining the minimum concentration of acid necessary under conditions of excess DNPH, the reaction stoichiometry was further studied to determine if the current conditions using a great excess 2,4-DNPH were necessary. To determine the ratios of DNPH:Carbonyl necessary to achieve complete reaction when using 0.5 M HCl to prepare the acidified 2,4-DNPH reagent, the standard concentration of 2,4-DNPH (120 mM) in the reaction mixture was reduced in sequential tests from 120 mM to 60 mM and 30 mM until reaction was not complete. At 30 mM DNPH, reaction was incomplete.

Acidified 2,4-DNPH: 10 mg (30 mM), 20 mg (60 mM) or 40 mg (120 mM) of recrystallized 2,4-DNPH was dissolved in 990  $\mu$ L of DMF and 10  $\mu$ L of 0.5 M HCl.

Reaction Mixture: 2 mg of nonanal, *t*-2-nonenal, or *t,t*-2,4-nonadienal, 150  $\mu$ L of acidified 2,4-DNPH, and 100  $\mu$ L ACN were vortexed for 30 seconds. The mixture was reacted for 20 minutes at room temperature (20-25 °C), diluted with 2 mL ACN, filtered through a 2-micron filter, and analyzed by HPLC.

### 5.3.8 Replacing Hydrochloric Acid with Formic Acid in 2,4-DNPH reagent

To test the effect of specific acids and the flexibility to change acids for mass spectrometry applications, hydrochloric acid was replaced with formic acid (98%) in the acidified 2,4-DNPH reagent. Nonanal, *t*-2-nonenal, and *t,t*-2,4-nonadienal were individually reacted to determine the extent of reaction of saturated and unsaturated C9 aldehydes (models for all aldehydes) when formic acid was used.

Acidified 2,4-DNPH reagent: 40 mg of 2,4-DNPH were dissolved in 850 microliters of DMF and 150 microliters of 98% formic acid.

Reaction mixture: 2 mg of aldehyde were dissolved in 100 microliters of ACN, 150 microliters of 2,4-DNPH reagent acidified with (98%) formic acid was added, the sample was vortexed and reacted for 20 minutes at room temperature (20-25 °C), diluted with 2 mL ACN, filtered through a 2-micron filter, and analyzed by HPLC.

### **5.3.9 HPLC Separation of Hydrazones – Base Conditions**

Carbonyl-DNPH derivatives were separated on an Agilent (Santa Clara, CA) 1100 Infinity HPLC system equipped with solvent degasser, quaternary pump, autosampler, temperature-controlled column compartment, diode array detector, and Restek Ultra C18 column (4.6 mm\*150 mm, 5  $\mu$ ). The injection volume was 5  $\mu$ L and the solvent system was A: acetonitrile / isopropanol (1:1); B: acetonitrile / isopropanol / 18 M $\Omega$  water (1:1:2). The elution gradient of separating hydrazones was 16.7% - 100% A in 17 minutes (1.2 mL/min), remain at 100% A for 22 minutes; return to 16.7% A in 5 minutes; total run time was 27 min at a flow rate of 1.2 mL/min. Absorbance of carbonyl-DNPHs was monitored at 360 nm; underivatized carbonyls were monitored at 206, 233 and 270 nm. These base separation conditions developed by Yao [12] were designed to elute the full aldehyde panel in 20 minutes and provide baseline resolution of all saturated aldehydes but incompletely separate some critical pairs (e.g. C sat aldehyde and C+2 enal).

### 5.3.10 HPLC Separation of Hydrazones – Alternate Gradients

Longer columns (250 mm, same column material) had been tested previously to improve resolution of critical pairs. Baseline separation was not achieved, and analysis times were tremendously extended so this approach was abandoned.

Two alternate changes to the HPLC separation gradient were tested to improve resolution of critical pairs. In the first adaptation, the time for increasing solvent strength from 16.7% to 100% A (1:1 ACN:IPA) was extended from 17 minutes to 27 minutes to provide more gradual solvent increase and longer time for separation. The second adaptation tested a different separation gradient: 1:1 ACN:IPA (A) and 18 M $\Omega$  water (B). The gradient was 40-80% A in 55 min. All gradients were tested on the following aldehyde mixture.

Aldehyde Mixture: 5 microliters of the following 16 aldehydes were dissolved in 1.6 mL of ACN: butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, undecanal, *t*-2-pentenal, *t*-2-hexenal, *t*-2-heptenal, *t*-2-octenal, *t*-2-nonenal, *t*-2-decenal, *t,t*-2,4-nonadienal, and *t,t*-2,4-decadienal

Acidified 2,4-DNPH reagent: 40 mg of DNPH was dissolved in 990 microliters of DMF and 10 microliters of 0.5 M HCl.

Reaction Mixture: 50 microliters of aldehyde mixture, 50 microliters of ACN, and 150  $\mu$ L of 2,4-DNPH acidified with 0.5 M HCl were vortexed for 30 seconds. The mixture reacted for 20 minutes at room temperature (20-25 °C), diluted with 2 mL ACN, filtered through a 2-micron filter, and analyzed by HPLC.

### 5.3.11 Separation of Hydrazones on Alternate Columns

To investigate whether a column with different binding patterns to alkyl chains vs double bonds might improve separation of critical pairs, three additional columns were tested for ability to separate the critical pair octanal and *t,t*-2,4-nonadienal. Columns tested included: Phenomenex Kinetex F5 pentafluorophenyl (PFP) column (2.6  $\mu\text{m}$  particle size, 100 Å pore diameter, 100 x 4.6 mm), a Restek Ultra Aqueous C18 column (3  $\mu\text{m}$  particle size, 150 x 4.6 mm), and a Restek Ultra C18 column (5  $\mu\text{m}$  particle size, 150 x 2.1 mm)

These columns were tested under extended gradient conditions: 1:1 ACN:IPA (A) and 18 M $\Omega$  water (B). The gradient was 16.7-100% A in 55 min. The PFP column was expected to bind the phenyl groups and allow increased separation by differential solubility of the aldehyde chains in the eluting solvent. The Ultra Aqueous column was expected to bind the double bonds of unsaturated aldehyde-hydrazones more strongly and prevent their movement with shorter alkyl chains. The Ultra C18 column was expected to separate in the same manner as our standard column but improve resolution with a smaller internal diameter.

Acidified 2,4-DNPH reagent: 40 mg of DNPH was dissolved in 990 microliters of DMF and 10 microliters of 0.5 M HCl.

Reaction mixture: 1 mg of *t,t*-2,4-decadienal and 1 mg of nonanal were dissolved in 100 microliters of ACN. 150 microliters of acidified DNPH reagent were added, the sample was vortexed and allowed to react for 20 minutes at room temperature (20-25 °C), diluted with ACN, filtered through a 2-micron filter, and analyzed by HPLC.

### 5.3.12 Potential Catalytic Activity of 3,5-DABA

3,5-DABA was added to the DNPH reagent to determine whether an alternate proton source could allow reaction at further reduced acidity while supporting complete reaction of unsaturated aldehydes.

2,4-DNPH reagent with only 3,5-DABA as the H<sup>+</sup> donor: 30 mg of 3,5-DABA and 40 mg of 2,4-DNPH were dissolved in 1 mL of DMF

2,4-DNPH reagent acidified with 0.1 M HCl plus 3,5-DABA to reduce acid amounts: 30 mg of 3,5-DABA and 40 mg of 2,4-DNPH were dissolved in 990 microliters of DMF and 10 microliters of 0.1 HCl.

Reaction Mixture: 2 mg of *t,t*-2,4-nonadienal were dissolved in 100 microliters of ACN, 150 microliters of 2,4-DNPH test reagent were added, the sample was vortexed and allowed to react for 60 minutes at room temperature (20-25 °C), diluted with ACN, filtered through a 2-micron filter, and analyzed by HPLC.

### 5.3.13 Calibration Curves and Method Validation of Carbonyl-DNPH Hydrazone Standards

Carbonyl standards of butanal, pentanal, *t*-2-pentenal, hexanal, *t*-2-hexenal, heptanal, *t*-2-heptenal, octanal, *t*-2-octenal, nonanal, *t*-2-nonenal, *t,t*-2,4-nonadienal, decanal, *t*-2-decenal, *t,t*-2,4-decadienal, and undecanal were derivatized by 2,4-DNPH to investigate the linearity, relative response factors (RFFs), limit of detection (LOD) and limit of quantification (LOQ) of the HPLC method. A series of aldehyde concentrations (0.5, 1, 2, 10, 20, 50, 100, 200, 400 microgram/mL) were analyzed to generate the calibration curves.



Acidified 2,4-DNPH reagent: 40 mg of DNPH were dissolved in 990 microliters of DMF and 10 microliters of 0.5 M HCl.

Reaction mixture: 2 mg of aldehyde, 100 microliters of ACN, and 150 microliters of acidified 2,4-DNPH reagent were mixed and vortexed for 30 seconds, allowed to react for 20 minutes at room temperature (20-25 °C), diluted to 1 mL with ACN, filtered through a 2-micron syringe filter, and analyzed by the standard HPLC separation method outlined in 5.3.3. The hydrazones were prepared and analyzed in triplicate.

#### **5.3.14 Determination of Limits of Detection and Quantification, Variability**

Peak areas were plotted against concentration of carbonyl standard and linearity was determined using the least squares method.

Variability of calibration curves between carbonyl standards was determined by calculating the relative standard deviation of calibration curve slope for each standard aldehyde.

Limits of Detection and Quantification were determined from calibration curves. The lowest concentrations within the linear range with a relative standard deviation of <2.5% was accepted, thus the LOD and LOQ for each aldehyde were equivalent.

#### **5.3.15 Data Analysis**

Chromatographic data acquisition and peak integration was performed using Agilent ChemStation software. Standard curves developed for each aldehyde using authentic pure compounds were run in triplicate.

## 6. RESULTS AND DISCUSSION

### 6.1 Effects of Acid and Solvent on the UV-Vis Absorbance of 2,4-DNPH

The spectrum of 2,4-DNPH in acetonitrile with standard  $\lambda_{\text{max}}$  at 360 nm is shown in Figure 11, top. Addition of acid leads to hypsochromic shifts (to lower wavelengths) in  $\lambda_{\text{max}}$ , proportional to acid concentration, indicating the protonation of 2,4-DNPH. With addition of 0.1 and 0.2 M HCl, the absorbance peak significantly decreased to 345 and 330 nm. Adding more acid (0.4 – 1.2 M HCl) shifted the  $\lambda_{\text{max}}$  further to 320 nm with gradual increases in absorbance. This spectrum is in good accordance with spectra reported by Bernheim et al. [56] for protonated 2,4-DNPH in an aqueous system.

When 2,4-DNPH was dissolved in DMF (Figure 11, bottom), acid shifted  $\lambda_{\text{max}}$  slightly from 360 to 350 indicating that some protonation of 2,4-DNPH occurred. However, the greater effect of acid was a significant decrease in absorbance of 2,4-DNPH, suggesting that DMF prevents protonation of 2,4-DNPH, possibly by preferentially binding the available protons through its oxygen [85]. This may allow complexation of protonated DMF with unprotonated 2,4-DNPH causing a change electron distribution and absorption and decreased absorbance of 2,4-DNPH.

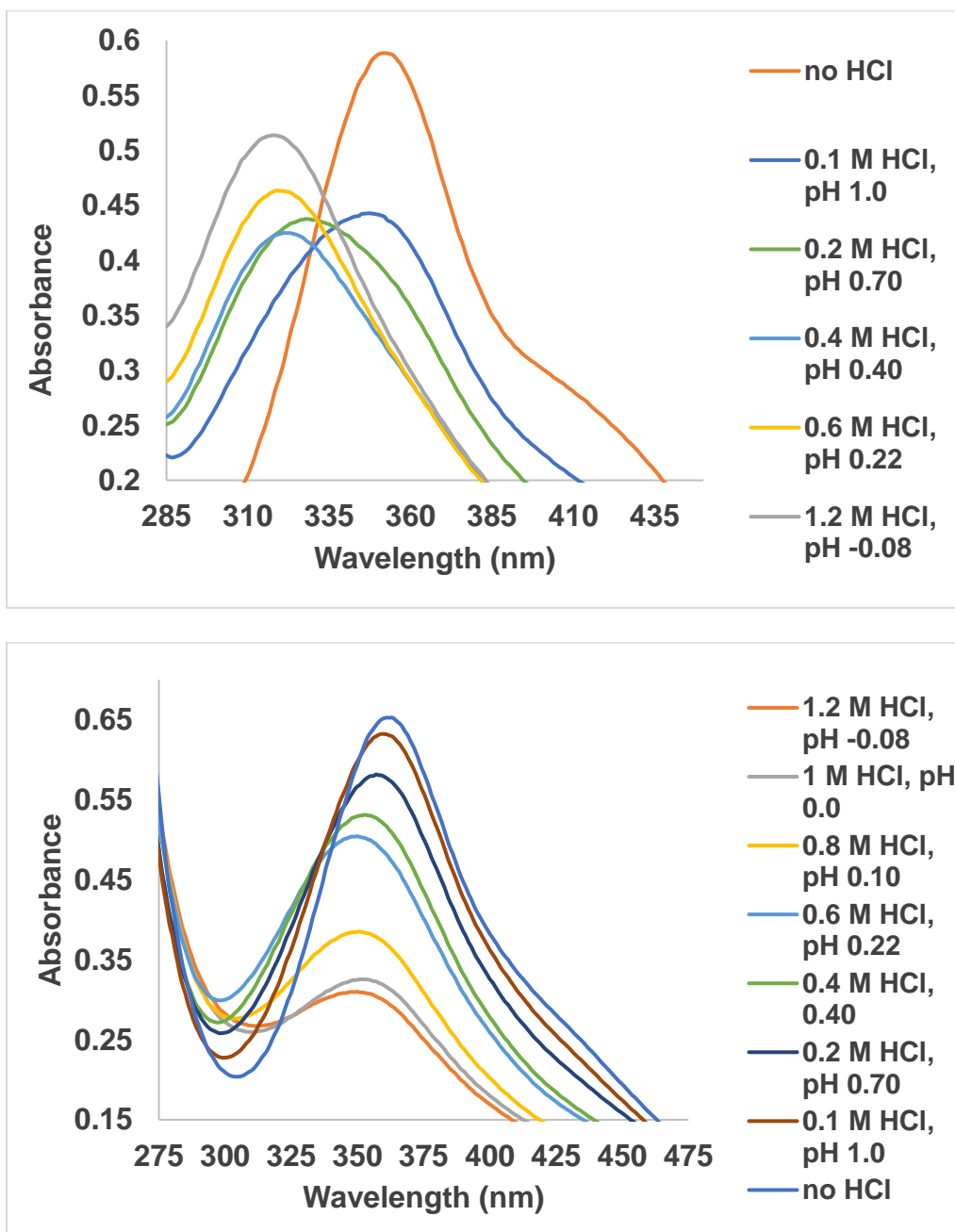


Figure 11. Effect of acid on absorbance of 2,4-DNPH. Top: in acetonitrile. Bottom: in dimethylformamide. 40 mg recrystallized 2,4-DNPH were dissolved in 990  $\mu\text{L}$  of solvent and 10  $\mu\text{L}$  HCl to give final concentrations of  $3.0 \times 10^{-5}$  M 2,4-DNPH with 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 or 0.1 M acid, with corresponding pH values.

The spectral data confirms that strong concentrations of acid reduce availability of 2,4-DNPH and verifies that minimizing acid concentration is a key requirement for accurate detection and quantitation of the reaction. Since the wavelength shift was not as distinct for 2,4-DNPH in DMF, it is an acceptable component of the solvent system, especially since it solubilizes a higher concentration of 2,4-DNPH than any other solvent and facilitates solubilization of triacylglycerols. DMF provides a functionality that this reaction needs, but the amount must be limited to just enough to dissolve lipids and 2,4-DNPH.

## **6.2 Effects of Acid on Kinetics of 2,4-DNPH Reaction with Saturated and Unsaturated Carbonyls**

Reaction conditions of Yao (2015) were used as a starting point for investigating effects of concentration and type of acid used to acidify the 2,4-DNPH reagent. Yao's reagent consisted of 40 mg of 2,4-DNPH dissolved in 10  $\mu$ L 95% sulfuric acid and 990  $\mu$ L dimethylformamide. The reagent was reacted with aldehyde standards dissolved in acetonitrile for twenty minutes at room temperature. For this study, the acid used to prepare the 2,4-DNPH reagent was changed to hydrochloric acid and overall acid concentration was reduced to determine the minimum acidity level that would still facilitate reaction within 20 minutes.

C-9 aldehydes of varying saturation (nonanal, *t*-2-nonenal, and *t,t*-2,4-nonadienal) were reacted individually with 2,4-DNPH reagent acidified with either 12.1, 1, 0.75, 0.5, 0.25, 0.1 or 0.01 M HCl and reaction kinetics and product formation were measured. The final pH and effective acid concentrations of the reaction mixtures for each concentration

of HCl added to acidify the 2,4-DNPH reagent are shown in Table 2. Values were determined as follows: 1 mL of the acidified 2,4-DNPH reagent was prepared by mixing 10  $\mu$ L hydrochloric acid with 990  $\mu$ L DNPH, then 150  $\mu$ L of the acidified reagent was reacted with 2 mg of carbonyl in 100  $\mu$ L of acetonitrile (total reaction volume 250  $\mu$ L) for 20 minutes at room temperature. The acid concentration in the reaction mixture was used to determine the effective pH in reaction by calculation from the equation  $\text{pH} = -\log [\text{H}^+]$ . This was assuming complete dissociation.

Table 2. Acid concentrations in acidified DNPH reagent and reaction mixture and effective pH in reaction (by calculation) for 2,4-DNPH reaction with carbonyls.

<b>[HCl] used to prepare 2,4- DNPH reagent (M)</b>	<b>[HCl] in 2,4- DNPH reagent (mM)</b>	<b>[HCl] in reaction mixture (mM)</b>	<b>Effective pH in reaction</b>
12.1	120	72.0	1.14
1	10.0	6.00	2.22
0.75	7.5	4.5	2.35
0.5	5.0	3.0	2.52
0.25	2.5	1.5	2.82
0.1	1.00	0.60	3.22
0.01	0.10	0.06	4.22

Peak areas of hydrazones produced from the reaction of each of the three C9 aldehydes were compared as a function of acid concentration used to prepare the 2,4-DNPH reagent and of the related final pH (Figure 12). Formation of hydrazones was monitored at 360 nm, while the loss of aldehyde was monitored at 233 nm for *t*-2-nonenal and 270 nm for *t,t*-2,4-nonadienal. Under these acidified conditions *t*-2-nonenal exists as a conjugated diene and *t,t*-2,4-nonadienal as a conjugated triene. The greatest formation of hydrazones for all three C9 aldehyde occurred when using 1 M HCl (pH 2.2, 10 mM acid in DNPH reagent). With 0.1 M HCl (pH 3.2), *t*-2-nonenal and *t,t*-2,4-nonadienal did not react to completion, and no hydrazone product was formed in reactions with 0.01 M HCl (pH 4.2). The lack of formation of hydrazone product using 0.01 M HCl is not due to a lack of 2,4-DNPH. All reactions occurred with an excess of the reagent. Further reaction times were not studied, because for our purposes we are interested in a 20-minute reaction time goal and the conditions that achieve this time limit.

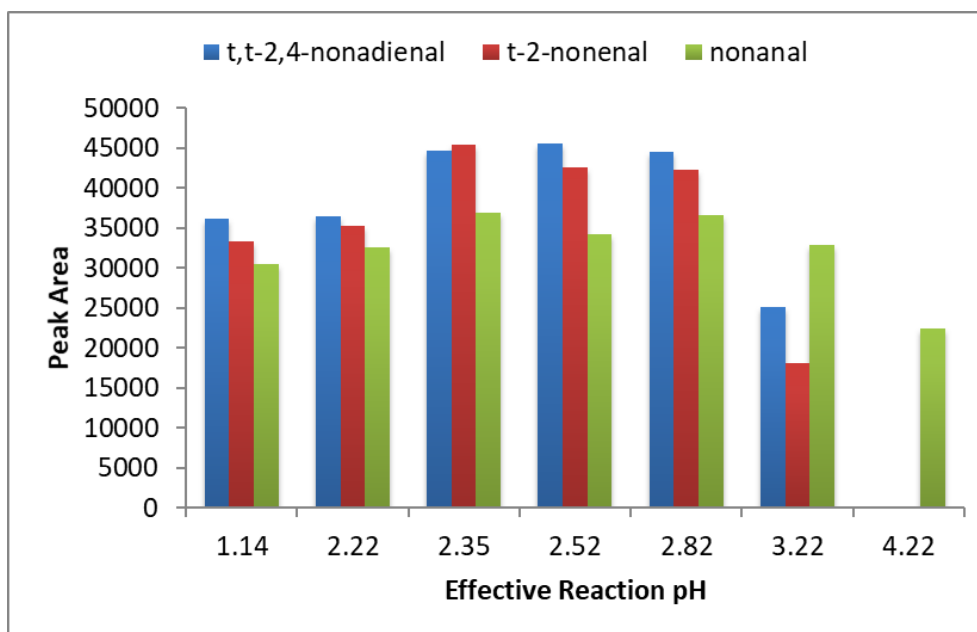
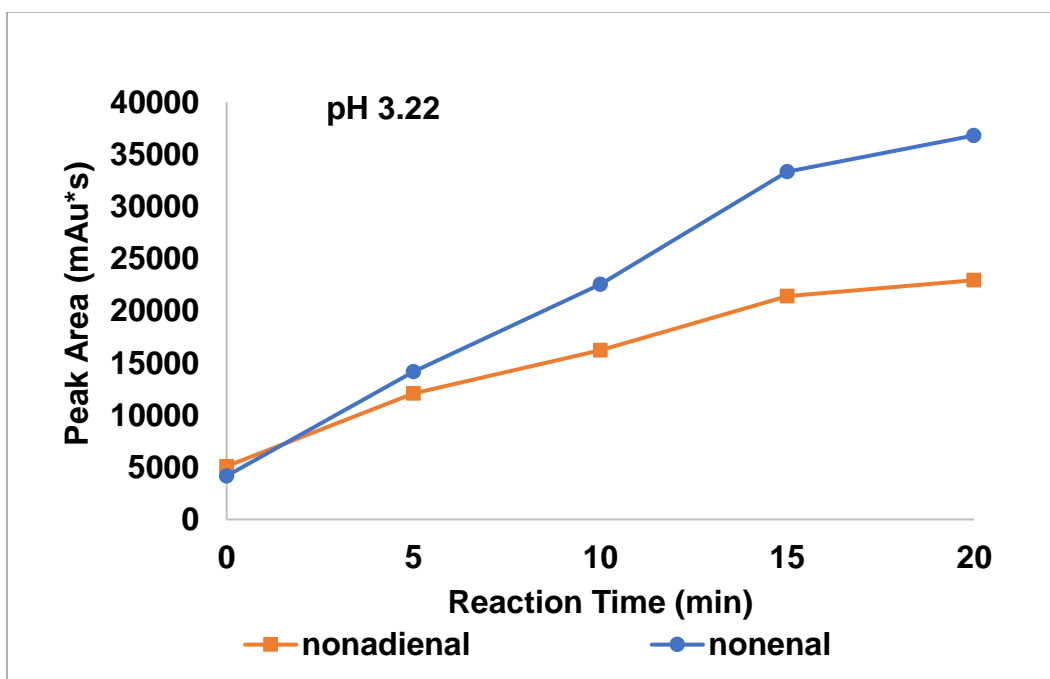


Figure 12. Effect of acidity (reaction pH) on hydrazone formation in reaction of nonanal, *t*-2-nonenal, and *t,t*-2,4-nonadienal with 2,4-DNPH. Peak areas reported.

Reducing the acid concentration ( $\text{pH} > 3$ ) reduced the rate as well as extent of unsaturated aldehyde reactions with 2,4-DNPH. At pH 3.22, hydrazone peak areas increased gradually over the course of 20 minutes for *t,t*-2,4-nonadienal and *t*-2-nonenal reacted with 2,4-DNPH reagent prepared with 0.1 M HCl (Figure 13, top). However, unreacted *t,t*-2,4-nonadienal as well as unreacted *t*-2-nonenal were still detected after 20 minutes, which was expected due to the low concentration of acid in the system.

With 1 M HCl ( $\text{pH} 2.22$ ), formation of hydrazones was immediate. Hydrazone peak areas were constant after five minutes of reaction (Figure 13, bottom), and reaction went to completion for the unsaturated aldehydes. In particular, unreacted *t,t*-2,4-nonadienal was not detected after five minutes of reaction.



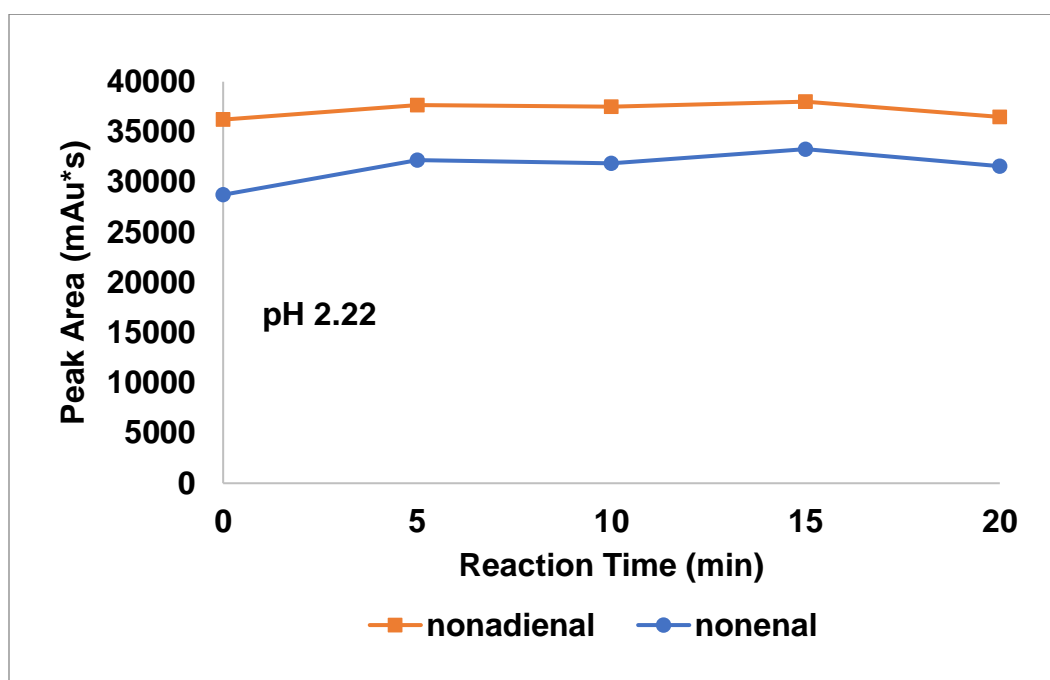


Figure 13. Kinetics of *t,t*-2,4-nonadienal and *t*-2-nonenal formation and product stability over time, measured at 360 nm with Top: 0.1 M HCl-DNPH. Bottom: 1 M HCl-DNPH.

While unreacted nonanal could not be detected even at 206 nm due to lack of a chromophore, the formation of hydrazone occurred within the time required to transfer sample to the HPLC and the peak area for the aldehyde remained constant from 5 to 20 minutes of reaction, between HCl concentrations of 0.1 and 12 M. Decreased product formation only began with reaction at pH 4.2 (DNPH prepared with 0.01 M HCl). This is consistent with the reported high reactivity of saturated aldehydes.

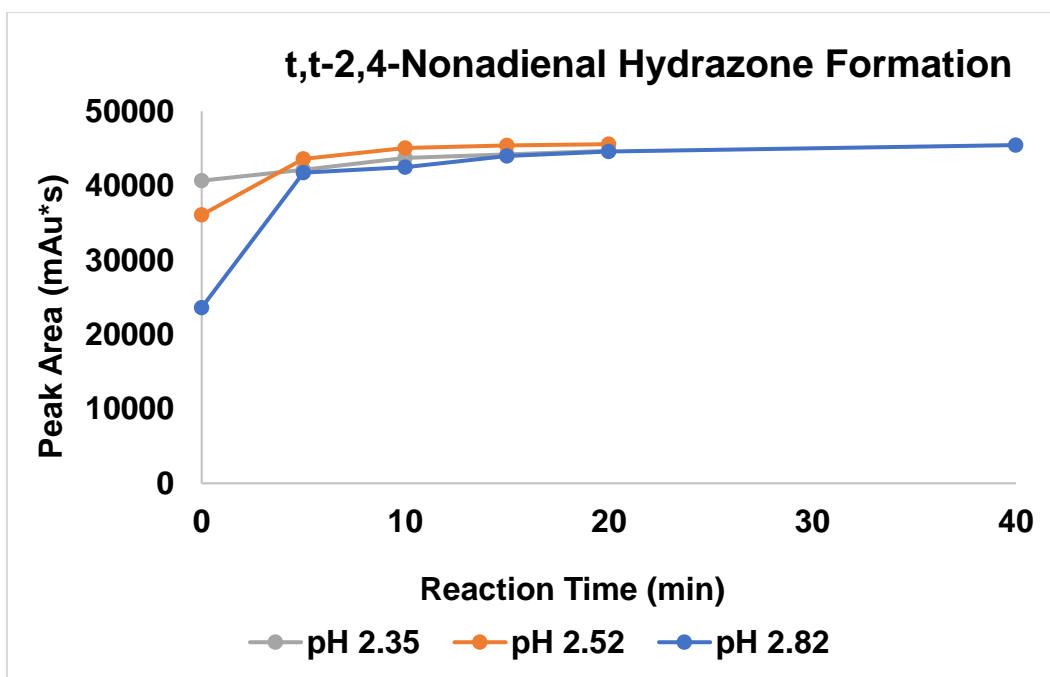
With final reaction pH 2.52 and 2.35 (HCl concentrations of 0.5 or 0.75 M, respectively, used to prepare the 2,4-DNPH reagent), *t,t*-2,4-nonadienal reacted fully within 10 minutes. Peak area reached a maximum within 10 minutes and remained stable thereafter (Figure 14, top), and there was no trace of unreacted aldehyde detected at 270 nm (Figure 14, bottom). With 0.25 M acid, the reaction required 40 minutes to reach



completion. Given a practical goal of <30 minutes reaction time, either 0.5 or 0.75 M HCl (reaction pH of 2.5-2.3) is an acceptable concentration for preparation of acidified 2,4-DNPH that will mediate complete reaction with *t,t*-2,4-nonadienal.

*t*-2-Nonenal required 15 minutes for complete reaction at pH 2.52 or pH 2.35, and hydrazone peak areas were stable after 15-20 minutes (Figure 15, top). No unreacted aldehyde was detectable at 233 nm after 15 minutes (Figure 15, bottom). There was a slight and consistent increase in hydrazone product at pH 2.35 (DNPH prepared with 0.75 M HCl). Since this difference is consistent at all time points compared to reaction pH 2.52, this may be due to a weighing error of the aldehyde, or it is possible that more hydrazone was produced at this reaction pH. With an ideal reaction time of less than 30 minutes, either 0.5 or 0.75 M HCl provides sufficient acid to drive complete reaction of *t*-2-nonenal without inactivating the 2,4-DNPH. At reaction pH 2.82 (DNPH prepared with 0.25 M HCl), *t*-2-nonenal required 40 minutes for the aldehyde to react to completion, too long for practical analyses.

The combined overall peak areas and kinetics of hydrazone formation and aldehyde disappearance over time all suggest that a reaction pH of 2.52 (0.5 M HCl used to prepare the 2,4-DNPH reagent) supports maximum hydrazone formation in shortest time. Under these conditions, complete reaction of all saturated and unsaturated carbonyls present occurs within 20 minutes. Thus, all further optimization of reaction parameters in this project was conducted using 0.5 M HCl to acidify the 2,4-DNPH reagent, giving a reaction pH of 2.5.



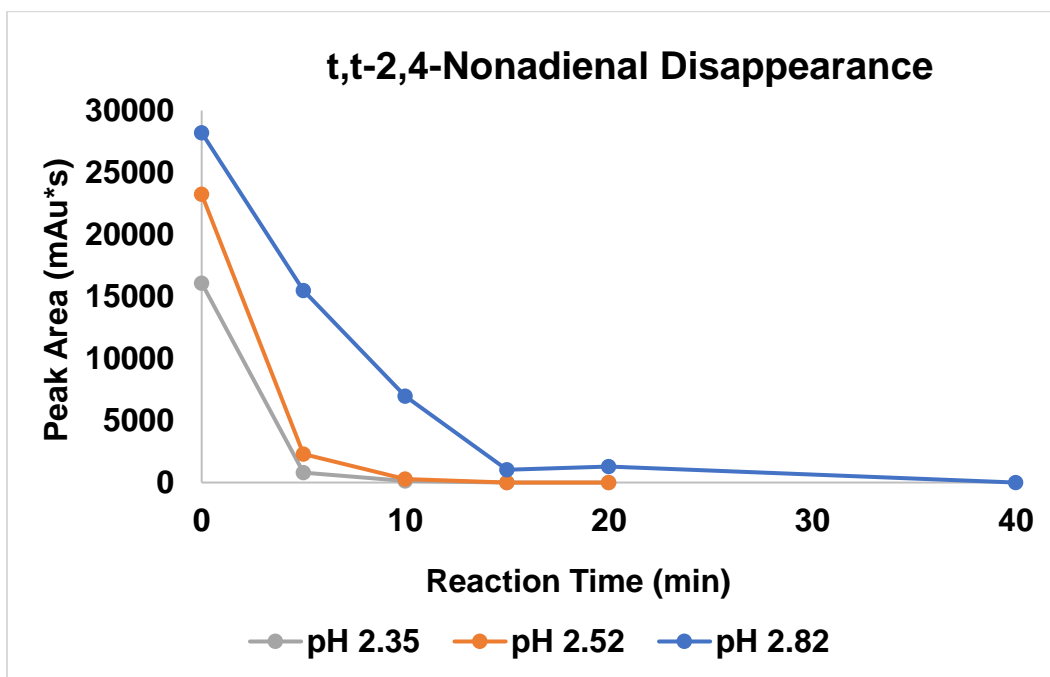
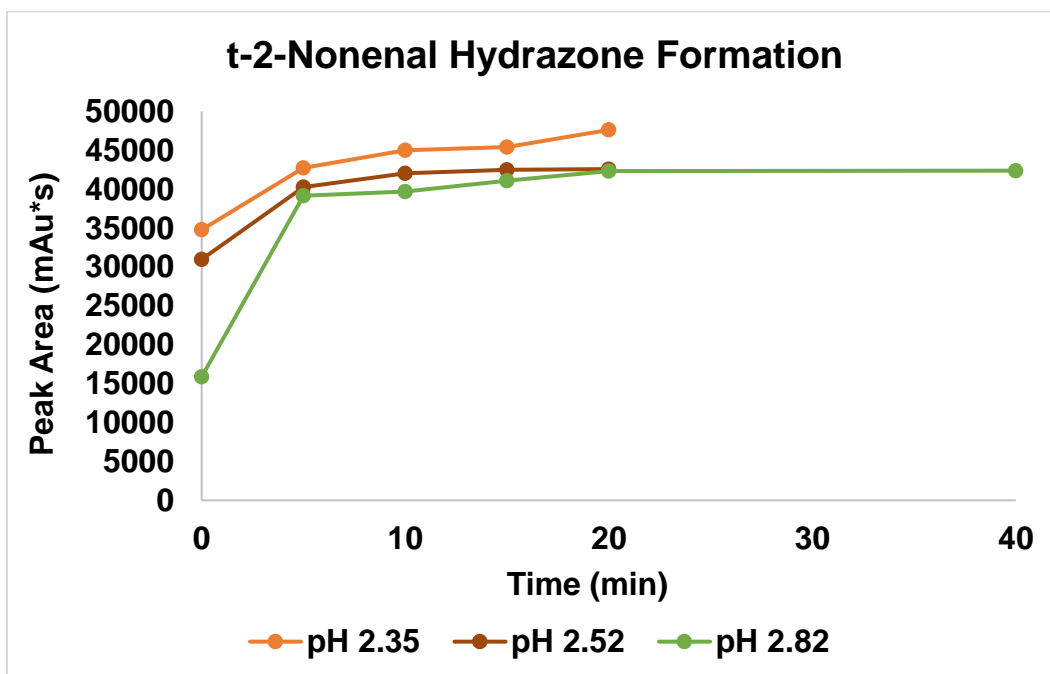


Figure 14. Effect of acid (pH) on kinetics of *t,t*-2,4-nonadienal reaction with DNPH. Top: formation of hydrazones. Bottom: disappearance of *t,t*-2,4-nonadienal.



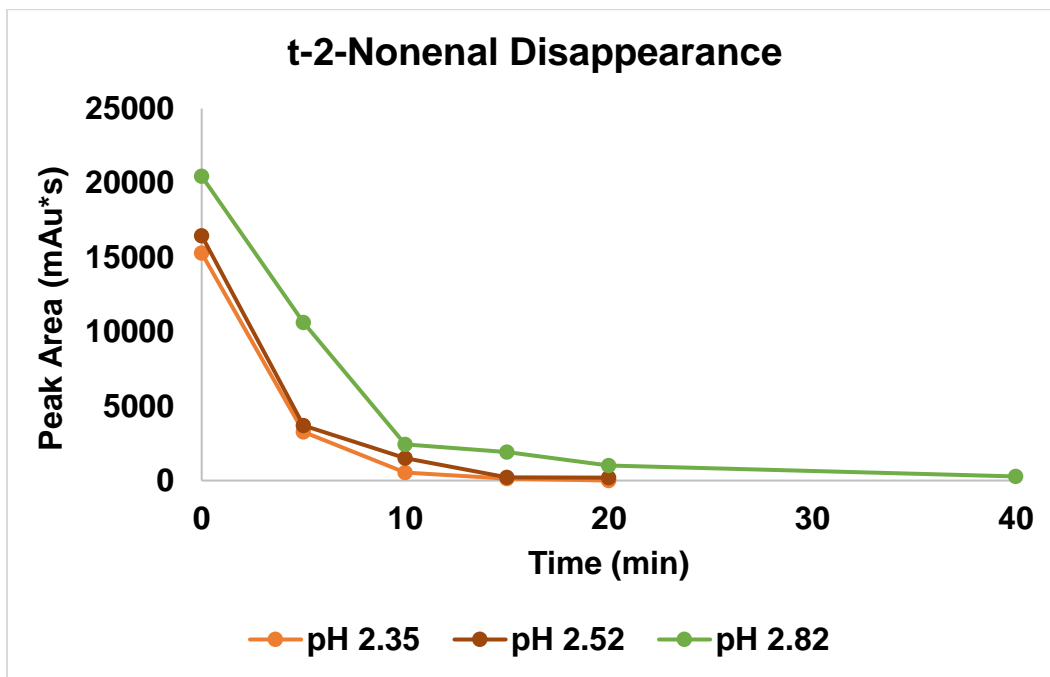


Figure 15. Effect of acid (pH) on kinetics of *t*-2-nonenal reaction with DNPH. Top: formation of hydrazones. Bottom: disappearance of *t*-2-nonenal.

### 6.3 Stability of Hydrazones

2,4-DNPH hydrazones are not stable at room temperature. To ensure that products formed would be stable throughout long analytical periods required for many samples on an HPLC autosampler, *t,t*-2,4-nonadienal hydrazone was used as a model sample, prepared at a reaction pH of 2.52. Samples were analyzed by HPLC every hour for 12 hours to determine stability of products.

The time curve for *t,t*-2,4-decadienal hydrazone (Figure 16) shows good stability over the 12-hour test period. Variation in E isomer peak area was larger at 6.37%, which can be explained by any acid remaining in the reaction system facilitating isomerization as samples sit in the autosampler queue. Variation in Z isomer peak area was 1.56%. Because E isomer peak area is significantly smaller than the Z isomer, the total peak area

variation was not significantly affected. Variation of total hydrazone peak area was 1.59%, indicating overall stability of the *t,t*-2,4-decadienal hydrazone.

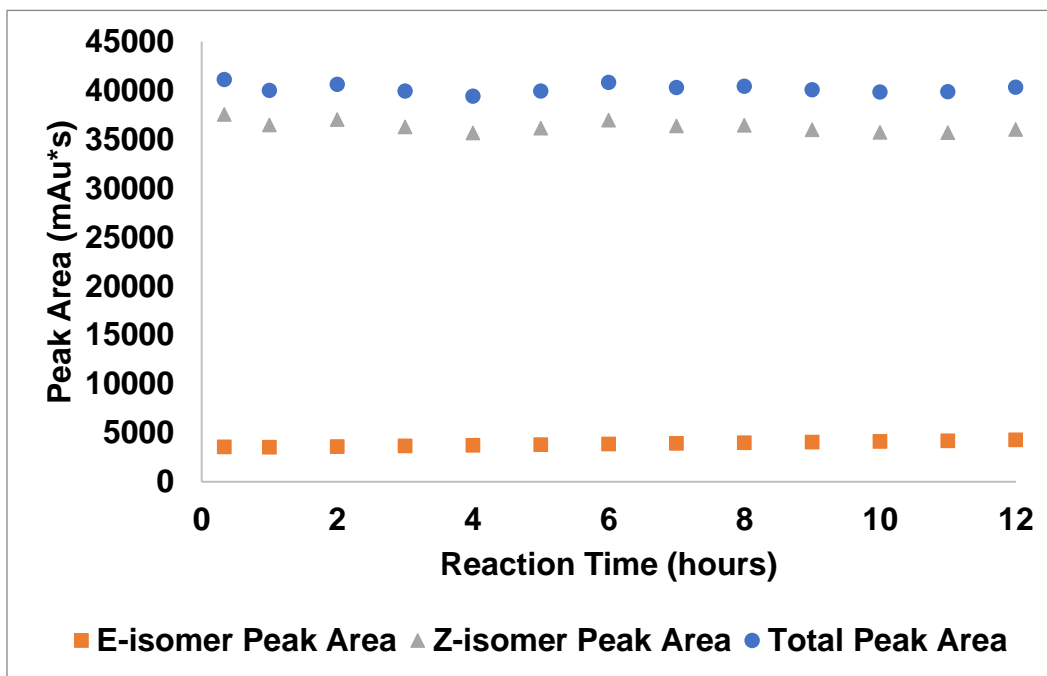


Figure 16. Stability of *t,t*-2,4-decadienal hydrazone over 12 hours, monitored at 360 nm.

#### 6.4 Reaction Stoichiometry of 2,4-DNPH:Carbonyl:H<sup>+</sup>

Many previous studies using the 2,4-DNPH reaction have noted that large excesses of 2,4-DNPH were required for full reaction, at least partially due to the large proportion of 2,4-DNPH protonated in high acid. Since oxidized lipids rarely have known carbonyl concentrations when analyzed, it is important to re-evaluate this stoichiometric requirement in the context of lower acid levels to ensure that sufficient DNPH is provided in analyses of lipids, especially when highly oxidized.

In the experiments described in this study, ratios of reacting 2,4-DNPH (120 mM) and aldehyde (48 mM) were 2.5:1 respectively, and reactions went to completion (all

aldehyde reacted) with reaction pH ranging 1.22-2.52. When the molar ratio of 2,4-DNPH to aldehyde was reduced to 1.25:1, where theoretically enough DNPH was present to react 1:1 with all the aldehyde (60 mM 2,4-DNPH to 48 mM aldehyde), reactions of *t*-2-nonenal (Figure 17 top) and *t,t*-2,4-nonadienal (Figure 17, bottom) with DNPH were incomplete. When less 2,4-DNPH than aldehyde was present (0.625:1, 30 mM 2,4-DNPH to 48 mM aldehyde), the reaction could not go to completion, and this was indeed observed in increased area in the unreacted aldehyde peak area (Figure 17).

The 2,4-DNPH reaction has always been written as a 1:1 complexation with carbonyls and results of the current study generally support this. A slight excess of DNPH is needed to allow for some potential loss by protonation. Because the reaction is diffusion controlled, some additional excess of DNPH is required to ensure efficient contact with carbonyls at the low carbonyl concentrations being analyzed. Use of DMF that increases of 2,4-DNPH solubility achieves such an excess by concentrating the DNPH in a small reaction volume. With DMF as a solvent and reaction pH above 2.5, a reaction stoichiometry of 2.5:1 (DNPH : carbonyl) is adequate for full reaction. This stoichiometry can be used as a guide when estimating concentrations of DNPH needed for quantification of carbonyls in unknown samples of oxidized lipids, i.e. DNPH concentrations will probably need to be increased as lipid oxidation progresses.

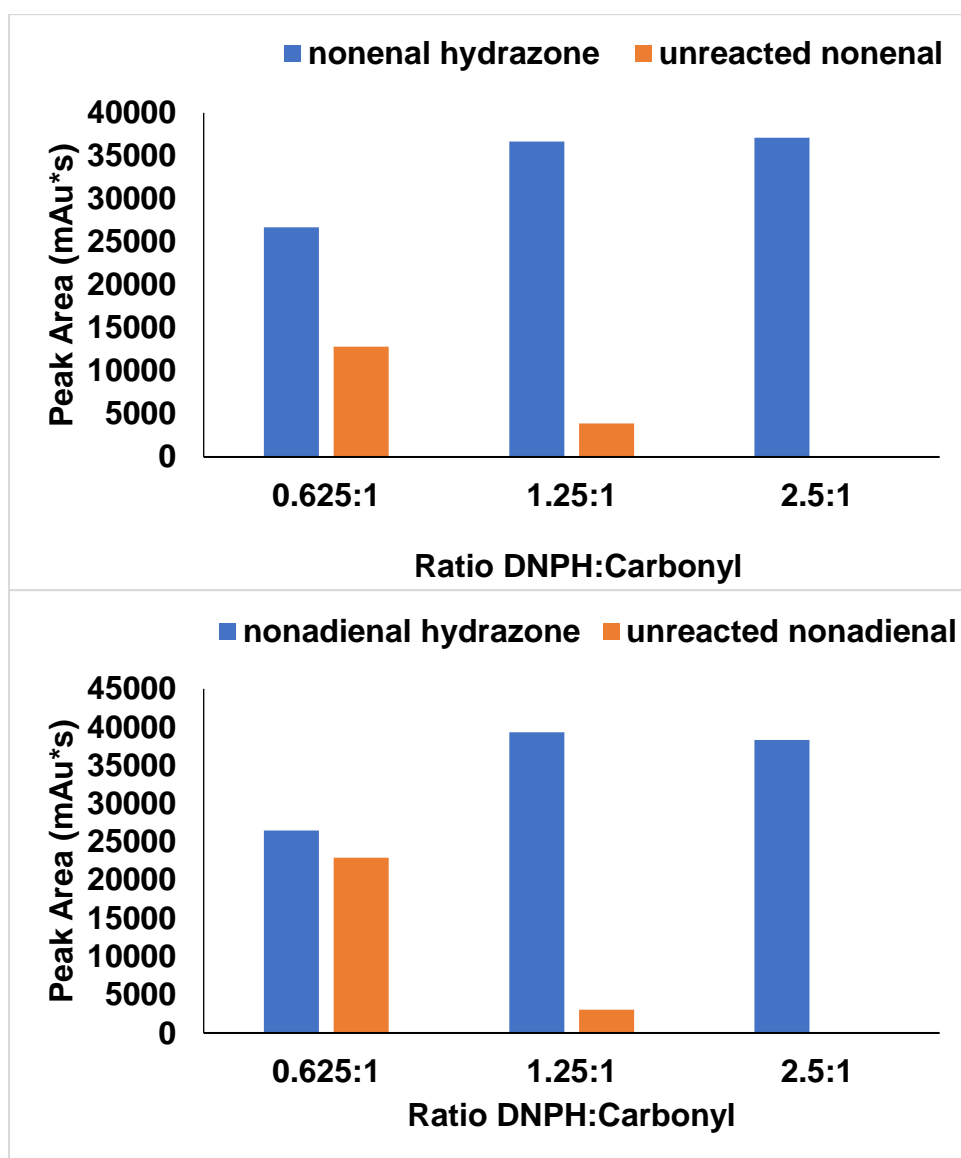


Figure 17. Peak areas of unreacted *t*-2-nonenal and hydrazone (top) and unreacted *t,t*-2,4-nonadienal and hydrazone (bottom) at varying ratios of 2,4-DNPH:Carbonyl. Unreacted carbonyls were measured at 233 and 270 nm respectively. An appropriate molar stoichiometry for complete reaction of unsaturated aldehydes at pH 2.52 is 2.5:1.0:0.0625 (120 mM 2,4-DNPH:48 mM Carbonyl:3 mM H<sup>+</sup>).

### 6.5 Improving Hydrazone Resolution with Gradient Modifications

A main limitation of the separation method developed by Yao [12] is that some pairs of aldehydes, called “critical pairs”, co-elute. For hydrazones, critical pairs are most often a saturated aldehyde and a di-unsaturated aldehyde two carbons longer, e.g. octanal and *t,t*-2,4-nonadienal, as well as nonanal and *t,t*-2,4-decadienal. The separation of a panel of aldehydes under standard separation conditions developed by Yao is shown in Figure 18. Co-eluting critical pairs are marked with an asterisk.

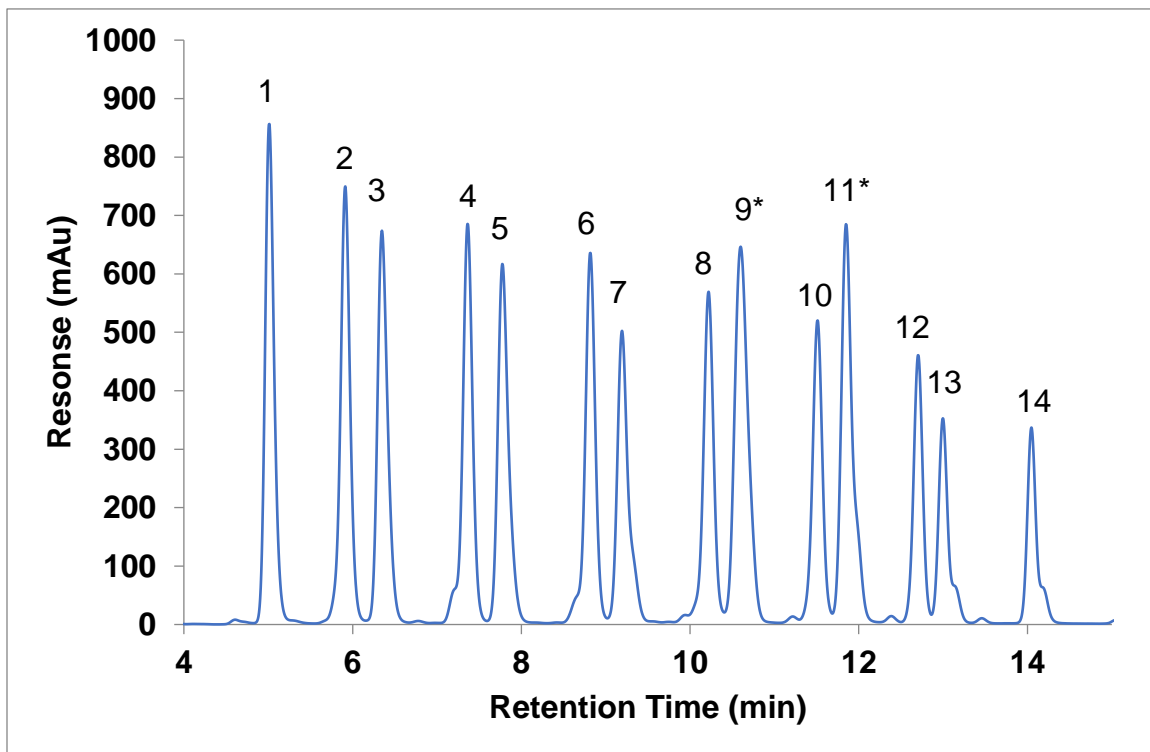


Figure 18. Chromatographic separation of aldehyde mixture using gradient proposed by Yao (2015) with Restek Ultra C18 column (4.6 mm\*150 mm, 5  $\mu$ ). Asterisks indicate critical pairs.



The elution pattern of the mixture of aldehydes in Figure 18 is as follows: butanal (1), *t*-2-pentenal (2), pentanal (3), *t*-2-hexenal (4), hexanal (5), *t*-2-heptenal (6), heptanal (7), *t*-2-octenal (8), octanal and *t,t*-2,4-nonadienal (9), *t*-2-nonenal (10), nonanal and *t,t*-2,4-decadienal (11), *t*-2-decenal (12), decanal (13), undecanal (14).

A UHPLC method using a solvent composition comparable to our base separation reported successful separation of hydrazone critical pairs with a gradient time of approximately one hour [5]. Following this example, standard separation conditions were first modified by extending the gradient time by 10 minutes to see if a longer separation time would improve resolution while keeping a reasonable analysis time. Changes in gradient were first investigated because of limited changes that could be made by interchanging or adding another solvent. Acetonitrile was kept as an eluting solvent due to its high polarity, and isopropanol was kept due to its polarity as well as its ability to solubilize lipids.

The differences between the base conditions and base conditions with increased gradient time are shown below. The gradient time, or amount of time in which 16.7% A increased to 100% A, was extended from 17 minutes to 27 minutes. However, this increased separation time still did not resolve critical pairs of octanal and *t,t*-2,4-nonadienal as well as nonanal and *t,t*-2,4-decadienal (Figure 19). Octanal and *t,t*-2,4-nonadienal continued to co-elute at 13 minutes, while nonanal and *t,t*-2,4-decadienal co-eluted at 15 minutes.

Base Separation Conditions		Base Separation with Increased Gradient	
Time (min)	%A	Time (min)	%A
0.0	16.7	0.0	16.7
17.0	100	27.0	100
23.0	100	32.0	100
27.0	16.7	37.0	16.7

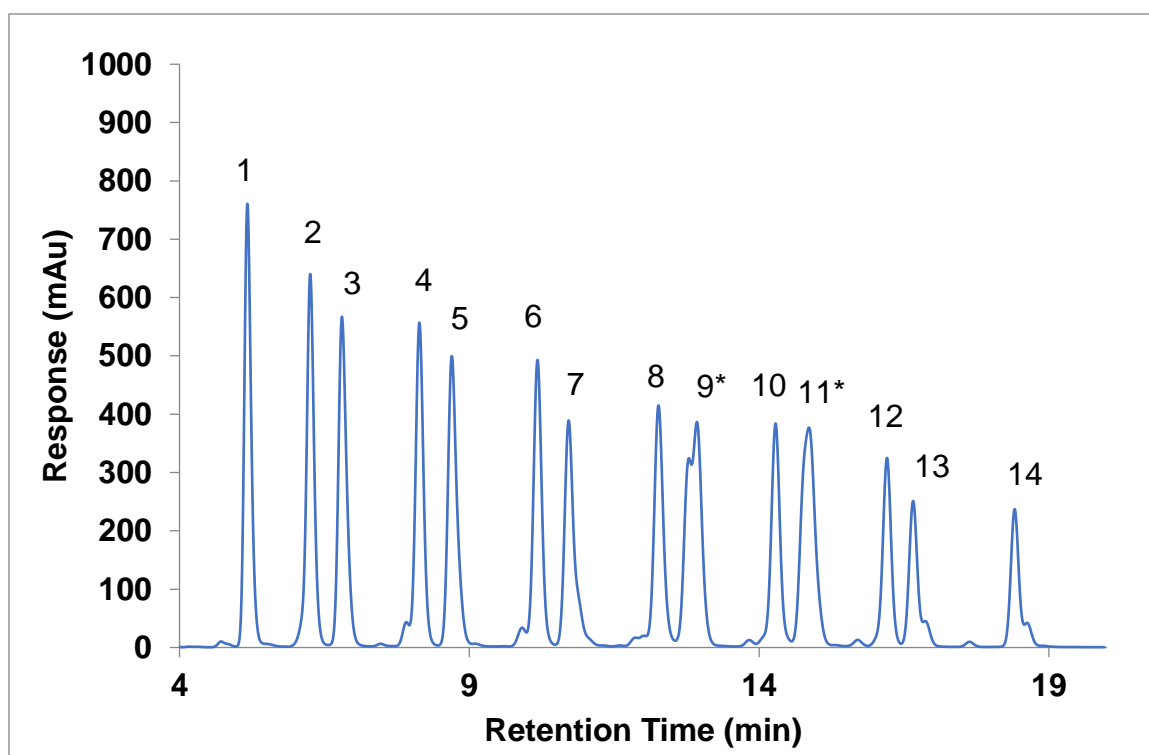


Figure 19. Chromatographic separation of aldehyde mixture using Restek Ultra C18 column (4.6 mm\*150 mm, 5  $\mu$ ). Gradient of 16.7% A to 100% A in 27 minutes; A: 1:1 ACN:IPA and B: 1:1:2 ACN:IPA:H<sub>2</sub>O. Asterisks indicate critical pairs.

The elution pattern of the mixture of aldehydes in Figure 19 is as follows: butanal (1), t-2-pentenal (2), pentanal (3), t-2-hexenal (4), hexanal (5), t-2-heptenal (6), heptanal

(7), t-2-octenal (8), octanal and t,t,-2,4-nonadienal (9), t-2-nonenal (10), nonanal and t,t-2,4-decadienal (11), t-2-decenal (12), decanal (13), undecanal (14).

A solvent and gradient used by Zhu et al. [5] was then investigated, changing methanol to isopropanol (IPA) for our purposes, i.e. 1:1 ACN:IPA (A) and 18 M $\Omega$  water (B). The gradient was 40-80% A in 55 min. The chromatographic separation is shown in Figure 20. Both sets of critical pairs were better resolved, but not with acceptable resolution for accurate quantitation, and the run time was doubled to one hour.

The elution pattern of the mixture of aldehydes in Figure 20 is as follows: butanal (1), t-2-pentenal (2), pentanal (3), t-2-hexenal (4), hexanal (5), t-2-heptenal (6), heptanal (7), t-2-octenal (8), t,t,-2,4-nonadienal (9), octanal (10), t-2-nonenal (11), t,t-2,4-decadienal (12), nonanal (13), t-2-decenal (14), decanal (15), undecanal (16).

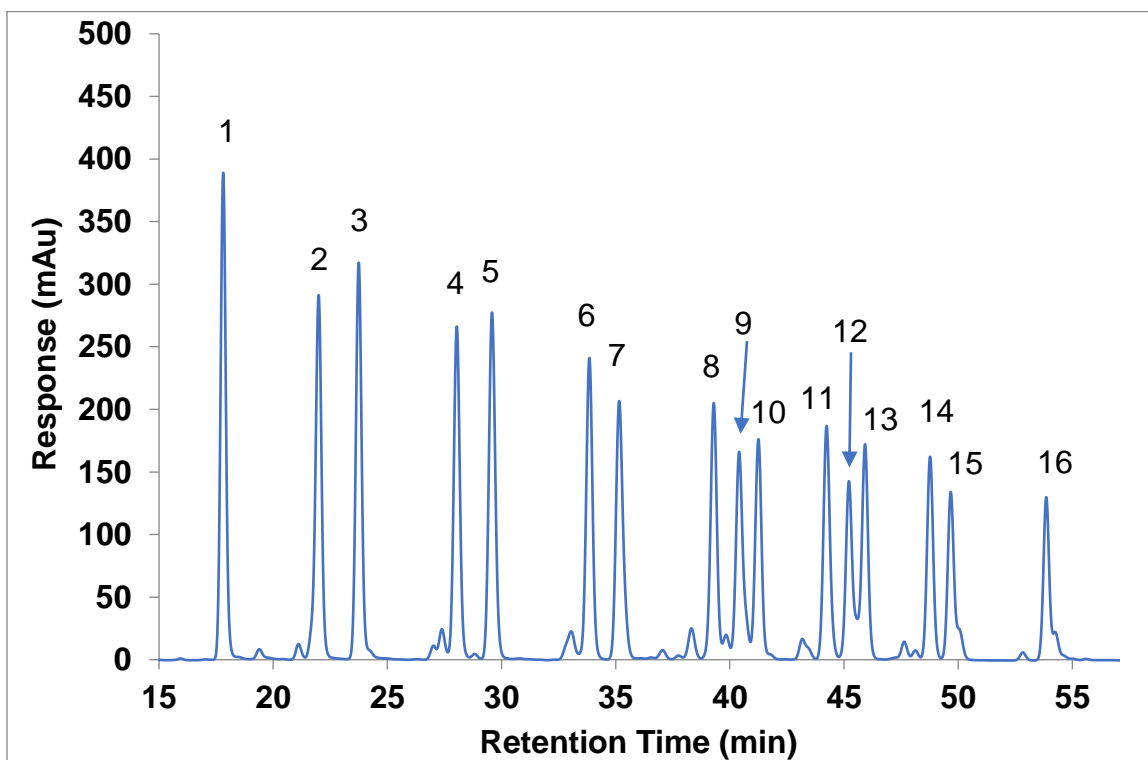


Figure 20. Chromatographic separation of aldehyde mixture using Restek Ultra C18 column (4.6 mm\*150 mm, 5  $\mu$ ) with 1:1 ACN:IPA (A) and 18 M $\Omega$  water (B), 40-80% A in 55 min.

Although changing the solvent composition and extending the gradient time to 50 minutes partially separated the two sets of critical pairs, the resulting elution required too much time for a single assay, considering that many samples must typically be analyzed in quality control labs and in shelf life studies in research. Accurate quantitation of all potential carbonyl oxidation products can only occur if all are detected in a short time. To achieve this, alternate approaches for resolving critical pairs must be considered such as alternate columns or UHPLC systems.

## 6.6 Alternate HPLC Columns

Taking a different approach to resolve critical pairs of hydrazones, several different columns were tested to see if they could improve the resolution of critical pairs. Two promising columns -- Kinetex pentafluorophenyl (4.6 mm\*100 mm, 2.6  $\mu$ ) and Restek Ultra C18 (2.1 mm\*150 mm, 5  $\mu$ ) -- were tested for ability to separate the critical pair of octanal and *t,t*-2,4-nonandienal hydrazones as model compounds. The Kinetex column was selected with the expectation that the addition of a polar modified and functionally bonded group to the C18 stationary phase would retain unsaturated aldehydes more strongly, and the smaller particles size would improve resolution. The Ultra C18 column with a smaller internal diameter was selected with the expectation that the reduction in i.d. would increase sensitivity and improve resolution.

Unfortunately, neither of the alternate columns could separate the critical pair. The Polar C18 column did not resolve this critical pair whatsoever (Figure 21). The Ultra C18 resolved the pair minimally (Figure 22), and this result did not separate as well as with the same column with a 4.6 mm internal diameter used in these studies. A Restek Ultra Aqueous C18 column were also tested and found to be ineffective (data not shown).

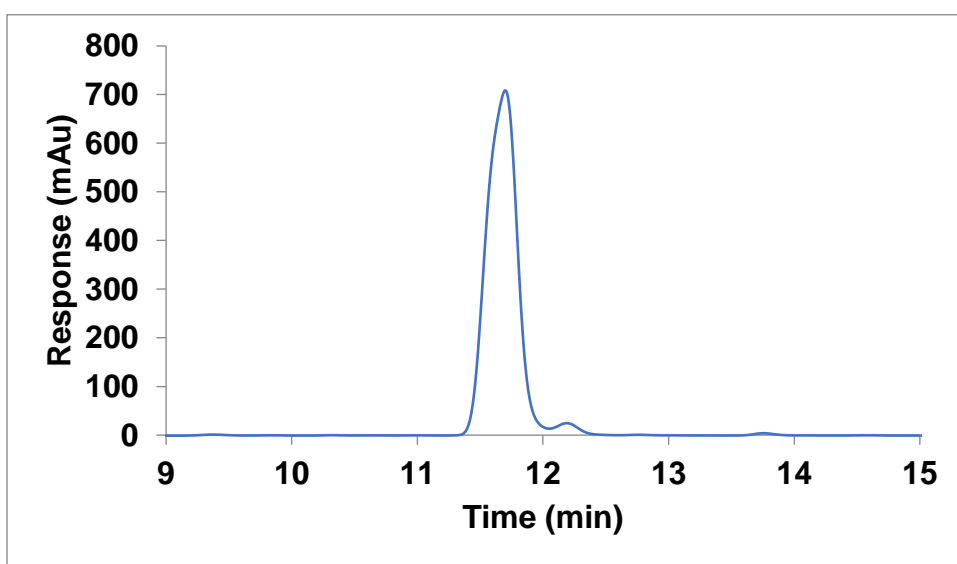


Figure 21. Chromatographic separation of octanal and *t,t*-2,4-nondienal hydrazones with Phenomenex Kinetex Polar C18 column (4.6 mm\*100 mm, 2.6  $\mu$ ).

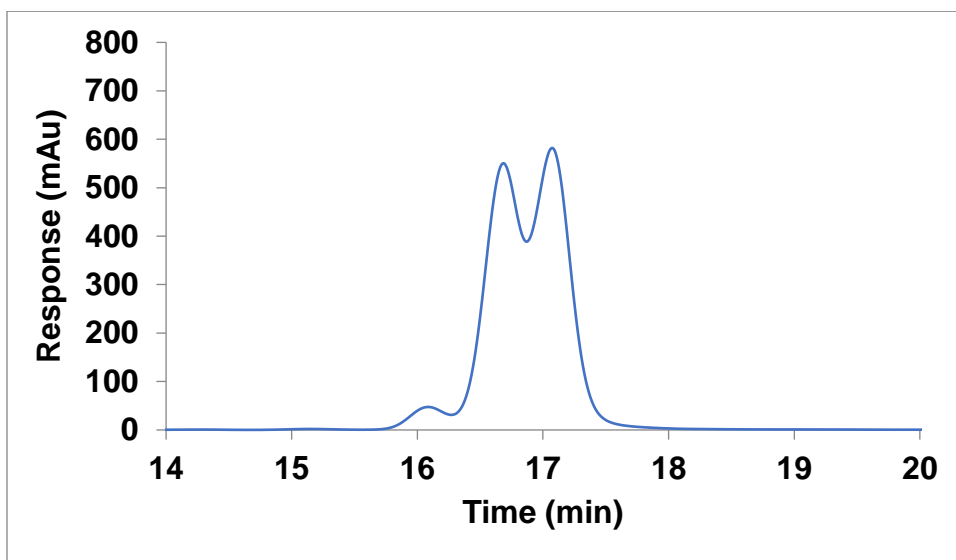


Figure 22. Chromatographic separation of octanal and *t,t*-2,4-nondienal hydrazones with Restek Ultra C18 column (2.1 mm\*150 mm, 5  $\mu$ ).

### 6.7 Replacing Hydrochloric Acid with Formic Acid

Formic acid was tested as a replacement acid that could minimize background interferences with mass spectroscopy detection. The reactivities of 2 mg of nonanal, *t*-2-nonenal, and *t,t*-2,4-nonadienal with 2,4-DNPH were comparable using 21 M (98%) formic acid or 0.5 M hydrochloric acid (equivalent  $[H^+]$ ) in preparation of the acidified DNPH reagent. Comparable hydrazone products were formed for all three aldehydes with either acid. Slightly higher hydrazone levels were formed for nonanal and *t,t*-2,4-nonadienal when HCl was the acid (Figure 23), but these differences are not statistically significant at  $p < 0.05$ .

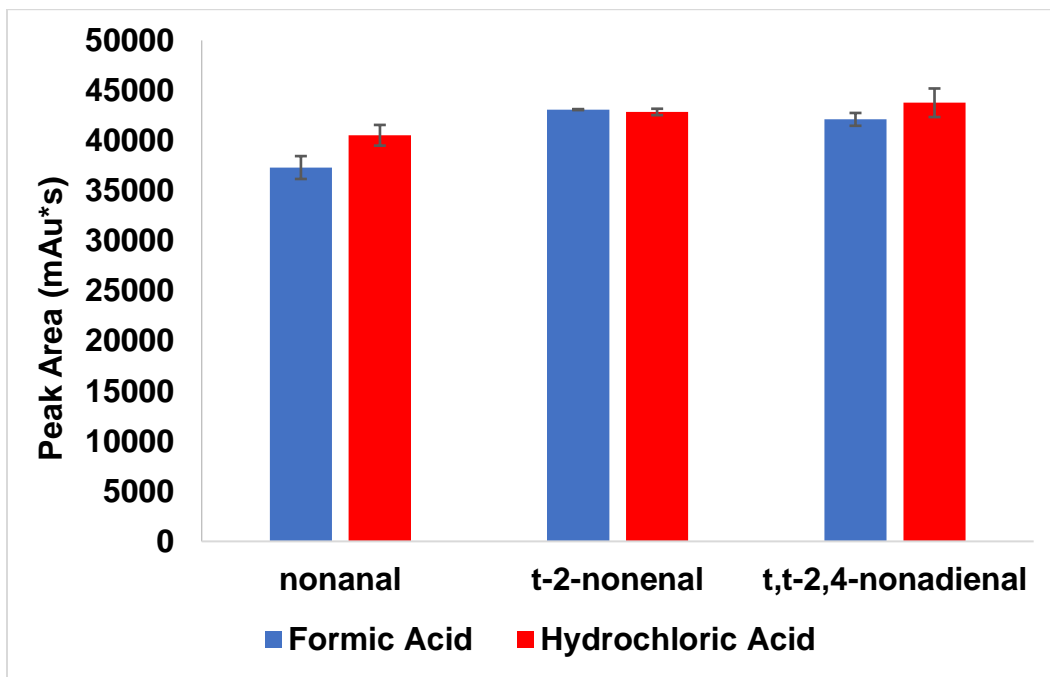


Figure 23. Effect of hydrochloric vs formic acid on hydrazone formation by nonanal, *t*-2-nonenal, and *t,t*-2,5-nonadienal.

Hydrochloric acid chemically is a better choice in reaction because it is a strong acid and  $\text{H}^+$  ions are completely dissociated and available for reaction. Hence, lower concentrations are needed and there is less anion for potential interference in any aspect of reaction or analysis. In contrast, formic acid is a weak acid with only partial dissociation of  $\text{H}^+$  ions so high concentrations (21 M) are needed to provide the same  $[\text{H}^+]$ . However, if an alternate acid is required to improve mass spectra or to eliminate  $\text{Cl}^-$  ions, formic acid appears to be an acceptable substitute based on comparable product formation.

## 6.8 Potential Catalytic Activity of DABA

Organic synthesis literature has suggested that 3,5-diaminobenzoic acid (DABA) can catalyze the formation of hydrazones without low pH [86]. To assess this possibility, 30 mg of DABA was added to DNPH reagent prepared without acid and reacted with *t,t*-2,4-nonadienal. This resulted in reaction concentrations of 120 mM 2,4-DNPH, 90 mM 3,5-DABA, and 48 mM aldehyde. No detectable product was formed under these conditions.

Since the reaction without acid did not occur, a low level of acid was added to see if 3,5-DABA could at least reduce the acid required for reaction. Accordingly, 30 mg of 3,5-DABA was added to 2,4-DNPH reagent prepared with 0.1 M HCl. After one hour, the reaction had still not gone to completion, as evidenced by the unreacted *t,t*-2,4-nonadienal peak measured at 270 nm just after 4 minutes (Figure 24). Adding 3,5-DABA to the reaction system did not show any catalytic effect compared to reaction at 3.22 using 2,4-DNPH reagent acidified with 0.1 M HCl.



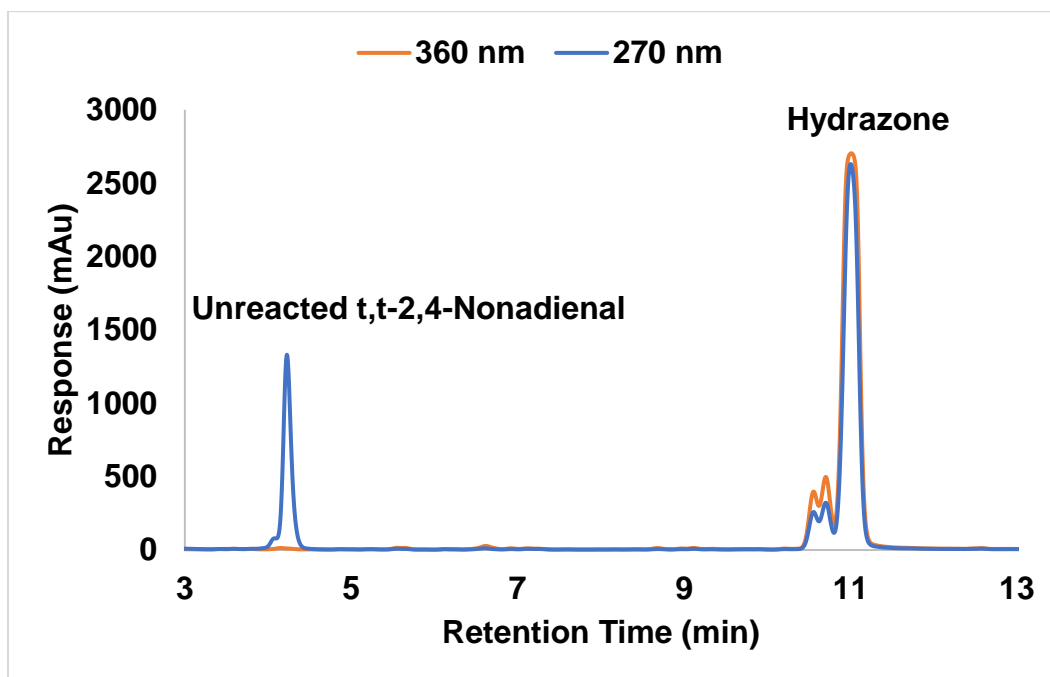


Figure 24. Chromatograms of *t,t*-2,4-nonadienal hydrazones formed after 1 hour with acidified 2,4-DNPH reagent containing 3,5-DABA as a proton source.

## 6.9 Testing Revised Reaction Conditions with Full Aldehyde Panel

### 6.9.1 Effect of carbonyl structure on reactivity and reproducibility

Reaction acidity shown to maximize reaction with model aldehydes was tested on a panel of saturated and unsaturated aldehydes ranging in chain length (C4-C11) to ensure that all aldehydes reacted to completion and that the peak areas of hydrazone response were reproducible with minimal standard deviation. All aldehydes were prepared in triplicate and reacted to completion at pH 2.52 with a molar ratio of 2.5:1 DNPH : aldehyde (120 mM 2,4-DNPH, 48 mM aldehyde). Under these reaction conditions, DNPH-hydrazone reactions are highly reproducible, as shown in relative

standard deviations (coefficients of variation) of less than 2% for hydrazone peak areas for all aldehydes (Table 3).

Table 3. Reproducibility of DNPH reaction with standard aldehydes. Relative standard deviation of peak area for corresponding hydrazones, N=3. Reaction at pH 2.52, 2.5:1 (120 mM:48 mM) 2,4-DNPH:Carbonyl.

Aldehyde	Relative Standard Deviation (%)
butanal	0.31
pentanal	0.38
hexanal	0.72
heptanal	1.09
octanal	0.97
nonanal	0.20
decanal	0.49
undecanal	1.35
t-2-pentenal	0.80
t-2-hexenal	1.56
t-2-heptenal	1.60
t-2-octenal	0.72
t-2-nonenal	0.68
t-2-decenal	1.06
<i>t,t</i> -2,4-nonadienal	1.96
<i>t,t</i> -2,4-decadienal	0.26

Linearity of response of the panel of aldehyde-DNPH hydrazones synthesized was determined from concentration curves developed from a series of concentrations (0.5, 1, 2, 10, 20, 50, 100, 200, 400 microgram/mL). Differences in reactivity with chain length and degree of unsaturation have been documented, but reduced acidity conditions of this

study should give more complete reaction of all carbonyls and more accurate quantitation of hydrazone products. Even so, because each hydrazone has a different optical extinction coefficient, it was expected that the calibration curves could still vary for each aldehyde as was shown by Yao [14]. Surprisingly, results here were different.

Curves still varied with aldehyde, but the range of slopes from calibration curves was much narrower and exhibited greater absolute values (Figure 25). The range of slopes determined from the normalized calibration curves reported here is 3749-5318 (Table 4), while Yao (2015) reported 2594-4783. Additionally, slopes of concentration response curves did not decrease with carbonyl chain length (Figure 26). Although slopes were somewhat lower for saturated aldehydes, most remarkable was the relatively narrow constant range of slopes over all chain lengths for each degree of unsaturation.

These differences in calibration curve slopes reflect increased accuracy and completion of reaction. Calibration curves are based on hydrazones synthesis. Therefore, improving reaction conditions to increase product formation clearly impacted detection response. Reducing the reaction acidity (raising the pH) generated a greater concentration of more stable hydrazones and enhanced reactions of longer chain and more unsaturated aldehydes. The result was a reaction that more equally and accurately detected all aldehydes in short reaction and analysis times.

Table 4. Calibration curves of concentration (mM) response from reaction of monomer carbonyl standards with 2,4-DNPH; separation by HPLC with diode array detection.

Carbonyl	Equation (mM)	R <sup>2</sup>
Butanal	$y = 4343.1x + 30.421$	0.9999

Pentanal	$y = 3736x + 13.223$	1
t-2-Pentenal	$y = 4491.8x + 24.735$	0.9999
Hexanal	$y = 4053.6x + 3.9329$	1
t-2-Hexenal	$y = 4531.7x + 16.528$	0.9997
Heptanal	$y = 4377.1x + 19.72$	0.9999
t-2-Heptenal	$y = 5318.5x + 56.04$	0.9991
Octanal	$y = 3749.6x + 0.1963$	1
t-2-Octenal	$y = 4751.8x + 56.967$	0.9997
Nonanal	$y = 3813.1x + 16.479$	1
t-2-Nonenal	$y = 4672.9x + 27.362$	1
<i>t,t</i> ,-2,4-Nonadienal	$y = 4403.8x + 26.13$	0.9999
Decanal	$y = 4043.3x + 3.7996$	1
t-2-Decenal	$y = 5095.6x + 6.3134$	1
<i>t,t</i> ,-2,4-Decadienal	$y = 4803.1x + 21.088$	0.9999

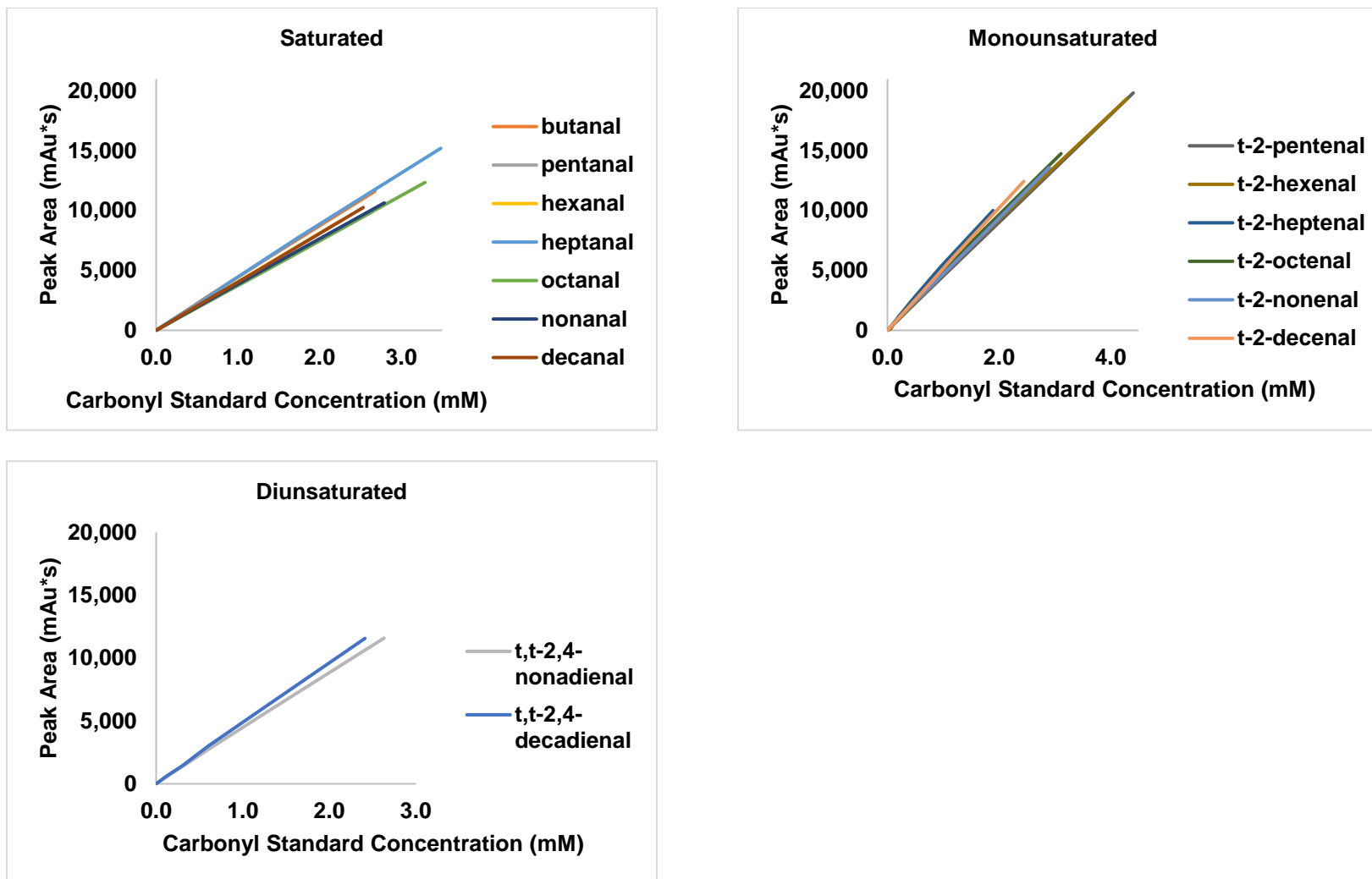


Figure 25. Calibration curves of carbonyl-DNPH standards.

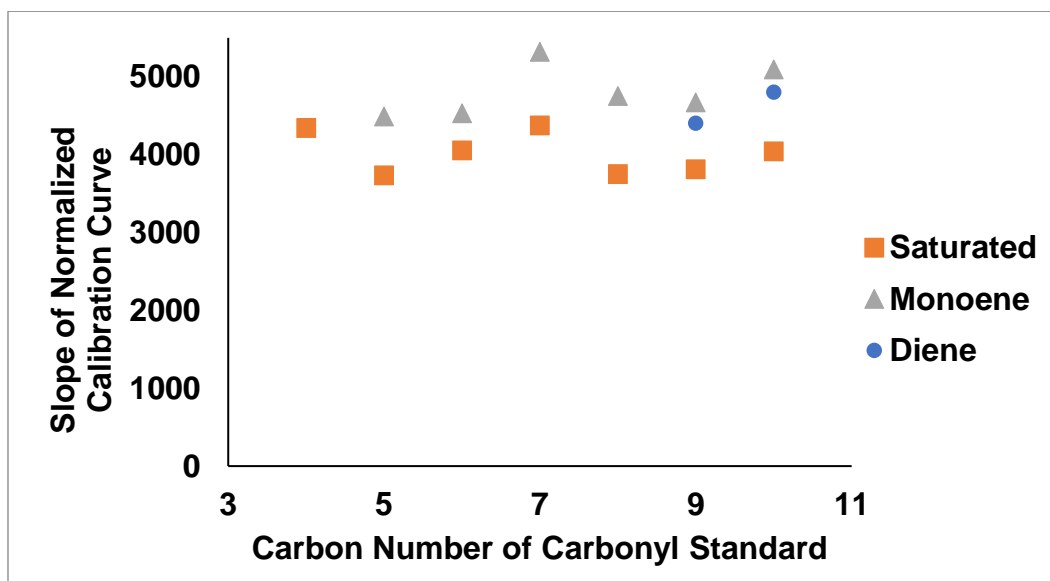


Figure 26. Relationship between carbon number of carbonyl standard and slope of hydrazone curve. The range of the y-axis is 2500 units. Variation of slopes plotted is 10%.

### 6.9.2 Limits of Detection and Quantification

Limits of detection were determined by visual evaluation of chromatograms and were the lowest concentrations of carbonyl standards that were reproducibly quantitated with an RSD < 2.5 %. In this study the limit of detection and limit of quantitation were equivalent. The LOD and LOQ values for each aldehyde standard and the relative standard deviation are shown in Table 5, with values in the  $\mu\text{g/L}$  concentration range and RSD values less than 2.5%. The RSD values are particularly good considering an analysis time of 13.5 hours per aldehyde to measure the response of all nine concentrations levels in triplicate.

Table 5. Limits of detection (LOD), and limits of quantification (LOQ) for reaction of monomer carbonyl standards with 2,4-DNPH followed by separation of product hydrazones by HPLC with diode array detection.

Carbonyl	LOD/LOQ (µg/L)	RSD (%)
Butanal	24.34	0.75
Pentanal	19.76	0.56
t-2-Pentenal	19.77	1.18
Hexanal	9.83	0.51
t-2-Hexenal	40.83	2.26
Heptanal	24.93	1.44
t-2-Heptenal	28.56	1.74
Octanal	21.20	1.18
t-2-Octenal	6.127	1.19
Nonanal	35.95	2.05
t-2-Nonenal	11.59	0.65
<i>t,t</i> -2,4-Nonadienal	35.70	1.83
Decanal	27.47	0.83
t-2-Decenal	24.29	1.57
<i>t,t</i> -2,4-Decadienal	24.22	1.67

## 7. SUMMARY AND CONCLUSIONS

This project addressed factors limiting the use of the 2,4-DNPH reaction to quantify soluble carbonyl, the first of which was reaction pH. The reaction acidity was optimized to the lowest level possible while still ensuring the complete reaction of unsaturated carbonyls. Preparing the acidified 2,4-DNPH reagent with 0.5 M HCl resulted in a reaction acid concentration of 3 mM and pH of 2.52. This acid level was shown to be appropriate for a panel of aldehydes varying in chain length (C1-C11) and saturation, i.e. it supported carbonyl complexation while limiting side reactions of DNPH protonation and carbonyl condensation that detract from carbonyl quantitation. At pH 2.52, all aldehydes represented in the panel reacted to completion, and the reaction, separation, and detection was reproducible under these conditions.

A second limitation was variable reactivity of carbonyls with different chain lengths and saturation/unsaturation. Saturated carbonyls formed hydrazones instantaneously and completely in strong acid and within 5 minutes with decreased acid concentration, but unsaturated carbonyls formed hydrazones more slowly over. Nevertheless, in samples with mixed saturated and unsaturated carbonyls, all aldehydes reacted to completion at pH 2.52 in 20 minutes with a molar ratio of 2.5:1 2,4-DNPH:Carbonyl.

3,5-DABA was ineffective as a possible substitute H donor. Alone it provided no catalytic effect, and when added to 2,4-DNPH reagent acidified with 0.01 M HCl (pH 3.22) *t,t*-2,4-nonadienal still did not react to completion. It is possible that the aldehyde would react to completion after a longer reaction time, but such conditions would not meet the practical requirements of this assay.



A third limitation in quantitating DNPH-lipid carbonyl hydrazones by HPLC was incomplete separation of some critical pairs of aldehydes. A pentafluorophenyl (PFP) column was unable to separate critical pairs even partially. This was worse performance than obtainable on the standard RP-C18 column where changes in solvent composition and increased gradient time did partially resolve critical pairs. However, increasing gradient time to 1 hour for separation of monomer hydrazones was unacceptable for practical analyses. If paired with the conditions for separating core aldehydes, the RP-C18 separation would take a total of 1 hour and fifteen minutes. Coupled with the reaction time, reagent preparation, and sample preparation, this would be an extensive amount of time and solvent to dedicate to one assay on most HPLC systems. Thus, complete resolution of critical pairs for quantitation remains a challenge to be solved.

Limits of detection and quantification were determined for hydrazones produced from the reaction of carbonyls and 2,4-DNPH, separated by HPLC and detected by DAD. Consistent with previous research, detection responses varied for the aldehyde, ranging from 6 to 41  $\mu\text{g/L}$  with no particular relationship between structure and response. Absolute values of detection responses were higher than previously reported, most likely due to optimization of reaction conditions, particularly pH.

Although not yet perfect, the reaction and separation conditions of pH 2.52 with a molar ratio of 2.5:1 2,4-DNPH:Carbonyl offer a useful practical method for quantitating monomer lipid carbonyl products.

## 8. FUTURE WORK

This project has clarified several aspects of the DNPH reaction with lipid aldehydes and separation of the resulting hydrazones, but there are still some areas worth investigating to further refine the process and improve quantitation of carbonyls:

1. The greatest improvement to this assay for academic research purposes would be to continue development with UHPLC. This is the only separation method that is reported capable of separating critical pairs of hydrazones on a C18 column under relatively similar conditions to the current method.
2. An alternative detection method that may improve quantitation is using a Corona Discharge Detector. If the detection limit is appropriate, the detection method may avoid the issue of significant variation in detection response between hydrazones.
3. An interesting adaptation to the current assay would be reductive amination of hydrazones to alkylated amines and separation on a RP-Amide column. Adaption of this method would require investigation that there are no interfering reactions with carbonyls on core aldehydes. Additionally, it is currently unclear if a RP-Amide column would be able to resolve alkylated amines of saturated and unsaturated carbonyls.
4. Gaining access to a mass spectrometer in our laboratory would be a huge improvement to identify compounds produced that are not detected or are too low in concentration to be detected by DAD. It is also essential to the confirmation and quantitation of carbonyls measured in an oxidizing lipid or food.

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