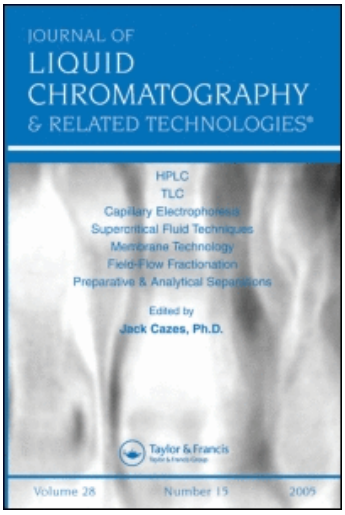

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Trace and Ultratrace Analysis by HPLC

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TRACE AND ULTRATRACE ANALYSIS BY HPLC

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Analyses performed at parts per million (ppm) or microgram (μg) levels are generally defined as trace analyses - an analytical landmark that was attained approximately 30 years ago. Ultratrace (ultra - beyond what is ordinary) analyses are defined as analyses performed below ppm or submicrogram levels.

A more rigorous definition was used earlier by the author for a limited review of those analyses performed at picogram or lower levels (1). The objective of the review was to determine if picogram analyses are viable or necessary. For example, it is necessary to perform low-level analyses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), as it can cause abortion in monkeys even at 200 parts per trillion (ppt) levels (2). Allowing for a one hundred fold margin of safety for human exposure, it can be calculated that the safe food level of TCDD would have to be less than 2 ppt. Another example highlights the effect of 0.43 parts

per billion (ppb) of polychlorinated biphenyls (PCB) in weakening the backbones of trout by interfering in collagen synthesis (3). The analysis of backbones of these fish revealed excess calcium levels and collagen and phosphorus deficiency. Since the fish were also deficient in vitamin C, a cofactor in collagen synthesis, this led to the conclusion that the trout used vitamin C for detoxification of PCB in lieu of using it for skeletal development.

The examples described above show innovative analytical research can lead to a better understanding and solution of some of the complex scientific problems. Furthermore, ultratrace analyses can assist in ensuring the safety and efficacy of drugs, cosmetics, environment, food, and water (4).

In the scientific literature, detectabilities are frequently expressed in a variety of units. For example, Johnson et al (5) have published separation in femtomoles of Dns derivatives of amino acids on a bonded phase -NH_2 column. A review of the data reveals that MAD of the amino acids varies from 2.0-5.8 ng. It is recommended that the gram be used as the unit for reporting in ultratrace analyses (Table 1), as it permits an immediate comparison of the detectabilities of the methods. A comparative view of the analytical quantities in terms of g, %, and ppm is provided in Table 1.

TABLE 1

Preferred And Commonly Used Analytical Units (4)

Preferred[a] Analytical Units		Commonly Used Units When Present/g	
g	Common Name	%	One Part per -
1×10^{-6}	Microgram (μg)	0.0001	Million
1×10^{-9}	Nanogram (μg)	0.0000001	Billion
1×10^{-12}	Picogram (pg)	0.0000000001	Trillion
1×10^{-15}	Femtogram (fg)	0.0000000000001	Quadrillion

[a]By the author

Furthermore, it is recommended that the following important analytical parameters be reported for each method:

Amount present in original sample/mL or g (APIOS).

Minimum amount detected in g (MAD).

Minimum amount quantitated in g (MAQ).

In addition, data such as accuracy, precision, linearity, and specificity of the methodology should be provided.

SAMPLING AND SAMPLE PREPARATION

The sample used for trace or ultratrace analysis should be representative of the "bulk material". The major considerations are (6):

1. Determination of the population or the "whole" from which the sample is to be drawn.
2. Procurement of a valid gross sample.
3. Reduction of the gross sample to a suitable sample for analysis.

It is desirable to reduce the analytical uncertainty to a third or less of sampling uncertainty (7). Poor analytical results can also be obtained because of reagent contamination, operator errors in procedure or data handling, biased methods, and so on. These errors can be controlled by proper use of blanks, standards, and reference samples.

Frequently preconcentration of the analyte may be necessary because the detector used for quantitation may not have the necessary detectability, selectivity, or freedom from matrix interferences (8). Significant losses can occur during this step because of very small volume losses to glass walls of recovery flask or disposable glass pipets and other glassware.

METHOD VALIDATION AND INTERLABORATORY VARIATIONS

Statistically designed studies should be performed to determine accuracy, precision, and selectivity of the methodology used for trace or ultratrace analyses. An example of such a study is the gas chromatography/mass spectrometry (GC/MS) quantitation of 2,3,7,8-tetrachlorobenzodioxin (9). The limit of quantitation is determined to be approximately 9 ppt. The calculated regression line for a standard solution gives a slope of 0.981 and any point on the line is 1-2 ppt over the range of values tested. The calculated regression line for beef fat analyses of 17 sample (aberrant value excluded) gave a slope of 0.89. The bias is a function of spiking level ranging from approximately +2 ppt for samples spiked at 9 ppt to -6 ppt for those spiked at 81 ppt.

Reliability of ultratrace data requires that the data withstand interlaboratory comparisons. Frequently, meaningful intercomparisons are difficult because of the nature of uniformity of sample, the ease of contamination during sampling and analyses, and the variety of limitation of analytical practices employed.

CHROMATOGRAPHIC METHODS

Techniques such as chromatography are frequently used to obtain separation of impurities from each other and the main compound (Table 2). Separations are based on properties such as adsorption, partition, ion-exchange, or molecular size. Frequently, because of its simplicity, thin layer chromatography is the technique of choice. It generally entails selection of a suitable solvent system for resolution of impurities on a silica gel plate. If the nature of impurities is not known, systems that are acidic, neutral, and basic are investigated. Universal detectors (UV or spray reagents) are preferred.

The volatile impurities can be resolved easily by means of gas chromatography (GC). If a compound is volatile it can be separated by GC from the other compounds, on the basis of its boiling point and/or polarity. Alternatively, derivatization can be used to obtain desired volatility or polarity. The combination of GC with selected ion monitoring provides a powerful tool for monitoring and characterizing impurities (4).

Nonvolatile or thermally labile compounds are best separated by high pressure liquid chromatography (HPLC). Although techniques such as adsorption and ion-exchange chromatography have been used occasionally, the technique of choice is reversed-phase liquid chromatography (RPLC). In RPLC, the stationary phase is nonpolar and the mobile phase with varying polarities is used for elution. This permits determination of a wide variety of compounds. Even ionic compounds can be handled by techniques such as ion suppression or ion pairing. A variety of detectors such as UV, fluorescence, electrochemical, or mass spectrometry are used. This paper deals only with select applications in HPLC.

HPLC is a very viable separation technique that is used in virtually every field of chemistry. This technique provides an excellent means of analysis and discovery of new compounds since compounds present even at ultratrace levels can be resolved from related compounds. One approach entails separation of potential compounds resulting as by-products, which cannot be resolved at normal inefficient crystallization techniques. Many of the by-products frequently have physicochemical properties and carbon skeleton similar to the parent compound with substituent(s) differing in position or functionality. Since it is not possible to theorize all by-products, some unusual compounds can be found and characterized with this approach. A more selective approach is based on

TABLE 2

Separation of Impurities by Chromatography (4)

Impurities	Parent Compound	Methodology	Reference Group
1. Isomers	Prostaglandin A_2	LC($AgClO_4$)	Prostaglandins
	Doxepine	GC	Antidepressants
	Pilocarpine	RPLC	Alkaloids
	Tetracycline	RPLC	Antibiotics
	Budesonide	RPLC	Steroids
	Oxytetracycline	TLC	Antibiotics
	Naproxen	Adsorp.,LC	Analgesics
	Diethylpropion	RPLC	Miscellaneous
2. Intermediates/ by-products	Levodpopa	RPLC	Amino acids
	Levothyroxine	RPLC	Amino acids
	Liothyronine	RPLC	Amino acids
	Indomethacine	TLC	Analgesics
	Meperidine	TLC	Analgesics
	Oxtetracycline	TLC	Antibiotics
	Griseofulvin	LC (Cyano)	Antibiotics
	Chloramphenicol	RPLC	Antibiotics
	Imipramine	TLC	Antidepressants
	Desipramine	TLC	Antidepressants
	Meprobamate	TLC	Antidepressants
	Chlordiazepoxide	Adsorp.,LC	Antidepressants
	Prostaglandin E_2	GC	Prostaglandins
	Fludrocortisone	RPLC	Steroids
	Hydrochlorothiazide	RPLC	Sulfonamides
	Folic Acid	RPLC	Vitamins
	Trihexyphenidyl	TLC	Miscellaneous
	Methotrexate	Column, LC	Miscellaneous
3. Arising from solvents	Amphetamine	GC/MS	Amines
	Trihexyphenidyl	TLC	Miscellaneous
4. Degradation products	Aspirin	RPLC	Analgesics
	Penicillin G	RPLC	Antiobiotics
	Cephalexin	LC (Amino)	Antiobiotics
	Procarbazine	RPLC	Antineoplastics
	Cyclophosphoramide	RPLC	Antineoplastics
	Benzocaine	RPLC	Local anesthetic
	Tetracaine	RPLC	Local anesthetic
	Hydrocortisone	RPLC	Steroids
	Dexamethasone	RPLC	Steroids
	Prednisone	RPLC	Steroids
	Digoxin	RPLC	Steroids
	Chlorpropamide	Adsorp.,LC	Sulfonamides
	Saccharin	RPLC, Ion-exchange	Miscellaneous
	Nitroglycerin	RPLC	Miscellaneous
	Diethylpropin HCl	RPLC	Miscellaneous
5. Contaminants	Ampicillin	GC	Antibiotics
	Cephalosporin	GC	Antibiotics
	Tetracycline	RPTLC/ bioautography	Antibiotics
	Penicillamine		Antibiotics

GC = gas chromatography; LC = liquid chromatogrpahy; MS = mass spectrometry; RP = reverse phase; TLC = thin layer chromatography

changes brought about in a chemical entity to evaluate its stability with reactions such as hydrolysis, oxidation, or photolysis. In this case, several theorized new and old compounds are produced. An innovative chromatographer can resolve and characterize both theorized and untheorized new compounds. Another interesting approach depends upon characterization of various degradation products produced in the matrixes used for pharmaceutical products. The compounds thus produced can be resolved from others by chromatography, and their structure determined by techniques such as elemental analysis, IR, NMR, or mass spectrometry.

Ultratrace analysis is generally performed by liquid/liquid partition chromatography where the reverse-phase separations (stationary phase is non-polar) are most popular. The detection and quantitations have been limited by the current availability of detectors. With a UV detector at 254 nm, the lower limit of detection is 3.5×10^{-11} g/mL for a compound such as phenanthrene. A fluorescence detector can increase the detectability to 8×10^{-12} g/mL. The same order of detectability can be achieved with detectors such as amperometric, electron capture, and photoionization. Further approaches to improvement of detection have been discussed (10).

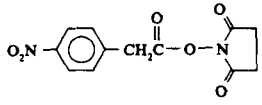
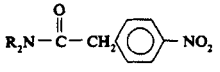
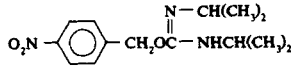
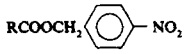
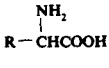
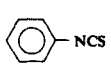
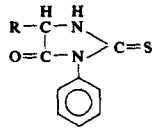
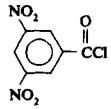
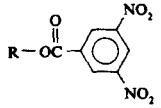
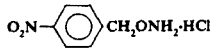
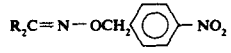
Derivatization HPLC has been used for improving detectability and thus allows better quantitation of many classes of compounds (Tables 3 and 4). Several examples of ultratrace analyses by HPLC can be found in the literature (4). Described below are several cases of such investigations from our laboratory.

A. Purity Evaluations

3,5-Dibenzylozyacetophenone is an important intermediate in the synthesis of a pharmaceutical compound of significant therapeutic value. This material has become commercially available, thus obviating the need

TABLE 3

Common UV-Absorbing Derivatives (4)

Compound	Reagent	Derivative
R_2NH		
RCOOH		
		
ROH		
		

for in-house synthesis. Prior to accepting this material, it was necessary to thoroughly evaluate it purity. The data by differential scanning calorimetry, an absolute purity determination method, was found insufficient for evaluating purity (11).

Similarly, TLC was found to be incapable of determining impurities at low level. An HPLC method was developed based on separation on μBondapak C_{18} column with a methanol:water (4:1) mobile phase followed by detection at 225 nm. With this method, Lab Sample 2 showed no impurities by TLC; however, it was found to contain 3 impurities by HPLC totaling 0.06% (Table 5). HPLC was clearly the method of choice. With this

TABLE 4

Common Fluorescent Derivatives (4)

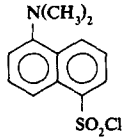
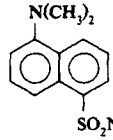
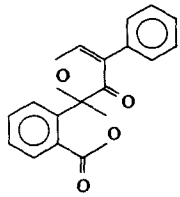
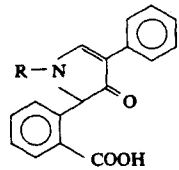
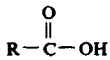
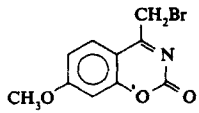
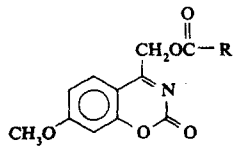
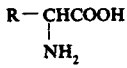
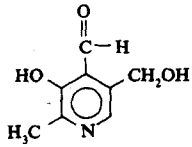
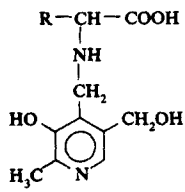
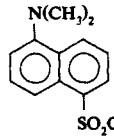
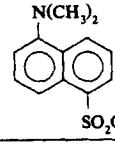
Compound	Reagent	Derivative
RNH_2		
		
		
		
$Ar-OH$		

TABLE 5

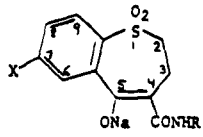
Purity Evaluations on 3,5-Dibenzyloxyacetophenone

Material	DSC (mole %)	TLC (% impurities)	Impurities by HPLC Percent	Impurities by HPLC Number
Lab Sample 1	99.3	1.5	--	--
Lab Sample 2	99.7	0.0	0.06	3
Reference Sample	99.5	--	0.12	5
Alternate Source	99.4	--	0.02	2

method, the lowest impurity content was found in the alternate source sample. MAD was found to be 3 ng. For the impurity present at 0.005% level, MAD was calculated to be 675 pg . The calculation is based on the fact that A(1%,1cm) value for the impurity is the same as 3,5-dibenzyloxyacetophone at 225 nm.

B. Discovery of New Transformation Product in a Complex Matrix

It was necessary to conduct a long-term toxicity study to evaluate the safety of a potential drug with the following structure (12):



Molecular Weight:	>400
Melting point:	224°C (decomp.)
Solubility:	Very slightly soluble in water; almost insoluble in acidic and basic solutions; soluble in methanol
pKa:	5.0

This compound was admixed with rat feed (Purina Laboratory Chow containing a minimum of 23% protein, 4.5% fat and maximum 6% fiber) with the following composition:

Meat and bone meal, dried skimmed milk, wheat germ meal, fish meal, animal liver meal, dried beet pulp, ground extruded corn, ground oat groats, soybean meal, dehydrated alfalfa meal, cane molasses, animal fat preserved with BHA (butylated hydroxyanisole), vitamin B_{12} supplement, brewers' dried yeast, thaimin, niacin, vitamin A supplement, D activated plant sterol, vitamin E supplement, dicalcium phosphate, iodized salt, ferric ammonium citrate, zinc oxide, manganous oxide, cupric oxide, ferric oxide, and cobalt carbonate.

A gas-liquid chromatographic method was first investigated for analysis of this compound. An electron capture detector provided detectability down to 5 pg for the active component; however, investigations revealed that degradation was occurring at the injection port. An HPLC method was then developed to circumvent this problem. The essential details of the method are as follows:

Column:	Bondapak C_{18}
Precolumn:	6 cm x 2.5 mm i.d., C_{18} Corasil
Solvent System:	70 MeOH/33 H_2O/1 HOAC
Flow Rate:	0.8 mL/minute

Sample Preparation

Sample + 5 mL 1N HCl + 20 mL EtOAC

Shake. Centrifuge. Inject 25 µL of EtOAC layer

Analysis of 20 week-old samples (Table 6) revealed that all samples had degraded significantly. The chromatogram showed the presence of another peak. The compound representing that peak was isolated by

TABLE 6
Analyses of 20-Week-Old Rat Feed Samples

Declared Content %	Found % of Label
0.06	69.3
0.02	58.1
0.004	49.4

TABLE 7

Selectivity of HPLC Method

Compound	Retention Time (minutes)
"Ketosulfone"	5.0
RNH_2	6.9
RNCO	10.1
"4-Hydroxy Compound"	13.0
Parent Compound	18.0

preparative HPLC and characterized by field desorption mass spectrometry, NMR, and other spectroscopic techniques to be the oxidation product ("4-Hydroxy compound" - Table 7).

The discovery of the "4-Hydroxy Compound" is primarily attributed to selective ultratrace HPLC methodology. It was possible to theorize the production of this compound by oxidation. Similarly, the formation of the amine (RNH_2) and "ketofulfone" can be attributed to the amide hydrolysis followed by decarboxylation. However, "4-Hydroxy Compound" was not available to check this proposal. Reliability had to be placed in development of a sufficiently selective HPLC method that would resolve ultratrace levels of this compound. After this was accomplished, it could be shown that in this matrix oxidation is highly facile as compared to hydrolysis. Its eventual synthesis led to a compound with potential pharmacological activity.

C. Determinations of Unstable Compounds

The problems discussed earlier for ultratrace analyses are further compounded when one is dealing with compounds such as hydrazobenzene and azobenzene. Discussed below are methods developed in our laboratory which circumvent some of the problems encountered with them (13).

A review of the literature revealed that a normal phase HPLC method has been reported for the analysis of hydrazobenzene and azobenzene (14). The method entails extraction of these compounds into n-hexane from 1N NaOH followed by analysis on Partisil-10 PAC column with a mobile phase containing 2.5% absolute ethanol. The published method suffers from the following shortcomings:

a. Hydrazobenzene and azobenzene show significant instability in 1N NaOH (Table 8).

b. Azobenzene can isomerize into cis- and trans- isomers. Their separation is not demonstrated or accounted for in the method.

c. Parent compound (I) can degrade directly or indirectly into hydrazobenzene and azobenzene (Figure 1).

d. Selectivity of transformation products given in Figure 1 is not demonstrated.

The properties of hydrazobenzene and azobenzene are given in Figure 2. Hydrazobenzene is known to be an unstable compound; it oxidizes easily to azobenzene and other compounds and has $t_{\frac{1}{2}}$ of 15 minutes in wastewater (15). Azobenzene, on the other hand, can isomerize or sublime even at 30°C (16).

To assure that the methodology would be reliable at ~10 ppm, suitable methods were developed for detecting these compounds at levels $\leq$ 1 ppm, i.e. ultratrace levels. To further assure reliability of analyses, an effort was made to meet the following requirements for ultratrace analysis (4).

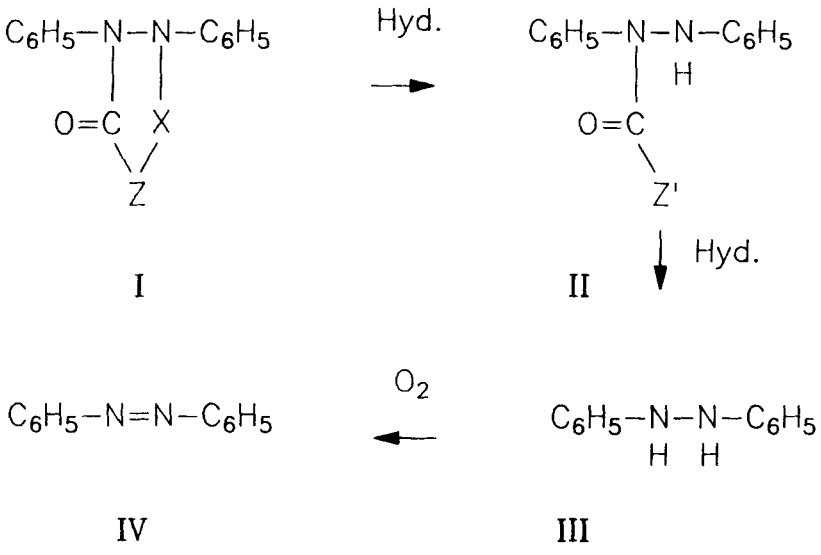


FIGURE 1

Degradation Pathway of Parent Compound

TABLE 8

Stability of Hydrazobenzene and Azobenzene

Medium	Time	% Loss	
		Hydrazobenzene	Azobenzene
0.1N NaOH	30 minutes	82.9%[a]	4.6%[b]
pH 9.2 buffer	30 minutes	0.9%[c]	None found[d]

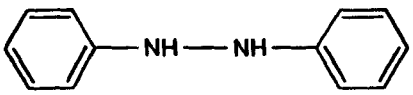
Original concentration in 10% acetonitrile:

[a] 11.8 µg hydrazobenzene/mL

[b] 15.7 µg azobenzene/mL

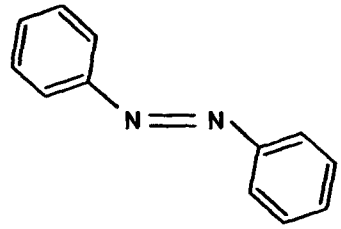
[c] 3.55 µg hydrazobenzene/mL

[d] 2.59 µg azobenzene/mL



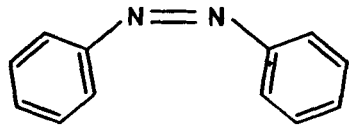
Hydrazobenzene

White crystals
Soluble in ethanol



trans–Azobenzene

Orange–red crystals
Soluble in ethanol, ethyl ether, benzene, acetic acid
Dipole moment 0.0D*



cis–Azobenzene

Orange–red crystals
Soluble in ethanol, ethyl ether, benzene, acetic acid
Dipole moment 3.0D*

Properties: Weast, R. C., *Handbook of Chemistry and Physics*, C.R.C. Press, Inc., New York, p. C -91,-314, 1985.

* Jannsen, J., *J. Chem. Ed.* 46, 117, 1969.

FIGURE 2

Physical Properties of Hydrazobenzene and Azobenzene

a. Sample used for analysis was representative of the whole lot.

b. Methodology incorporated optimum separation and detection techniques.

c. Component of interest was allowed to suffer a minimum loss during various analytical operations.

d. Adequate steps were incorporated in the analytical method to account for losses that might occur due to sample preparation or degradation.

Furthermore, to assist other researchers in evaluating whether these methods could be useful for their investigations, the following analytical parameters were included:

Amount Present in Original Sample (APIOS)

Minimum Amount Detected in g (MAD)

Minimum Amount Quantitated in g (MAQ)

The method entails taking a sample weight anticipated to contain ~10 ppm of hydrazobenzene or azobenzene and shaking it with 30 mL of pH 9.2 THAM buffer. This is followed by extraction with 10 mL of n-hexane. After centrifugation, 5 mL of the n-hexane layer is evaporated to dryness, at room temperature, with nitrogen and the residue is solubilized in 1.0 mL of acetonitrile. Twenty-five microliters are immediately injected into HPLC equipped with Partisil 10 μ C_8 column (25 cm x 4.6 mm) and a dual channel detector (254 and 313 nm). Elution is carried out with a mobile phase composed of acetonitrile:acetate buffer, pH 4.1 (11:14). Both hydrazobenzene and azobenzene standards are treated similarly.

Investigations revealed that the optimum pH for extraction for both hydrazobenzene and azobenzene is 9.2. At this pH, these compounds can be easily extracted from the parent compound and are also quite stable (Table 8). The cis- and trans- isomers of azobenzene and hydrazobenzene can be resolved well with the reversed-phase HPLC method (Figure 3). Previous investigations had confirmed the selectivity of this method as it resolves compound I (Figure 1) from other transformation products (17). Data on spiked samples are given in Table 9. Average recoveries of 91% and 114% were obtained for hydrazobenzene and azobenzene respectively with relative standard deviation (R.S.D.) of 3-11%. The methods were found useful for quantitating ≦1 μg/g of these compounds with

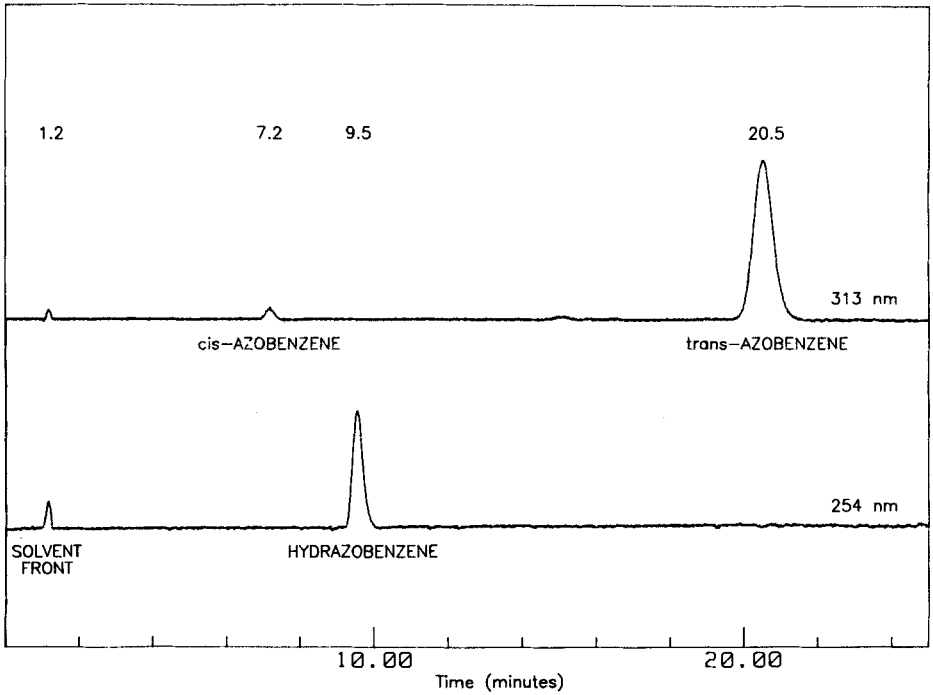


FIGURE 3

Chromatograms of Azobenzene (cis- and trans-) and Hydroazobenzene (0.05 μg of each compound injected and monitored at 313 nm and 254 nm

respect to the parent compound (MAD = 6-7 ng). The high recovery obtained for azobenzene is partly due to conversion of hydrazobenzene (~9%). Further improvements are being investigated.

D. Metal Catalysis at Ultratrace Levels

1,3-Benzenediols substituted with an amino-alkyl moiety are of significant pharmacological interest. Stability investigations on substituted 5-amino-ethyl-1,3-benzendiol sulfate (AEB), under various

TABLE 9

Recovery Data of Spiked Samples

APOIS: ≤10 μg/g of Parent Compound

Sample	Hydrazobenzene Found	Azobenzene Found
Parent Compound	89.0 ± 8.6% (n = 7)	123 ± 2.6% (n = 6)
Capsules	89.6% ± 10.8% (n = 5)	98.7 ± 10.2% (n = 3)
Tablets	95.8 ± 5.4% (n = 3)	121 ± 4.2% (n = 3)
Average	91%	114%
R.S.D.	±5 - 11%	±3 - 10%
MAD (μg)	0.006 (6 ng)	0.007 (7 ng)
MAQ (μg/g)	≤1	≤1

exaggerated conditions, revealed that AEB is susceptible to degradation in aqueous solution in the presence of metals (18). Metal cations such as copper, iron, and calcium have been shown to accelerate degradation of AEB under and oxygen atmosphere, with concomitant discoloration. The effectiveness of these metals in terms of AEB degradation is in the following order (Table 10): $Cu^{+2}>Fe^{+3}>Ca^{+2}$. Copper (Cu^{+2}) effectively catalyzes AEB degradation down to 10 parts per billion (ppb) level in the presence of oxygen.

A significant increase in the rate of AEB degradation occurs when the concentration of Cu^{+2} is increased from 10 ppb to 1,000 ppb (Table 11). The increase in the rate of degradation is less pronounced at higher concentrations such as 1 to 10 ppm on parts per million basis. Kinetic studies have been performed to determine the effect of pH (in the range of interest) and temperature on degradation of AEB by monitoring

TABLE 10

Metal Catalysis of AEB in Oxygen Atmosphere

Metal	Concentration	% AEB Remaining After Storage at 90°C (O_2*)			
		5 Hours	16 Hours	22 Hours	40 Hours
Cu^{+2}	100 ppb	102	--	74.6	--
	500 ppb	--	--	55.7	--
	1 ppm	88.5 (7 hrs)	--	42.5 (24 hrs)	--
Fe^{+3}	100 ppb	--	89.3	--	47.9
	500 ppb	--	76.9	--	33.0
	1 ppm	--	70.7	--	22.4
Ca^{+2}	1 ppm	--	90.7 (17 hrs)	--	61.2 (41 hrs)
Fe^{+3}	1 ppm (EDTA; large excess)	--	100	--	83.6
	1 ppm (N_2 atmos.)	--	102	--	99.7
Cu^{+2}	1 ppm	--	--	99.8	--

*All studies were conducted under oxygen atmosphere, unless noted otherwise; solutions were adjusted to pH 3.0 with hydrochloric acid.

TABLE 11

Determination of Optimum Concentration of Copper for Kinetic Studies in Oxygen Atmosphere

Copper Concentration (ppm)	% AEB Remaining	
	After 18 Hours at 90°C	After 41 Hours at 90°C
10	35.2	8.7
5	45.0	17.7
1	64.7	31.2
0.5	69.9	39.6
0.01	89.8	59.1

TABLE 12

Kinetic Studies of AEB Solution (1 mg/mL)
in an Oxygen Atmosphere
(1 ppm Cu)

Time (hours)	% AEB Remaining								
	pH 3.0			pH 4.0			pH 5.0		
	90°C	70°C	50°C	90°C	70°C	50°C	90°C	70°C	50°C
7	88.5	--	--	95.9	--	--	96.9	--	--
24	42.5	85.3	--	93.8	98.6	--	96.5	98.4	--
48	21.7	56.8	101	86.4	98.7	99.3	91.2	101	100
120	2.5	--	--	31.0	--	--	64.0	--	--
123	--	28.2	--	--	--	--	--	--	--
144	--	--	63.8	--	63.8	96.0	--	--	99.1
164	--	19.3	--	--	--	--	--	--	--
167	--	--	--	23.4	--	--	--	--	--
213	--	12.4	51.1	--	49.7	--	--	--	--
216	--	--	--	--	--	--	40.0	--	--
332	--	--	31.9	--	--	74.2	--	97.1	--
452	--	--	23.1	--	24.0	61.0	--	--	--
787	--	--	--	--	--	47.7	--	66.5	--
1147	--	--	--	--	--	--	--	48.3	--
1892	--	--	--	--	--	--	--	--	94.0

the degradation rate with a selective high performance liquid chromatographic method (Table 12). The method entails separation on μBondapak C_{18} column (30 cm x 4.6 mm i.d.) with a mobile phase containing 0.0028M 1-octanesulfonic acid in 36 methanol:64 water:1 acetic acid followed by detection at 280 nm.

The results of these studies are given in Table 12. The pH of the solution has greatest effect on AEB degradation. The rate of degradation

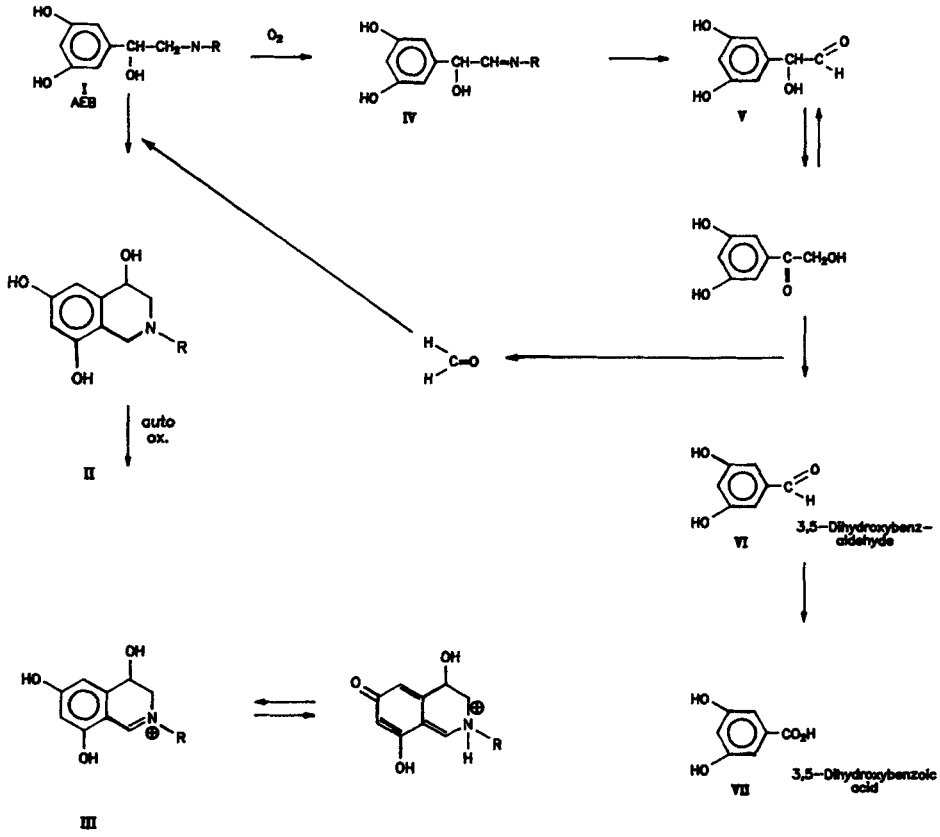


FIGURE 4

Propoased Degradation Pathway Under Stressed Conditions
[Adapted from Acta Pharm. Suec., 9, 141 (1972)]

is fastest at pH 3 and slowest at pH 5. At pH 4 and 5 the degradation reaction appears to proceed in two steps (k_1, k_2) instead of an apparent single step at pH 3. The postulated second step (k_2) is relatively fast. At pH 3 (90°C), k_1 is indistinguishable from k_2 and its calculated value is 17 times higher than that at pH 5 (Table 13). It should be noted that

TABLE 13

Calculated Apparent First Order Rate Constants
For Kinetic Study
(Data in Table 12)

Temperature	pH	k_1 (hour^{-1})	k_2 (hour^{-1})
90°C	3.0	*	0.0318
	4.0	0.0029	0.0140
	5.0	0.0019	0.0049
70°C	3.0	*	0.0103
	4.0	0.0003	0.0042
	5.0	0.00008	0.0008
50°C	3.0	--	0.0041
	4.0	0.00026	0.0014
	5.0	0.00003	--

*$k_1 = k_2$

since k_1 values are small and based on few data points, these extrapolations must be used judiciously. Based on k_2 data at 90°C, it would appear that a solution at pH 5 is ≅6 times more stable than one at pH 3.

Two degradation products (II and III; Figure 4) were prepared in our laboratory. Their presence could not be demonstrated in the solutions degraded under exaggerated conditions; however, very small peaks for resorcylic acid (VII) and 3,5-dihydroxy benzaldehyde (VI) were found by HPLC. This suggests that oxidative degradation is favored (Figure 4).

SUMMARY

The importance of trace and ultratrace analysis by HPLC in solving several complex scientific problems is shown with several examples from our laboratory. These examples include analyses of both stable and unstable compounds at nanogram to picogram levels, discovery of new compounds, and catalytic studies at ultratrace levels.

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