REACTION CHROMATOGRAPHY

I. GAS-LIQUID/THIN-LAYER CHROMATOGRAPHIC DERIVATIZATION TECHNIQUE FOR THE IDENTIFICATION OF CARBONYL COMPOUNDS*

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Identification of oxygenated flavor- or aroma-producing compounds from food and plant essential oils is sometimes complicated by the small quantities present. In lieu of positive spectral proof, tentative gas chromatographic identifications necessarily require confirmative evidence by derivative behavior. A simple and inexpensive technique readily available in most laboratories has been developed for obtaining such evidence for carbonyl compounds. In an extension of the gas–liquid/ thin layer chromatographic (GLC/TLC) derivatization technique reported by CASU AND CAVALOTTI[1], 2,4-dinitrophenylhydrazones (DNPHs), p-nitrophenylhydrazones (PNPHs), and 2,4-dinitrophenylsemicarbazones (DNPSCs) were found to be suitable carbonyl compound derivatives which form readily on TLC plates at the exhaust of a gas chromatograph. TLC behavior of these derivatives in several systems and subsequent possible manipulations are described.

The technique involves isolation of the carbonyl compounds from the essential oil by the Girard T technique[2], GLC of the regenerated compounds, and derivative formation on TLC plates of each eluting maximum at the chromatograph exhaust port. After development of the TLC plates in a suitable system, the purified derivative spot is scraped from the plate, eluted from the adsorbent, and subsequently examined in other TLC or GLC systems. Also, U.V.-visible spectra may be determined, or melting point taken. The Girard T isolation is a desirable but not essential feature in the process. The utility of these techniques has been demonstrated in the isolation and identification of carbonyl compounds from the essential oil of the cotton bud[3].

EXPERIMENTAL

Reagents and apparatus

Gas chromatograph. Barber-Colman Model 5000, modified with exhaust splitter and Luer-Lok exhaust line discharging vertically downward, equipped with a 10 ft. × ¼ in. column packed with Apiezon L (20 %, w/w) on Gas Chrom P.

2,4-Dinitrophenylhydrazine and p-nitrophenylhydrazine (Matheson, Coleman,

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and Bell) and 2,4-dinitrophenylsemicarbazide (K & K Laboratories) were used without further purification.

Reagents for gas chromatographic derivatization on thin layer plates were prepared as follows:

DNPH. Reagent was dissolved in H_3PO_4 and ethanol according to JOHNSON[4].

PNPH. p-Nitrophenylhydrazine (0.5 g) was added to 30 ml ethanol, 6 ml of methylal, and 5 drops of concentrated H_3PO_4 catalyst.

DNPSC. 2,4-Dinitrophenylsemicarbazide (0.15 g) was dissolved in 20 ml of boiling ethanol (95 %), and 6 drops of concentrated HCl were added. On cooling, some of the reagent precipitated out of solution, but this was ignored as it caused no ill effects.

TLC plates (250 μ adsorbent depth) were prepared with Brinkman apparatus on 20 × 20 cm × 3 mm glass. Silica Gel G (SGG) and polyamide powder (Merck) were used as adsorbents. In addition, SGG plates were coated with Carbowax 600 by immersing in 20 % (v/v) Carbowax 600–acetone solution, removing, and allowing the acetone to evaporate at room temperature. Polyamide and Carbowax coated SGG plates were used without further treatment; the SGG plates were activated at 115° for 1 h before use.

All aldehydes and ketones examined were commercial samples available in our laboratory or obtained from previous work with the cotton bud essential oil[3].

Formation of standard derivatives

DNPHs and PNPHs were prepared according to SHRINER AND FUSON[5]. Purification was by recrystallization[5], except for some of the PNPHs which were oils. These were purified by preparative TLC utilizing system A (see later).

DNPSCs were prepared by the method of McVEIGH AND ROSE[6]. Saturated aliphatics were recrystallized from 95 % ethanol and the unsaturated aliphatic and aromatic derivatives from a mixture of N,N-dimethylformamide (DMF) and 95 % ethanol. In the latter case, a minimum of DMF was added to the boiling ethanol to dissolve the derivative. Melting points and micro-Kjeldahl nitrogen analyses were obtained to establish purity and identity.

Formation of derivatives from gas chromatograph

A thin layer plate was placed on a lab jack so the starting line of the plate was under the exhaust port of the gas chromatograph. With the jack, the distance of the plate from the exhaust port could be adjusted as desired. For best results, the plate was kept as close as possible to the port during derivatization without allowing it to touch. A drop of reagent (*ca.* 10 μl) was spotted on the starting line of the plate just before elution of each GLC maximum, and the eluant was allowed to flow into the center of the reagent spot. Extra reagent was added to the spot during elution as needed to keep it moist. Ordinarily eight GLC maxima were reacted on each plate on 2 cm centers, and a drop of unreacted reagent was added at a vacant site for comparison. If the eluted sample was larger than *ca.* 2 μl, it was usually necessary to derivatize on more than one spot to prevent reagent caking and overloading of the adsorbent. The temperature of the exhaust port was maintained at 185° for maximum yield of derivative.

With all three reagents, 50 μg or less of a carbonyl compound injected into the

gas chromatograph could be derivatized by this method in sufficient amounts to allow development, elution, and rechromatography on other systems.

Thin-layer chromatography

Samples were spotted 1.5 cm from the bottom edge of the plate (starting line) and 2 cm apart in every case. All plates were allowed to develop to a height of 10 cm from the starting line. Tanks containing solvents were equilibrated before introduction of plates.

Systems used to separate the derivatives were:

(A) Benzene–petroleum ether (38–50°) (4:1); solid adsorbent—SGG.

(B) Methanol–water (95:5); solid adsorbent—polyamide powder.

(C) Heptane–benzene (4:1); solid adsorbent—SGG coated with Carbowax 600.

(D) Benzene–ethyl acetate (4:1); solid adsorbent–SGG.

All DNPHs were readily visualized on the plates by their bright yellow to deep orange-red color. The PNPHs and DNPSCs of saturated aliphatic aldehydes were not visible under ordinary light but were readily distinguishable under U.V., since they are normally strong absorbers. The aromatic and α,β-unsaturated aldehyde PNPHs fluoresce yellow to yellow-green under U.V. light.

Test mixture (Desaga, Heidelberg) was spotted on each plate and R_F values, based on the Butter Yellow spot in the mixture, were determined for each sample spot. Standard derivatives and those prepared from the gas chromatograph were spotted side by side for comparison.

All derivatives prepared from the GLC were made on SGG plates. The DNPHs and PNPHs were developed in system A and the DNPSCs in system D. A reagent blank was run on each plate with the derivatives. The major derivative spot from each sample, normally 80+ % of the total quantity of derivatives formed, was scraped from the plate, and the derivative was eluted from the silica gel with solvent. GLC unresolved carbonyl impurities in the commercial standard, reagent impurities, and pyrolytic or other artifacts from the reaction gave rise to the several small spots usually observed to accompany the major derivative spot. There was no difficulty in establishing which spot was the derivative since it was the darkest and largest with the exception of the unused reagent. The DNPHs and PNPHs were eluted with methylal; the less soluble DNPSCs were eluted with methylal–DMF mixtures. These derivatives were respotted and developed in other systems and R_F values calculated.

RESULTS AND DISCUSSION

Several general features are desirable or necessary for a suitable GLC/TLC derivatization system of a compound class. The derivatives must form rapidly in good yield, since the exposure to the heating effect of the exhaust gas is brief. They also must be separable from unexpended reagent or post-reaction modified reagent by some TLC system, and must either be visible or capable of being visualized by non-destructive techniques so they may be transferred to other systems or otherwise examined. At least two distinctly different derivatives would be desirable for more positive identification of compounds of a given functionality type.

Compounds which are labile under the reaction conditions may or may not be suitable for characterization by this method, depending on the relative rates of

decomposition and derivatization. This is true, of course, for the same reaction in a test tube, but on the TLC plate, catalytic action of the adsorbent may favor one reaction path over the other. Terpenoids of known acid lability thus may be unsuited to this method, since all the reagents used here are acidic. However, parallel GLC/TLC derivatization of a standard and unknown gives reproducible and closely comparable results. In summary, if a derivative can be formed of a compound on a macro scale, and if additional requirements outlined above are met, GLC/TLC derivatization appears to be feasible.

Adsorbents for the reaction preferably should be physically strong and adherent to the glass plate support to withstand repeated spotting, handling, strong reagents, and elevated localized temperatures. Alumina and silica gel both serve well, but silica was preferred for its handling properties in elution of the spots. Both DPNHs and DNPSCs appear to be stable on silica gel; the PNPHs undergo changes with time, as explained later.

The three derivatives reported here satisfy most of the requirements mentioned. The sensitivities were found to be roughly equal, and they were equally sensitive for aromatic and aliphatic compounds. The carbonyl compound detection threshold is below 50 μg for all three. Aromatic and conjugated unsaturated aliphatic derivatives were noticeably deeper yellow or yellow-red than the saturated aliphatics.

All three derivatives ran at higher R_Y values on the reaction plate systems than the reagent, with the exception of aromatic DNPSCs. These latter offered no problem, however, for several reasons explained more fully later.

The reproducibility of R_Y values was not very good since they varied as much

TABLE I

TLC R_Y[a] VALUES OF 2,4-DINITROPHENYLHYDRAZONES (DNPH)
Solvent systems: (A) Silica Gel G; benzene–petroleum ether (38–50°) (4:1). (B) Polyamide; methanol–water (95:5). (C) Carbowax 600/Silica Gel G; heptane–benzene (4:1).

DNPH compound	R_Y		
	A	B	C
Hexanal	1.04	1.12	0.70
Heptanal	1.07	1.02	0.89
Nonanal	1.16	0.85	1.10
Isovaleraldehyde	0.95	1.35	0.64
2-Hexenal	0.93	0.86	0.54
2-Heptenal	1.00	0.80	0.65
2-Octenal	1.05	0.74	0.75
2-Nonenal	1.08	0.65	0.84
trans-2, cis-6-Nonadienal	1.02	0.81	0.53
Benzaldehyde	0.92	0.54	0.13
p-Tolualdehyde	1.22	0.33	0.25
Acetone	0.33	1.24	0.35
2-Butanone	0.87	1.33	0.70
2-Nonanone	1.08	1.04	1.16
6-Methyl-5-hepten-2-one	1.03	1.21	1.03
Acetophenone	0.98	1.41	0.42
l-Carvone	1.19	0.64	1.03
Menthone	1.16	0.84	1.17

[a] R_Y is travel ratio of unknown to Butter Yellow dye.

TABLE II

TLC R_Y[a] VALUES OF p-NITROPHENYLHYDRAZONES (PNPH)

Solvent systems: (A) Silica Gel G; benzene–petroleum ether (38–50°) (4:1). (B) Polyamide; methanol–water (95:5).

PNPH compound	R_Y	
	A	B
Propionaldehyde	0.22	1.13
Butyraldehyde	0.27	1.10
Valeraldehyde	0.29	1.02
Hexanal	0.32	0.94
Heptanal	0.36	0.86
Nonanal	0.41	0.73
Decanal	0.43	0.63
Undecanal	0.46	0.61
Dodecanal	0.48	0.57
Isobutyraldehyde	0.33	1.15
Isovaleraldehyde	0.31	1.13
Crotonaldehyde	0.23	0.80[b]
2-Hexenal	0.35	0.76[b]
2-Nonenal	0.43	0.56[b]
Benzaldehyde	0.31	0.52[b]
p-Isopropylbenzaldehyde	0.41	0.51[b]
Salicylaldehyde	0.15	0.35[b]
Cinnamaldehyde	0.26	0.28[b]
2-Butanone	0.22	1.15
2-Nonanone	0.35	0.87
6-Methyl-5-hepten-2-one	0.27	1.08
Acetophenone	0.31	0.58[b]
l-Carvone	0.55	0.55[b]
Menthone	0.50	0.80

[a] R_Y is travel ratio of unknown to butter Yellow dye.
[b] Fluorescent under U.V. radiation.

as ± 15 % (relative) from one plate to another for the same compound. However, standard derivatives and those prepared from the gas chromatograph gave values which agreed within 3 % (relative) when run side by side. R_Y values in Tables I–III indicate the relative behavior of various compounds in different systems. The compounds reported for each derivative type were run on a single plate in each system several times, so the values given are internally consistent.

DNPHs of some representative compounds and their R_Y values in three TLC systems are listed in Table I. R_Y values for system A were not calculated from the reaction plates but from plates on which the derivative had been respotted and developed. In all cases the derivatives ran out ahead of the reagent on the reaction plate, but excess reagent on this plate tended to retard the derivatives somewhat, giving false R_Y values.

If the exhaust port is held too close to one spot on the plate for too long, the derivative appears to pyrolyze or darken considerably. This is particularly true of highly unsaturated compounds. The reagent also may cake, in which case the solvent does not move through it easily and the derivative may not move off the starting line.

Elution of the DNPHs from the reaction plate with a suitable solvent provides

them in purified form for examination by several methods. Other TLC systems[7,8], and several paper chromatographic systems[9,10] have been reported to give good separations of these derivatives. In addition, they may be subjected to gas chromatography[11] and visible spectrophotometry[12] or melting points may be taken after solvent evaporation.

Table II lists R_F values for PNPHs of several representative compounds. Values for system A, in which the reaction plates were developed, were calculated from a second plate after elution from the reaction plate, respotting, and development.

The PNPHs appear to be rather unstable and darken on the SGG plates on standing in air. Additionally, several spots were observed for each sample in system A. For this reason, the major spot from each sample was scraped from the reaction plate and eluted immediately after development. To avoid possible degradation, even in solution, the PNPHs should be examined as soon as possible after elution.

So far as can be found, this work is the first report of TLC of PNPHs, although

TABLE III

TLC R_F[a] VALUES AND MELTING POINTS OF 2,4-DINITROPHENYLSEMICARBAZONES (DNPSC)
Solvent systems: (B) Polyamide; methanol–water (95:5). (D) Silica Gel G; heptane–benzene (4:1).

DNPSC compound	R_F		$M.p.$[b] ($^\circ C$)
	B	D	
Valeraldehyde	1.00	0.54	185–187
Hexanal	0.88	0.59	155–157
Heptanal	0.77	0.61	150–151
Nonanal	0.68	0.65	138–139
Decanal	0.54	0.71	133–135
Undecanal	0.51	0.67	130–132
Dodecanal	0.42	0.68	134–136
Isobutyraldehyde[c]	1.09	0.53	207–208
Isovaleraldehyde	1.11	0.56	198–200
2-Methylvaleraldehyde	1.01	0.64	171–173
Crotonaldehyde[c]	0.82	0.51	228–230
2-Hexenal	0.69	0.62	204–206
2-Nonenal	0.54	0.68	177–179
Citral[d]	0.61	0.80	171–174
	0.52	0.64	
Citronellal	0.87	0.70	140–142
Benzaldehyde[c]	0.27	0.52	225–254 (dec.)[e]
p-Isopropylbenzaldehyde	0.21	0.57	267–269
Salicylaldehyde	0.22	0.31	258–261
Cinnamaldehyde[c]	0.19	0.52	228–230 (dec.)
2-Butanone	0.84	0.54	248–249 (dec.)
2-Nonanone	0.54	0.67	191–192
6-Methyl-5-hepten-2-one	0.59	0.63	221–222
Acetophenone[c]	0.27	0.58	264–266 (dec.)[f]
l-Carvone	0.32	0.90	222–225
Menthone	0.59	0.80	212–214

[a] R_F is travel ratio of unknown to Butter Yellow dye.
[b] Fisher John's block, uncorrected.
[c] Reported by McVEIGH AND ROSE[6].
[d] Commercial; contains geranial and neral by GLC
[e] McVEIGH AND ROSE[6] report 232°.
[f] McVEIGH AND ROSE[6] report 245° (dec.).

paper chromatographic systems are available in which alkaline sprays are reported to be of some value in identification, especially under U.V. light[13]. Gas chromatography[14] and visible spectrophotometry[15] also may yield useful data for compound identification. Melting points may be taken in many cases, but some PNPHs are oils or are very difficult to crystallize. This may create problems in preparation and purification of standards, but preparative TLC purification of these derivatives appears to be satisfactory.

Of the DNPSCs listed in Table III, only five have been reported previously[6], and no reports could be found of TLC, paper chromatography, or GLC of these derivatives. The melting point and R_F values in two systems for each standard derivative are listed in the table. The DNPSC reagent reacts rapidly with carbonyl compounds and provides an excellent derivative which may be recrystallized from 95 % ethanol or DMF–ethanol mixtures. On the SGG plates the reaction is almost quantitative, consuming most of the reagent. Neither pyrolysis nor caking was observed with any of the DNSPCs investigated.

In system D, the R_F values of the DNPSCs of aliphatic carbonyl compounds were greater than the R_F of the reagent, except for crotonaldehyde which had a value about equal to that of the reagent. The R_F values of the aromatics were usually less than that of the reagent in this system. No solvent combination was found which would move these latter out of the reagent, which tended to streak badly from the starting line up to R_F 0.50. However, the aromatic compounds investigated reacted so thoroughly that little excess reagent was left to contaminate the derivative spot. In system B, the R_F of the reagent was greater than any of the derivative values, and very little streaking of the reagent was observed. This was of value, since any remaining reagent in the aromatics from reaction system D was removed in this second system.

It has been reported that the visible spectra of DNPSCs are very characteristic of the various carbonyl compound classes[16].

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SUMMARY

A technique for identifying small quantities of carbonyl compounds present in essential oils is described. These compounds are derivatized on thin-layer plates as they are eluted from the exhaust port of a gas chromatograph. Subsequently, they may be examined by thin-layer chromatography, gas–liquid chromatography, paper chromatography, or several other methods for confirmation of identity. 2,4-Dinitrophenylhydrazones, *p*-nitrophenylhydrazones, and 2,4-dinitrophenylsemicarbazides were utilized in this study, and thin-layer chromatographic data are given for each. In addition, melting points are listed for some previously unreported 2,4-dinitrophenylsemicarbazides.

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