A METHOD FOR THE DETECTION AND ISOLATION OF TRACES OF ORGANIC FLUORINE COMPOUNDS IN PLANTS

ROBERT H. WADE, JAMES M. ROSS AND HARRIS M. BENEDICT

Physical Sciences Laboratories, Southern California Laboratories, Stanford Research Institute, South Pasadena, Calif. (U.S.A.)

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INTRODUCTION

During the course of several years of investigating atmospheric fluoride pollution problems we have felt the need for two separate fluoride analytical method. First, a rapid semiquantitative method for the determination of excess amounts of fluoride in vegetation has been needed; such a method, recently developed in these laboratories, is to be reported elsewhere[1]. Second, a method for the detection and isolation of microgram quantities of organic fluorine compounds from plant materials is the subject of the present paper. The method is presented in two parts: a rapid procedure for screening large numbers of plant extracts and materials for the presence of any organic fluorine compounds, and a chromatographic process for further isolation and identification of materials found in the screening method.

Background

Fluoride analyses of vegetation are confined for the most part to modifications of the WILLARD AND WINTER[2–4] method. Although this method is reliable and reproducible, it is time consuming, requires relatively large samples (1–5 g vegetation containing at least 5 μg of fluoride) and, unless special precautions are taken, is not likely to account for fluorine present in the organic form. At best it gives a total fluoride (organic and inorganic value. The isolation and identification of fluorine compounds in plant materials to be used as salad crops or as feed for grazing animals is important because of the known fluoride uptake from polluted atmospheres and because of the very wide range of toxicities, especially of organic fluorine compounds. These toxicities range all the way from inert, innocuous materials (such as the Freons) to poisons (such as fluoroacetic acid and some of the fluoro-olefins where the LD_{50} is in the range that makes detection difficult or impossible). For example, inhalation of the refrigerant Freon CF_2Cl_2 (Freon 12) of 20 % concentration does not even produce unconsciousness, although analgesia and confusion result. Recovery is complete within ten minutes[5]. In contrast, the lethal dose of fluoroacetic acid for man is estimated at 2–10 mg per kg[6,7] body weight. The toxicity of inorganic fluoride is intermediate between these extremes; the toxic dose of sodium fluoride is estimated between 5 and 10 g, an amount unlikely to be ingested from vegetable contamination. Regular intake of sublethal doses of inorganic fluoride, however, may produce a definite and damaging series of chronic symptoms[8–11]. It was the concern over these chronic symptoms and

the possibility of the presence of even more toxic organic fluorine compounds in fluoridated salad and forage crops that initiated this research.

In attacking these problems, forage plants (alfalfa and orchard grass) and leafy vegetables (chard, spinach and romaine lettuce) were fumigated with concentrations of about 0.60–0.83 $\mu g/m^3$ of hydrogen fluoride during their entire growth period. These concentrations produced no fluoride-type markings on the leaves, although a relatively high concentration of fluorides accumulated in them (30–100 p.p.m.).

The problems of separating organic from inorganic fluorides were first attacked qualitatively by making several series of extractions, using solvents of widely varying polarity. Regardless of whether the solvents were employed in order of increasing or decreasing polarity, most (over 90 %) of the fluoride was found in the most polar solvent (water), indicating the fluoride to be essentially inorganic. However, sufficient fluoride was found in the non-polar solvents to allow at least the possibility of the presence of organic fluorine compounds.

DISCUSSION

The isolation and identification of organic fluorine compounds from plant or animal materials is complicated by two factors. First, not only is the fluorine present in very small amounts, but it has previously been shown to be present largely in the inorganic form. As a consequence, organic fluorine in fluoride-fumigated plants is present in even smaller amounts, if at all, and hidden by the small but nevertheless swamping amount of inorganic fluoride. Second, organic fluorine compounds do not in themselves constitute a functional class; for example, fluoroacids behave essentially like other acids and fluoroalcohols have the properties of alcohols in general. Consequently, the chemistry of organic fluorine compounds cannot be used in any way to isolate them as a group nor as tests to indicate their presence. Therefore, every fraction, extract, or compound must be completely freed of inorganic fluoride and then quantitatively analyzed for residual fluorine.

Quantitative analytical methods are all based on the determination of fluoride in ionic form. A large variety of methods for conversion of organic fluoride to analyzable ionic form habe been reported. Many conventional methods are summarized by SIMONS[12]. None of them are applicable in the microgram range and all are too tedious and time consuming for large numbers of samples. In 1955, SCHÖNIGER[13] introduced an oxygen flask combustion method for reducing many of the elements in organic compounds to ionic or other analyzable forms. From this rapid and inexpensive method, a very suitable procedure for the determination of fluorine in organic compounds has been developed. It is particularly applicable to the quantitative determination of fluorine in spots developed on paper chromatograms. However, in a search for minute amounts of unknown organic fluorine compounds in the presence of much larger amounts of inorganic fluoride, new procedures were needed.

METHOD

The various solvent and aqueous extracts of fluoridated vegetation were concentrated on a rotovacuum apparatus and first analyzed for total fluoride.

Sub-micro determination of fluorine

A modification of the procedure reported by SOEP[14] was used for quantitative sub-micro determination of fluorine. We found, as did SOEP, the decoloration of the zirconium-Eriochrome Cyanine R complex to be proportional to fluoride concentration. We also investigated the more sensitive aluminum-morin complex[15] and a number of other metal-dye complex reagents such as sodium 2-(*p*-sulfophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonate-zirconium lake (SPADNS)[16] before standardizing on the Eriochrome complex as most satisfactory for the determination of from 0–3 μg of fluoride. A new direct color alizarin complexan method of fluoride analyses reported by BELCHER *et al.*[17–19] was not found satisfactory for this work but was used in the semiquantitative procedure to be reported elsewhere[1].

Apparatus

A 125-ml iodine flask modified with a platinum wire attached to the stopper according to SCHÖNIGER[13] plus a microstopcock attached to the bottom of the flask (Fig. 1).

Volumetric flasks, 50 ml size.

Micropipettes, graduated variety 1–5 λ, 5–25 λ and 20–100 λ sizes.

Portable hair dryer for drying spotted strips of paper.

Beckman model DK Ratio Recording Spectrophotometer used at 546 mμ.

Cuvette from Lumetron Photoelectric Colorimeter.

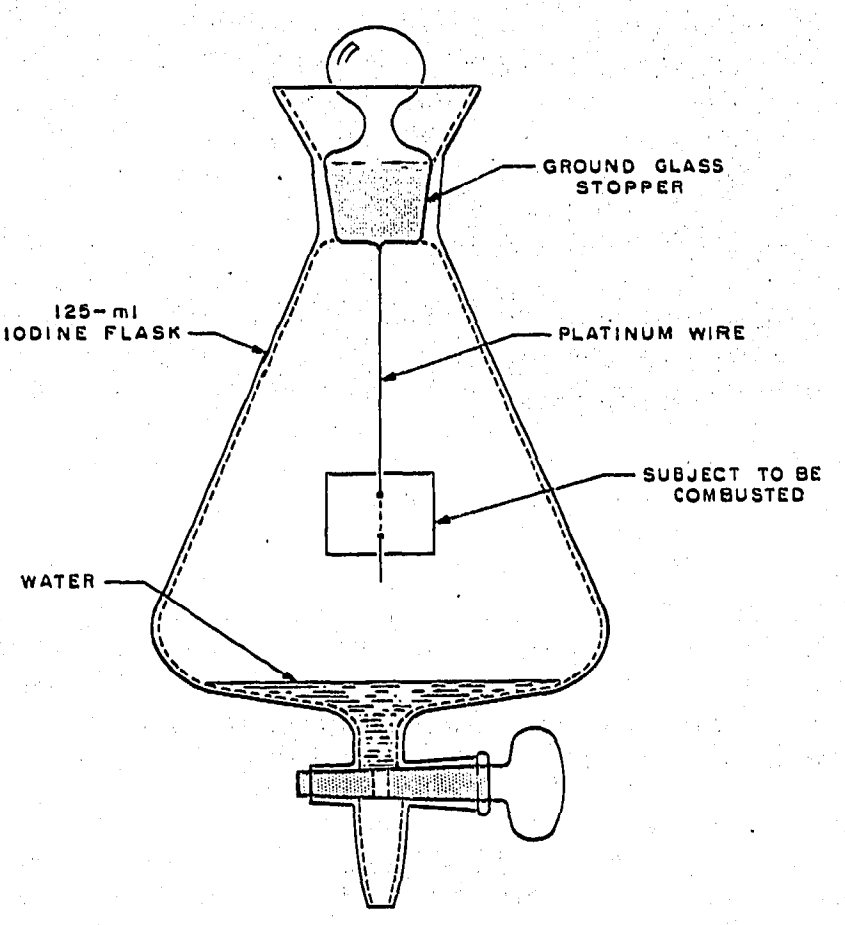


Fig. 1. Combustion apparatus.

The 1-cm cell was small enough to allow adequate filling with 5 ml of solution and was used sideways to give a 32-mm light path. A simple support was provided to hold it in the light path of the Beckman instrument.

Reagents

Solution A: 528.8 mg of Eriochrome Cyanine R dissolved and diluted to 250 ml with water.

Solution B: 76.3 mg of zirconyl chloride octahydrate dissolved in 202 ml of hydrochloric acid (concentrated) and diluted to 500 ml with water.

Reagent: One volume of A mixed with two volumes of B, fresh daily.

Organic fluorine standard: 15.5 mg of sodium difluoroacetate dissolved and diluted to 100 ml with water giving 0.5 μg of fluorine/μl of solution.

Laboratory distilled water was upgraded by distilling through an all-quartz, two-stage water purification apparatus.

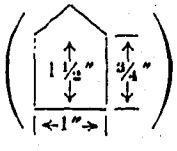
All materials were stored in polyethylene bottles.

PROCEDURE

A portion of each extract (about 100 μl) is spotted on a small piece of filter paper. The paper is dried with hot air from the hair dryer and fixed to the platinum wire of the stopper. After adding 2 ml of water, the flask (Fig. 1) is thoroughly flushed with oxygen and the paper strip is ignited from a small pilot flame and plunged into the flask. We found that when the paper is ignited from the top (in the position to be lowered into the flask) a much larger piece containing more sample can be smoothly combusted. When the flame is at the bottom of the strip as it is plunged into the flask it immediately flashes up consuming the whole of the paper and usually causes some of the paper to fall to the water incompletely burned. Also, the more rapid combustion often causes the stopper to be dislodged with resultant loss of gases. The flask is shaken vigorously, drained, shaken with a second 2 ml of water, and rinsed with about 0.5 ml of water into a 5-ml volumetric flask. The freshly mixed reagent is added (0.40 ml) and the total volume diluted to 5.0 ml. The extinction values are measured at 546 mμ after one hour, using water in the reference beam. The values obtained are compared with a calibration curve prepared similarly from the organic fluorine standard ($CF_2HCOONa$) to obtain the fluorine content. Under these conditions the curve is linear from 0 to about 2.5 of 3 μ of fluoride.

Separation of organic fluoride

Rapid screening procedure. If the above total fluoride analysis (organic or inorganic) shows fluorine to be present, a separation must be accomplished to determine if any of it is in the organic form. The method used for this separation was developed from chromatographic procedures for separation of inorganic anions and from the elution concentration scheme of DAVIS, DUBBS AND ADAMS[20] and was designed to screen large



numbers of plant extracts. First, a small section was cut from a 1-in. roll of Whatman No. 1 chromatographic paper and a small volume of extract (volume dependent on previous total fluoride analysis) spotted along the base of the wedge. The sample can be placed either in a spot or along a line across the base. We did not find it necessary to elute contaminants on the paper and to trim off the tip before using, as did DAVIS *et al.*, because, although materials were found there, they did not interfere with the subsequent fluoride analysis. We found it convenient to use full length microscope slides rather than cut them in half as did DAVIS *et al.* After clamping with No. 2 ring clips, obtainable in any stationery store, ten units can be placed in a standard staining jar without support (Fig. 2). A chromatographic solvent or solvent mixture in which

Fig. 2. Short-strip separations of organic from inorganic fluoride.

fluoride ion has been determined not to move ($R_F = 0$) is placed in the jar. The solvent immediately fills the space beteen the two slides, providing a continuous source to the base of the strip. The edges of the dish are covered to reduce evaporation from other than the tips, and the strips allowed to stand overnight. The solvent evaporates from the tip allowing a continual flow of fresh solvent and thereby causing even the slow moving (R_F low) substances to move to the tip and be concentrated there while inorganic fluoride remains on the original spot. Several solvent mixtures for the separation of the halides and other anions are listed by MCOMIE AND POLLARD[21]. We used as standard the upper layer of a mixture of equal parts of *n*-butanol and 1.5 *N* ammonia, well shaken and allowed to stand at least overnight before separation. This solvent mixture was exhaustively tested to be sure that the R_F factor for inorganic fluoride was consistently zero. In addition, papers spotted with sodium fluoride were

run alongside every batch of plant extracts and the fluoride was found in all cases to remain on the original spot. After overnight development, a small portion of the tips and the original spot are separately combusted in the oxygen flask and analyzed for fluoride as above. Under these conditions, inorganic fluoride remains exactly on the original spot and all of the organic fluorine compounds tested move quickly to the tip (Table I). Any fluoride found in the combustion of the tips (with butanol solvent) is therefore definite indication of the presence of a fluoro-organic. As confirming evidence of organic fluorides, duplicate strips were run using water as solvent rather than butanol–ammonia. Here, of course, inorganic fluoride quickly moves to the tip. The movement of fluoro-organics with water is not so straightforward and depends on the nature of the compounds. Highly polar, water-soluble compounds would move with the solvent and non-polar insoluble materials would remain in the original spot. The butanol and water strips together give an indication of the presence and nature of an organic fluorine compound. Table I indicates the results of organic and inorganic fluorides run on short-strip analysis.

TABLE I

SHORT-STRIP FLUORIDE ANALYSIS

Compound or extract	*Solvent*	*Fluoride (μg)*	
		Tip	*Spot*
Perfluorobutyric acid	Butanol	3.2	0.3
Perfluorobutyric acid	Water	3.2	0.2
Sodium fluoroacetate	Butanol	3.0	0
Sodium fluoroacetate	Water	3.5	0.1
Sodium difluoroacetate	Butanol	0.8	0.2
Sodium fluoride	Butanol	0	2.9
Sodium fluoride	Water	3.6	0.1
2,4-Dinitrofluorobenzene	Butanol	0.6	0.3
2,4-Dinitrofluorobenzene	Water	0.9	0

Profile analyses of chromatograms for organic fluorine compounds. It should be obvious, of course, in the short-strip analysis with butanol–ammonia, that essentially all of the organic compounds present move to the tip and not merely the fluoro-organics. Thus, an absolute separation of inorganic from organic fluorine compounds has been obtained, but no indication of the number or nature of the organic compounds present.

If a positive indication of fluoro-organics is obtained in the short-strip analysis, a number of conventional paper chromatographic strips are run. For example, a portion of an extract showing an indication of an organo-fluorine compound in the short-strip analysis is spotted on a 1 in. × 18 in. strip of Whatman No. 1 chromatographic paper. The spot is dried and the strip hung in a chromatographic tank overnight in equilibration with the vapors of the lower butanol–1.5 *N* ammonia layer mentioned above. The strip is then developed several hours with the upper layer of the same solvent mixture and thoroughly dried. Theoretically, the strip could be treated with a suitable reagent to make visible the organic fluoride sought. Unfortunately several factors preclude this: first, as mentioned above, there are no color reactions for the

general fluoro-organic class; second, the compounds, if any, are present in extremely small amounts; third, the paper chromatogram is rather completely stained yellow and green with chlorophyll and other pigments.

Instead, the chromatogram is subjected to a procedure we have chosen to call a profile analysis. It must be quantitatively analyzed for fluorine by sections. A short chromatogram of 12–15 cm is cut crosswise in ten strips, each representing 0.1 R_F factor. A large strip might be cut in twenty strips of R_F 0.05 each. Each strip is combusted in the oxygen flask and analyzed for fluoride. A profile of the fluoride content of the strip is then obtained by plotting the fluoride analyses *versus* the R_F factor (Fig. 3). In the chromatogram represented in Fig. 3A a sample of sodium di-

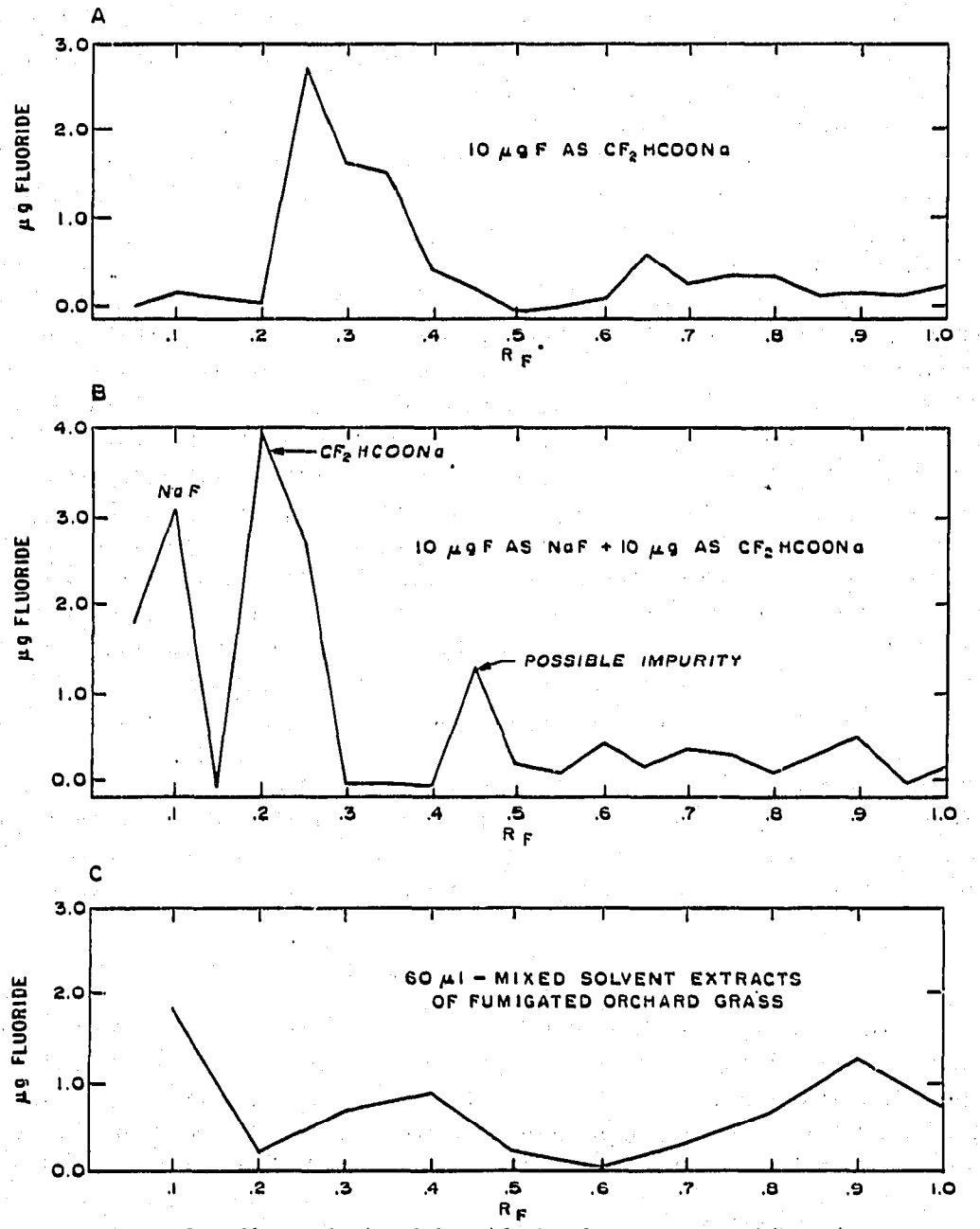


Fig. 3. Profile analysis of fluoride in chromatographic strips.

fluoroacetate containing 10 μg of fluorine was spotted and developed as described. The dried paper was analyzed in twenty strips, each representing 0.05 R_F units. As indicated in the figure, the R_F factor for $CF_2HCOONa$ is about 0.25. The strips at R_F 0.25, 0.30 and 0.35 indicate a total of about 7 mg of fluorine, or 70 % recovery. In the chromatograph represented in Fig. 3B, a mixture of sodium fluoride and sodium difluoroacetate were spotted together and developed, again in butanol–ammonia, but a little longer for better separation. The inorganic fluoride is found in the R_F 0.05 and 0.10 strips and the organic fluorine again found at R_F 0.20–0.25. The strip at R_F 0.15 is free of fluorine, indicating complete separation of the two compounds. Fig. 3C shows the profile analysis of a solvent extract of hydrogen fluoride fumigated orchard grass indicating the presence of inorganic fluoride and possible traces of two organic fluorine compounds. Since the strip are cut from the chromatograph by R_F number and not by visual location of any spot, a single compound will usually be found in two or three adjacent strips rather than a single one.

The final step is to isolate the material in pure form. An obvious method is to prepare a number of chromatograms under identical conditions, determine the location of the unknown on one chromatogram by profile analysis and extract the material from similar R_F portions of the remainder. This method was applied as follows. A portion of fumigated orchard grass tips (fluoride previously shown to concentrate there) was extracted in a soxhlet with petroleum ether followed by ether and several other solvents. The ether extract was found to contain 1.2 μg total fluoride per 100 μl. A short-strip analysis indicated the fluoride to be approximately 70 % organic. Therefore, a portion of the ether extract was spotted on a 1 in. × 18 in. strip of paper, equilibrated, and developed with butanol–ammonia. Combustion of the resulting chromatogram in twenty strips (0.05 R_F units) gave a peak at R_F 0.0–0.1 for inorganic fluoride and a larger peak at R_F 0.7–0.9 indicating organic fluoride. To confirm the finding of organic fluoride at R_F 0.7–0.9, eighteen strips $3^1/_4$ in. wide were each spotted with 500 γ of the extract and developed in butanol–ammonia as before. The 0.7–0.9 portion of each strip was cut out, taking care to eliminate most of the chlorophyll portions which run with the solvent front. These portions were extracted with ether in a soxhlet and the extract concentrated to about 2 ml. This extract was found to contain 6–8 μg total fluoride per 100 μl. Short-strip analysis indicated it all to be organic.

Larger quantities of these fluoridated salad and forage crops were investigated by chromatography using heavier preparative papers and cellulose column techniques. Although microgram quantities of organic fluorine compounds were found, the levels were far too low for isolation and identification or to cause concern over toxic materials.

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SUMMARY

A method for the detection and isolation of sub-microgram quantities of organic fluorine compounds from plant materials in the presence of much larger amounts of inorganic fluoride is presented. The procedure consists first of a rapid screening step for use with large numbers of vegetable samples and extracts and, second, of a chromatographic step to isolate and characterize any fluoro-organics found. These methods are developed in light of specific chemical characteristics of organic fluorine compounds as a general class. A modification of SOEP's quantitative sub-micro fluoride analytical method is presented as applicable to these isolation methods. Microgram quantities of organic fluorine compounds were found in the plant materials investigated but at a level too low for isolation and identification.

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