FLUORESCENT SOLVENT IMPURITIES AS CHROMATOGRAM ARTIFACTS

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(Received May 2nd, 1966)

The use of fluorescence under ultraviolet light for the detection of certain organic compounds on paper chromatograms and thin-layer chromatograms has become a common practice. As divergent a range of substances as carcinogens[1], narcotics[2], coumarins[3], pesticide residues[4], and biochemical intermediates[5] has been examined through this technique, and its application to natural estrogens[6] and mycotoxins[7] is of particular current importance.

During the course of an investigation of the natural fluorescent constituents of common vegetables[8], we noted certain recurring patterns of fluorescent spots on paper- and thin-layer chromatograms which appeared to be independent from those of vegetable origin. Investigation of the residue remaining after distillation of the several common reagent-grade solvents employed in the research revealed them to be the source. The superficial similarity of these impurities to the natural products under examination prompted a more detailed study of their occurrence and properties.

EXPERIMENTAL

Chromatography

Chromatoplates were prepared in the usual way by application of a 1:2.5 slurry of Merck Silica Gel G to glass plates 20 mm square with a Brinkman 0.6 mm fixed-thickness spreader. After drying at 110° for 30 min, the prepared plates were stored in a desiccating cabinet.

One liter of each of the commercial solvents listed in Table I was concentrated to a volume of 5 ml under vacuum on a rotary evaporator below 40°. An aliquot of 100 μl of each residue was applied to a chromatoplate and chromatographed with toluene–dioxane–acetic acid (6:2:1). When the solvent front had moved a distance of 14 cm from the origin, the plate was removed from the development chamber, dried, and examined under ultraviolet light (366 mμ). After recording the fluorescent zones, the plate was sprayed with a solution of 4-nitrobenzenediazonium fluoborate (0.05 % in 20 % aqueous sodium acetate) for detection of phenols.

Samples of the residues from reagent methanol (a) and reagent ethyl acetate (d) were subjected to gas chromatography. An Aerograph A-90-C gas chromatograph equipped with a thermal conductivity detector was employed; the 24 in. × 1/4 in. copper column was packed with 30/60 mesh Chromosorb P impregnated with 20 % Dow 11 silicone oil, allowed to equilibrate at 250° for 48 h with a helium flow rate of 100 ml/min, and operated at 200°. Based on retention time, each major fraction

was collected from the gas chromatograph exit port by insertion of the small end of a Pasteur pipette into a Neoprene septum covering the port; cooling of the pipette by wrapping it in wet filter paper proved to be advantageous.

Samples from both (a) and (d) residues were applied in bands to chromatoplates and chromatographed in toluene–dioxane–acetic acid (6:2:1) as described. Each fluorescent zone was eluted with chromatographically pure methanol; the eluate was concentrated, reapplied to a chromatoplate, and chromatographed in hexane–acetone (2.5:1). Elution was repeated, and the zones were rechromatographed in chloroform–ethyl acetate (3:1). After a final elution with pure methanol, ultraviolet spectra were measured in 1 cm quartz cells with a Beckman DK2-A recording spectrophotometer. In order to confirm purity, a part of each solution was then concentrated and subjected to gas chromatography. The single component was collected, dissolved in methanol, and again examined in the spectrophotometer.

Infrared spectra of the pure fractions were measured on 1.5 mm KBr pellets in a Perkin-Elmer Model 221 spectrophotometer equipped with a 4X microbeam condenser system.

Solvent purification

Ethyl acetate (d) (1 l) was distilled from an all-glass system containing a 12 in. Vigreaux column to minimize mechanical entrainment of droplets in the vapor. Half of the distillate was evaporated on a rotary evaporator to a volume of 1 ml, while the other half was allowed to percolate through a 1 in. × 12 in. column of 50–200 mesh activated coconut charcoal and then likewise evaporated. Another sample of (d) (500 ml) was allowed to percolate through the charcoal without prior distillation; others of equal volume were allowed to percolate through activated Florisil, powdered silicic acid, and activated alumina, respectively; and yet another remained untreated. Each effluent was evaporated to a volume of 1 ml. A 100 μl aliquot of each residue was chromatographed in toluene–dioxane–acetic acid as described, and the intensity of the fluorescent spots was compared under ultraviolet illumination.

RESULTS AND DISCUSSION

The isolation of minor constituents from plant and animal products generally requires that rather large volumes of volatile extraction solvents be removed as the initial step in concentration. It is inevitable that non-volatile solvent impurities, even those present in part-per-million amounts, will reach a considerable level if sufficient extraction solvent is evaporated. If these impurities closely resemble the plant products in properties important to detection and isolation, such as fluorescence, chromatographic characteristics, response to color reagents, and spectra, their presence may lead to gross errors in interpretation of the results of natural product investigations.

For example, approximately 500 g of commercial potato flour was extracted with "reagent grade" methanol for 24 h in a Soxhlet extractor, and the residue remaining after vacuum evaporation of the solvent was examined by thin-layer chromatography for natural fluorescent constituents. The purported presence of 6-methoxy-7-hydroxycoumarin (scopoletin), reported by REPPEL[9], was of particular interest. Highly fluorescent bands appeared on the chromatograms at R_F 0.00, 0.80

TABLE I

COMMERCIAL SOLVENTS EXAMINED FOR FLUORESCENT CONSTITUENTS

Solvent	*Grade*	*Commercial container*
(a) Methanol	Reagent	Can (4 gal)
(b) Methanol	Reagent	Bottle
(c) Methanol	Spectro	Bottle
(d) Ethyl acetate	Reagent	Can (4 gal)
(e) Diethyl ether	Redistilled	Bottle
(f) Ethanol	Redistilled	Bottle

and 0.95, while weaker blue fluorescence was observed at R_F 0.45 and 0.63. The R_F value for scopoletin in the same solvent (toluene–dioxane–acetic acid) was found to be 0.42. Further investigation revealed, however, that the fluorescent substances were artifacts derived from the extraction solvents; neither scopoletin nor any other methanol-extractable fluorescent compound could be identified as occurring naturally in potato flour.

About a dozen common organic solvents are used in quantity for research in our laboratory on pesticide residues and natural products. Among them are methanol, ethanol, acetone, ethyl acetate, ether, benzene, and hexane (or petroleum ether). Each of these was found to contain minute traces of fluorescent impurities which were concentrated upon evaporation of the solvent. Even lots sold for fluorimetric use, when concentrated, revealed them, as did "reagent" and "spectral" grades. The contents of six containers were examined in detail (Table I); of these, reagent grade ethyl acetate yielded the largest amounts of fluorescent impurities (about 10 mg/l).

The data summarized in Table II show that each of the high-purity solvents examined gave rise to several highly fluorescent residual substances upon evaporation. On chromatograms, under ultraviolet light, these impurities could easily be mistaken for coumarins or cinnamic acid derivatives because of the similarity in the intensity and color of their fluorescence as well as their R_F values. Even their behavior toward spray reagents such as 4-nitrobenzenediazonium fluoborate was very similar to that observed with the plant phenolics.

TABLE II

MAJOR FLUORESCENT IMPURITIES IN SOLVENT RESIDUES

Solvent	R_F*				
	I	*II*	*III*	*IV*	*V*
a	0.00	0.45	0.63	0.80	0.95
b	0.00			0.80	0.95
c					0.95
d	0.00	0.51	0.67	0.80	0.95
e		0.43	0.70		0.98
f	0.00		0.70		0.98

* Silica Gel G plate in toluene–dioxane–acetic acid (6:2:1).

Each of the residues produced a band (Fraction V) at R_F 0.95 which exhibited a bright blue-white fluorescence and an orange-red color with the diazonium reagent. Upon gas chromatography, a single sharp peak was produced at a retention time of 5.3 min (Fig. 1) which, when collected and chromatographed on a thin-layer plate, exhibited the original fluorescence and R_F. The isolated substance was a yellowish oil, and, although the chromatographic separations appeared well defined, it could represent a mixture. The ultraviolet spectrum (Fig. 2) showed absorption at 270 mμ typical of a conjugated or aromatic structure. Despite the color reaction with the phenol reagent, however, the infrared spectrum did not support a phenolic structure due to a lack of characteristic strong absorption in the 1140–1230 cm^{-1} region. Although appropriate bands appeared in the 1500–1600 cm^{-1} region, the absence of notable absorption below 1000 cm^{-1} also precluded the presence of an aromatic ring.

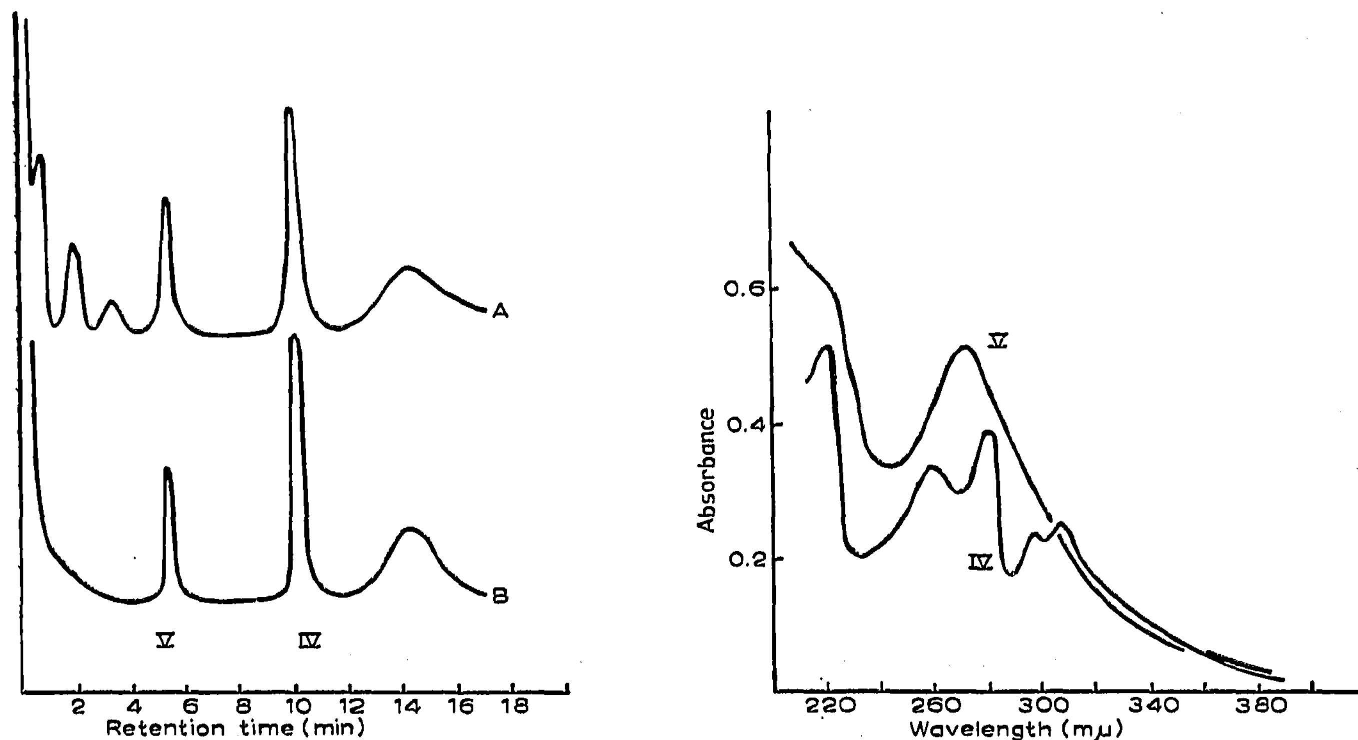

Fig. 1. Gas chromatogram of solvent impurities from ethyl acetate (A) and methanol (B).

Fig. 2. Ultraviolet spectra of fractions IV and V from methanol and ethyl acetate.

Another major fluorescent band (Fraction IV) was noted in residues from solvents a, b, and d. The substance moved at R_F 0.80 when chromatographed on silica plates; the same R_F and violet fluorescence were observed with samples collected from the gas chromatograph at a retention time of 10.0 min (Fig. 1). In this case, the ultraviolet spectrum (Fig. 2) exhibited absorption at 260, 270, 297 and 307 mμ reminiscent of aromatic compounds having much the same chromatographic and fluorescent properties and response to the diazonium reagent (orange-red color). Again, however, the infrared spectrum did not support the presence of a substituted aromatic ring.

The identity of the fluorescent impurities was not explored further. Their origin is obscure; the fact that Fraction V appears in all of the solvents, despite the

varied processes by which they are manufactured, suggests that these trace contaminants probably are extractives from some plastic, lubricant, adhesive, or other extraneous material with which commercial solvents may come in regular contact. Their presence and level may vary from batch to batch of solvent, although concentrated samples of newly purchased ethyl acetate revealed the gas chromatographic pattern of Fig. 1 two years after the major part of the presently described study had been completed.

In our investigation of fluorescent vegetable constituents[8], we found that the levels of solvent impurities resulting from the concentration of organic plant extracts could not be ignored. Plant material generally was extracted with at least 2 l of solvent which subsequently was evaporated to a small volume. In many instances, the resulting concentration of solvent impurities produced fluorescent spots or bands on chromatograms which were as intense as many of the principle natural plant products. The remarkable further similarities in color reactions, gas chromatographic characteristics, and ultraviolet spectra may well have given rise to many of the reports of unidentified fluorescent plant constituents in such investigations as those of CRAFT AND AUDIA[10].

TABLE III

REMOVAL OF FLUORESCENT IMPURITIES FROM ETHANOL AND ETHYL ACETATE

Treatment	*Relative fluorescence*
Untreated	100
Distillation	15
Activated carbon percolation	15
Distillation and carbon percolation	5
Silicic acid percolation	60
Activated alumina percolation	60
Activated Florisil percolation	30

In our experience, the major part of the fluorescent impurities may be removed from common solvents rather easily (Table III). Percolation of the "pure" commercial solvent through activated carbon was approximately as effective as careful redistillation for removal of much of the interference, while combination of the two methods provided a product almost free from fluorescent substances. Percolation through silica gel, activated alumina, or Florisil appeared to be ineffective.

ACKNOWLEDGEMENT

This work was supported in part by a research grant (EF-00306) from the U.S. Public Health Service, Bureau of State Services.

SUMMARY

Common laboratory solvents such as methanol and ethyl acetate—even reagent and "spectral" grades—contain non-volatile fluorescent impurities which are concentrated during normal extraction procedures. These substances exhibit chromato-

graphic, spectral, and chemical properties which may cause them to be confused with certain natural products such as coumarins and cinnamic acids. They may be removed by distillation and/or adsorption.

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