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Simplified Method of Volatile Leaf Oil Analysis for Identification of *Citrus* Cultivars

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Abstract. **Volatile oils were extracted from aqueous leaf suspensions of sweet orange [*Citrus sinensis* (L.) Osb.] cultivars Hamlin, Navel, and Valencia and grapefruit (*Citrus paradisi* Macf.) cultivars Marsh and Ray Ruby. Pressurized air was used as the sparging gas, and volatile oils were collected in a C-18 cartridge. Gas-liquid chromatography was used to separate and quantify 17 volatile components. Significant quantitative differences for individual components made it possible to distinguish sweet orange from grapefruit (four components), 'Marsh' from 'Ray Ruby' grapefruit (two components), 'Hamlin' from 'Valencia' or 'Navel' orange (six components), and 'Valencia' from 'Navel' (three components). The simplicity and sensitivity of the procedure suggest potential use for *Citrus* taxonomic, genetic, and breeding research.**

Gel electrophoresis of leaf isozymes has been useful for genetic and taxonomic studies of *Citrus* species (Soost et al., 1980; Torres et al., 1978, 1985). Most *Citrus* species can be distinguished with this method, and progeny of interspecific crosses can be screened for hybrids among predominantly nucellar seedling progeny. However, few or no differences are found within species with this technique, thus limiting its usefulness to the breeder. Chromatographic analysis of the volatile oils from leaf material is another useful method for biochemical characterization of *Citrus* species, hybrids, and related genera (Pieringer et al., 1964; Scora and Kumamoto, 1983; Scora et al., 1969). However, the chromatographic procedures, as described, are more elaborate than gel electrophoresis, so-the latter is used more commonly.

Several techniques are available 'for the preparation of volatile oil samples to be analyzed by gas chromatography, the most common method being steam distillation (Parliment, 1986). The large quantity of leaves needed to recover sufficient amounts of oil and, therefore, the few samples that can be evaluated limit the method's usefulness for breeding and genetic studies. Another method of sample preparation uses headspace techniques (Parliment, 1986), of which several variations exist. Generally, a carrier gas sweeps "through a suspension or over some specific plant material, and the volatile compounds are carried by the gas and collected elsewhere in the setup. This trapping is necessary to concentrate sufficient quantities of oil for analysis. The volatiles are released from the trap by a special temperature treatment and injected into the gas chromatography. Weinbaum et al. (1982) used seedling leaf tissue and a headspace sampling method without concentrating volatiles to identify hybrids in the progeny of an interspecific *Citrus* cross. Only a few volatile compounds in these gas samples were present in detectable concentrations. Parliment (1981) described a method for concentrating volatile materials from solutions by passage over a reverse-phase packing material that retains the volatile organics; trapped compounds can be desorbed in concentrated form with appropriate solvents.

The principles of using a carrier gas and adsorption on a reverse-phase packing material were used to design a simplified method for collecting volatile leaf oils from *Citrus* for breeding, genetic, and taxonomic studies. An experiment was performed with several sweet orange ('Valencia', 'Hamlin', and 'Navel') and grapefruit cultivars ('Marsh' and 'Ray Ruby'). Trees of all cultivars were planted together in a block in Apr. 1986 at the Citrus Research and Education Center, Lake Alfred, Fla., and grown under uniform cultural conditions. All trees were budded on 'Carrizo' citrange [C. *sinensis* × *Poncirus trifoliata* (L.) Raf.], except 'Ray Ruby' grapefruit, which was budded on 'Swingle' citrumelo *(C. paradisi* × *P. trifoliata).* Two trees per cultivar were randomly selected from the grove. Forty mature, healthy leaves were randomly collected from the same growth flush of each tree, cut in pieces (excluding the main leaf veins), and mixed together. Three subsamples were made for each tree by combining 1.0 g leaf material (≈ 30 pieces) with 10 ml distilled water in glass test tubes (25 × 150 mm). The samples were processed with a tissue homogenizer for 40 sec and immediately capped for storage at –12C. Tubes were placed in a 50C water bath to melt the suspension before volatile oil sample extraction.

Figure 1 is a schematic diagram of the apparatus used for the extraction of the leaf oils. The fundamental component of this new method is the use of a SEP-PAK C-18 cartridge (Waters Associates, Milford, Mass.) to trap the volatile oils released from the suspension (S. Nagy and M. Klim, personal communication). The C-18 cartridges were prewashed with 3 ml ethanol (HPLC-grade). Pressurized air, filtered through activated charcoal, was used as the carrier gas. A pressure regulator and divider were used to provide uniform air flow rates to each sample.

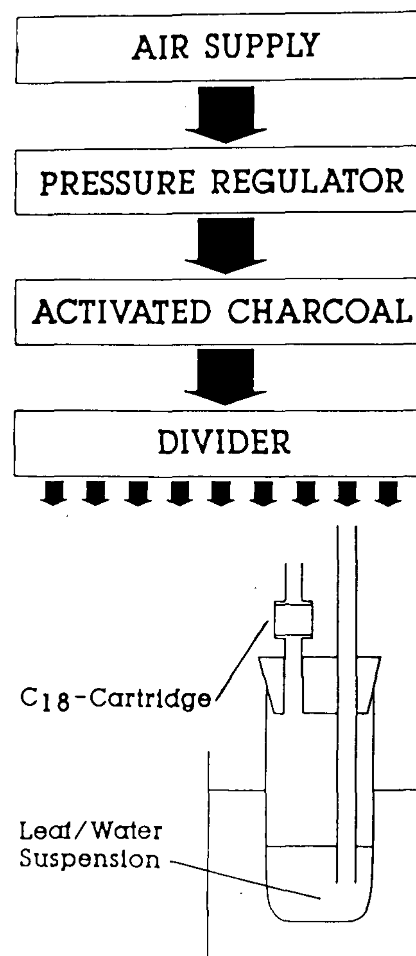

Fig. 1. Schematic diagram of apparatus used to extract *Citrus* volatile leaf oils. Pressurized air, filtered through activated charcoal, was bubbled through the leaf-water suspension for 2 h. Volatile oils were trapped by the C-18 cartridge. The tube is suspended in a water bath held at **50C.**

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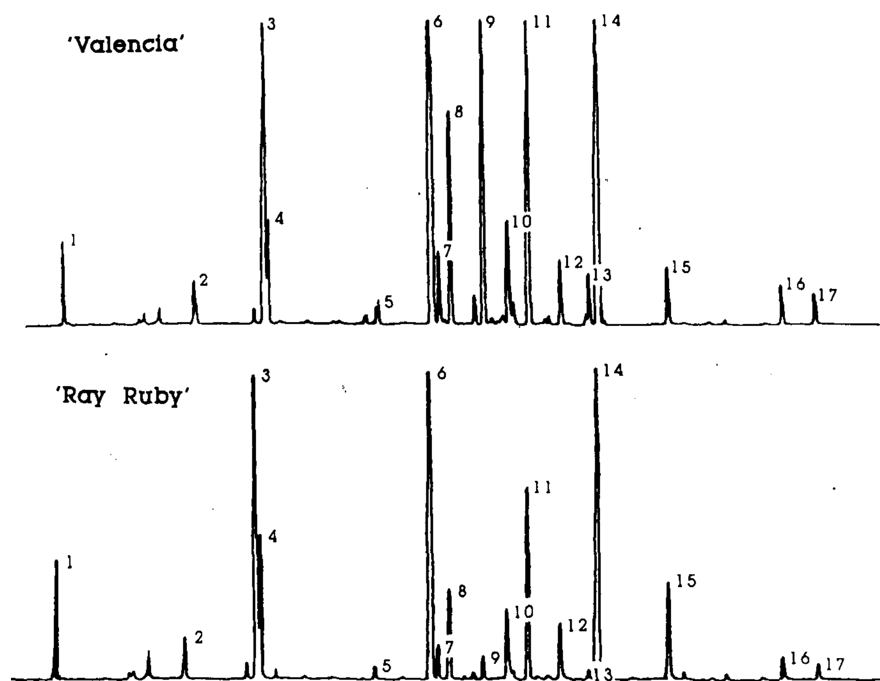

Fig. 2. Typical gas chromatograms of volatile leaf oil extracts from **(top)** 'Valencia' sweet orange and (bottom) 'Ray Ruby' grapefruit. Note differences between these cultivars at peaks 8, 9, and 13.

Air flow rates were carefully monitored during the procedure by observing the rate of bubble emergence through the sample suspensions. Eleven tubes were connected for each of three extractions, one subsample per tree plus one distilled water blank to monitor the purity of the carrier gas. During sample extraction, the tubes were placed in a 50C water bath to enhance the release of volatile oils. After air was passed through the system for 2 h, cartridges were disconnected, and condensed water was removed from their bases with tissue paper. The volatile compounds trapped by the C-18 cartridge were eluted with HPLC-grade ethanol. The concentration of volatiles was very high in the first few drops of eluent, but concentration decreased rapidly because only a few compounds could be detected in the second milliliter of ethanol (data not presented). In this experiment, only the first 0.2 ml of ethanol eluted through the C-18 material was collected in microcentrifuge tubes. The solutions were stored in the refrigerator and subsequently used for chromatographic analysis.

The analysis of the volatile leaf oils was performed using a Hewlett Packard (HP) Model 5890 gas-liquid chromatography (Avondale, Pa.). Proper mixing of the leaf oil extract before injection was essential because the mixture separated in layers during storage. Two injections of 2 µl leaf oil extract were made from each subsample. Injection temperature was set at 40C and held for 1 min. The temperature was increased to 180C at a rate of six degrees per minute. Hydrogen was used as the carrier gas with a split ratio of 80:1. Detection was by flame ionization. Injector and detector temperatures were set at 220 and 240C, respectively. The chromatogram was printed on an HP Model 3393 integrator that calculated peak area and retention time for each peak. Peak area values were used to calculate the relative percentage of each compound compared to the total of the 17 selected compounds. Several other leaf oil components were detected at concentrations too low to be reliable in the calculations and, therefore, were not considered.

Analysis of variance (ANOVA) was performed using the relative percentage values determined for each peak; the subsample mean square values were used as error terms. Significant differences were detected between trees within cultivars for some leaf oil components. In these specific situations, the sample mean square values for tree were used as error terms. Mean values were separated by Duncan's multiple range test with appropriate error terms, as indicated by the F statistics of the ANOVA.

Table 1 lists the mean relative percentage of 17 leaf oil components for each of the five cultivars and the statistical significance of F values for cultivar and trees within cultivar from analysis of variance. These values are based on 12 observations per cultivar (two trees × three subsamples × two injections). Significant differences between the two trees within a cultivar were detected for five of the components measured (peaks 1,4, 9, 10, and 15), but 14 of the 17 peaks showed significant differences among cultivars.

Mean separations revealed several differences between sweet oranges and grapefruit. Peaks 2, 8, 9, and 13 were useful in distinguishing grapefruit from sweet orange. Although there was significant variation among trees within cultivars at peak 9, cultivar differences were of greater statistical significance; in fact, the greatest contrast between sweet oranges and grapefruit was associated with peak 9. Leaf oil chromatographic profiles produced with this method from 'Valencia' sweet orange and 'Ray Ruby' grapefruit helped to visualize the differences between these species (Fig. 2).

Differences among cultivars within each species group were also evident. 'Marsh' and 'Ray Ruby' grapefruit produced similar patterns, but peaks 3 and 8 had significantly different mean values. It was possible to distinguish 'Hamlin' sweet orange from 'Valencia' and 'Navel' because of significant differences in mean relative percentage at six peaks (3, 12, 13, 14, 16, and 17). 'Valencia' and 'Navel' differed from each other at only three peaks (12, 16, and 17). The peaks that were important in distinguishing different

Table 1. Retention time and mean percentage of 17 volatile leaf oils among five *Citrus* cultivars.

Cultivar	Peak numbers																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	Retention time (min)																
	4.1	7.0	8.5	8.6	11.1	12.2	12.4	12.6	13.4	13.9	14.3	15.0	15.6	15.8	17.3	19.7	20.5
	Mean percentage[z]																
Valencia	1.58 b	1.11 b	15.27 c	3.08 c	0.65 a	31.66 a	1.18 a	3.85 a	6.16 a	2.49 ab	7.05 a	2.05 b	1.04 a	18.38 a	2.13 a	1.35 b	0.98 b
Hamlin	2.51 ab	1.39 b	20.90 b	3.47 bc	0.79 a	38.43 a	1.50 a	4.20 a	4.88 a	3.14 a	6.21 ab	0.86 c	0.83 b	8.88 b	0.92 a	0.62 c	0.47 c
Navel	1.97 b	1.05 b	17.02 c	3.24 bc	0.58 a	28.56 a	1.05 a	3.74 a	6.35 a	2.08 ab	6.64 a	2.83 a	1.11 a	17.62 a	2.59 a	2.07 a	1.50 a
Marsh	2.82 ab	2.63 a	21.76 b	4.96 ab	0.70 a	34.80 a	1.52 a	2.97 b	0.33 b	2.54 ab	6.16 ab	1.88 b	0.12 c	12.74 ab	3.05 a	0.56 c	0.45 c
Ray Ruby	3.69 a	2.38 a	27.14 a	5.73 a	0.60 a	30.42 a	1.33 a	2.47 c	0.24 b	1.80 b	5.31 b	1.74 b	0.08 c	14.23 ab	2.05 a	0.43 c	0.36 c
$F_{T/C}$[y]	***	NS	NS	**	NS	NS	NS	NS	***	***	NS	NS	NS	NS	***	NS	NS
F_C[x]	**	***	***	**	NS	NS	*	***	***	*	***	***	***	***	NS	***	***

[z]Mean separation within peak numbers by Duncan's multiple range test, $P = 0.01$.
[y]Significance levels of F values for trees within cultivars from ANOVA of each peak.
[x]Significance levels of F values for cultivar from ANOVA of each peak.
[NS,*,**,***]Nonsignificant or significant at $P = 0.05$, 0.01, or 0.001, respectively.

cultivars within a species showed no significant variation between trees of the same cultivar.

It is possible that some enzymic oxidation of volatiles occurred during extraction. Helium or nitrogen would have been preferred carriers to prevent oxidation; however, pressurized air was used because it was inexpensive and readily available. Cost and convenience are important considerations when evaluating many individual samples for breeding or genetic studies. As noted above, air was purified by passage through active charcoal and monitored, and no spurious peaks were noted. Further, oxidative enzymes in tissue homogenates could have been inactivated by addition of an organic solvent, such as isopropanol; this step was deliberately avoided to exclude another variable from the method. Our preliminary results demonstrated reproducibility, and if some oxidation of volatiles did occur, it did not hamper the ability to differentiate cultivars in this study.

This experiment was designed to evaluate the potential of a new method of leaf oil extraction and analysis for breeding and genetic studies of Citrus. The reproducibility of these results over a range of situations remains to be tested, but our results suggest that this method may successfully distinguish *Citrus* species and cultivars. Environmental and physiological factors can cause significant changes in leaf oil contents (Scora and Torrisi, 1966; Weinbaum et al., 1982). Therefore, this method will be most useful when sources of variation can be minimized or eliminated. However, the small amount of leaf tissue needed, the simplicity of sample preparation, and the demonstrated sensitivity suggest great potential for use of this method in *Citrus* breeding, genetic studies, and cultivar identification.

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