interaction of linear effect of added sterol × sterols analyzed was not quite significant at the 5% level. These results indicate that exogenously applied sterols are not transferred to the smoke condensate in the same quantities as endogenous sterols and that other added compounds such as alkaloids influence the amount recovered from the tobacco leaf, as well as that transferred to the condensate.

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Determination of Methyl Anthranilate in Grape Beverages by High-Pressure Liquid Chromatography and Fluorescence

Alun T. Rhys Williams and Walter Slavin

Methyl anthranilate has been determined to less than 0.05 μg/mL in grape beverages with no sample preparation beyond dilution. Using a fluorescence detector to provide very high sensitivity and selectivity for methyl anthranilate, the other products in commercial grape beverages produced no interferences in the method. The fluorescence detector was also shown to be useful in identifying other compounds in the samples. As little as 300 pg of methyl anthranilate was detected by this method.

Methyl anthranilate, a methyl ester of o-aminobenzoic acid, is the principal flavorant of Concord grape beverage products. A very recent AOAC method uses steam extraction, finishing with fluorescence (Casimir et al., 1976). In this paper we have applied reversed-phase liquid chromatography to the separation of methyl anthranilate from the other compounds in the commerical grape beverages. To enhance the sensitivity for this compound and provide selectivity from the many other compounds available in the commercial products, we have used a new fluorescence detector (Slavin et al., 1977).

We believe that considerable analytical time is saved by use of this method compared with any of the current procedures for methyl anthranilate because no sample preparation is required. In addition, this procedure appears to be more sensitive than the method of Casimir et al. (1976).

METHOD AND EQUIPMENT

The Perkin-Elmer Model 601 liquid chromatograph was used with a reversed-phase, ODS Sil-X-1 column used in an isocratic mode. The Perkin-Elmer Model LC-1000 was

Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire HP9 1QA, Great Britian (A.T.R.-W.) and The Perkin-Elmer Corporation, Norwalk, Connecticut 06856 (W.S.).

Table I. Chromatographic Conditions

Column	ODS Sil-X-1, 0.26 × 25 cm
Mobile phase	3% acetonitrile in water, buffered to pH 6 in 35 mM PO_4, 2 mL/min
Temperature	60 °C
Detectors	LC-1000 Fluorescence Detector Excitation: 330 nm Emission: 430 nm X50M, 5mV, Fast LC-55 Variable Wavelength UV 217 nm, 0.02 AUFS

used as the fluorescence detector. A Model LC-55 variable-wavelength UV detector was coupled between the fluorescence detector and the column to provide a general indication of the presence of peaks that could not be detected by fluorescence. Model 56 recorders were used with both detectors. The Perkin-Elmer Model 200 UV spectrophotometer was used to determine the optimum UV absorbance.

In Table I we show the general chromatographic conditions that were used for this analysis. About 3% acetonitrile is necessary in the aqueous eluent. The desired concentration of acetonitrile was achieved by using 10% acetonitrile in pump A and water in pump B. The mixing dial was set to 30%. This was done because it was occasionally useful to vary the acetonitrile content. The
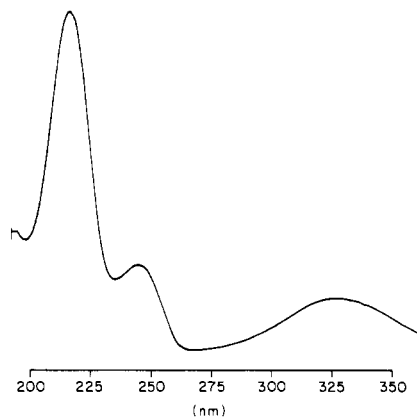
Figure 1. The UV spectrum of methyl anthranilate in 3% acetonitrile in water at pH 6. The absorbance maximum is shown to be 217 nm.
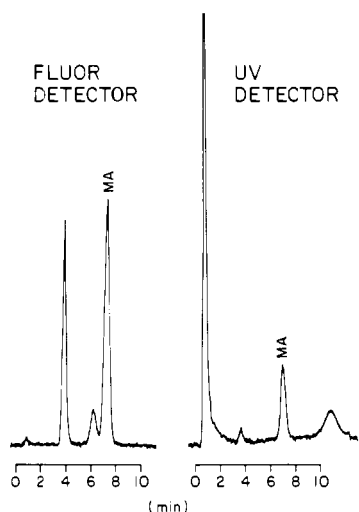

Figure 2. Comparison between fluorescence and UV detectors. The sample is 4 μL of a 1:1 dilution of a grape beverage to which the standard was added. MA is methyl anthranilate.

Figure 3. Methyl anthranilate additions to a 1:1 dilution in water of a Welch grape soda sample. Peak 1 is an unidentified compound and peak 2 is methyl anthranilate. Chromatogram A is the methyl anthranilate itself to confirm the retention time. B is the 1:1 dilution of the sample in water to which no methyl anthranilate has been added. C is the same dilution to which 4.1 μg/mL of methyl anthranilate has been added. D is the same dilution to which 8.2 μg/mL of methyl anthranilate has been added. E is the same dilution to which 16.4 μg/mL of methyl anthranilate has been added.

Figure 4. Working curve for methyl anthranilate by the method of additions. The data are from Figure 3.

solution was buffered at pH 6 for optimum fluorescence of the methyl anthranilate. A 35 mM PO_4 buffer was used.

During the course of the analysis we observed that the retention time for methyl anthranilate changed gradually from about 7.5 to 6.3 min. To minimize this problem in routine practice, the columns should be run overnight with a very low flow of solvent, thus continuously providing equilibrium conditions for the analysis.

Certain commercial samples contain compounds which are strongly retained by the column. If no precaution is taken, these compounds will elute during subsequent analyses, providing considerable confusion. These compounds from previous injections are identifiable because the peaks are wider than is to be expected. To obviate this problem, the column may be swept between runs with a strong solution of acetonitrile to remove the retained compounds from the head of the column.

TEST OF THE METHOD

Figure 1 shows a UV scan of methyl anthranilate. The standard material is from Eastman Chemicals, recrystallized and made up in the aqueous acetonitrile solvent used for the LC. The spectrum shows that the maximum absorbance is at 217 nm. A UV detector limited to 254 nm would be inadequate for this analysis.

In Figure 2 are chromatograms of 4 μL of a 1:1 dilution of Welch grape soda with 16 μg/mL of standard added. The fluorescence detector and the UV detector are compared. The fluorescence detector is at least three times
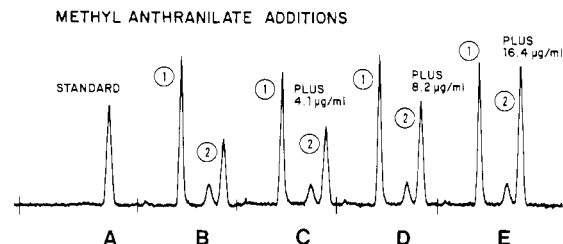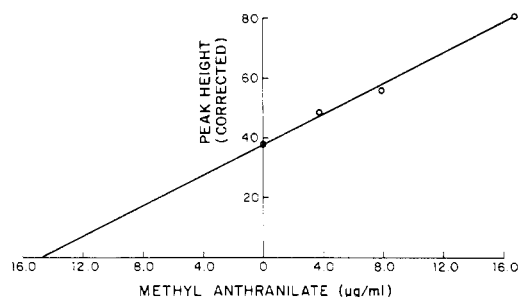
more sensitive than UV and has the additional advantage of being selective for fluorescing compounds like the methyl anthranilate. The retention times shown in Figure 2 are typical of this study. They apply equally to Figures 3, 5, and 6.

Figure 3 shows chromatograms for the methyl anthranilate standard and a dilution of a Welch grape soda sample, showing increasing additions of methyl anthranilate. The additions were made by preparing a solution containing 82 μg/mL of methyl anthranilate, recrystallized from the Eastman material as recommended by Casimir et al. (1976). In the first chromatogram the standard is shown to confirm the retention time. The successive chromatograms each represent 3 μL of a dilution of the grape soda sample with 0, 0.5, 1.0, and 2.0 mL of the 82 μg/mL solution added to 10 mL of the grape soda sample, which was then diluted to 20 mL with water. Thus the additions are 4.1, 8.2, and 16.4 μg/mL, expressed in terms of the original soda sample.

These additions are plotted against the fluorescence signal in Figure 4, using the method of additions to extrapolate the amount of methyl anthranilate present in the original sample (chromatogram B). Since there may be small variations in the amount of sample injected and in the dilutions, peak 1 is used as an invariant internal standard between the runs. The ratio of the amplitude of peak 1 between the second chromatogram (B) and each successive chromatogram is used as a correction for the observed peak height of the methyl anthranilate peak (2) in each analysis. This corrected peak height is plotted against added methyl anthranilate in Figure 4. The extrapolation of this linear curve to zero peak height provides an indication of the amount of methyl anthranilate present in the original samples, 14.6 μg/mL in this particular sample.

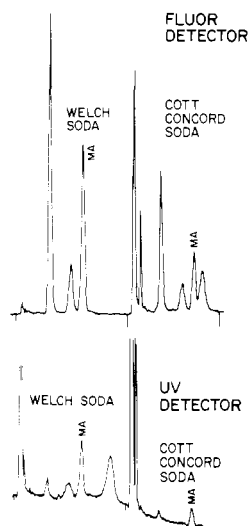The detection limit of methyl anthranilate can be calculated from the curves in Figure 2. The 4-μL mixture

Figure 5. The analysis of two grape beverage products. The examples shown represent both fluorescence and UV detector chromatograms for products typical of those analyzed. Six microliters of the sample were injected at the head of the column. Quantitation was accomplished by use of standards. Analytical results are shown in Table II.

Table II. Beverage Samples Tested

Name	Label description	MA concn, μg/mL
Cott Concord Grape Soda	Natural and artificial	3.0
Welch's Grape Juice	Vitamin A, C, B_1, B_2, niacin, etc.	1.1
Welchade Grape Drink	With some vitamins	<0.05
Welch's Grape Soda	Artificial flavors	8.8

contained 16 ng/μL, or 64 ng. Using the criterion of twice the noise level, the detection limit is about 0.3 ng (300 pg). Assuming a sample size of 10 μL of undiluted beverage, we can detect 0.03 μg/mL of sample.

Casimir et al. (1976) were able to detect 0.1 μg/mL of methyl anthranilate in a 10-mL sample of the Concord grape juice, after steam distillation. Using 10-μL samples directly on the column, we can easily detect 0.05 μg/mL of methyl anthranilate, with no sample preparation being required.

ANALYSIS

Using the conditions previously described, a group of commercial beverage products was analyzed with the fluorescence detector and with the UV detector at 217 nm. The fluorescence and UV detector responses on two of the products are shown as examples in Figure 5.

It happened that we used a new column for this group of samples. It is noteworthy that the new column provided retention times very similar to those found in the earlier work and that the sensitivity was very similar.

The methyl anthranilate content varies greatly between the different samples. The samples and the label descriptions are shown in Table II. Methyl anthranilate ranges from not detectable in the Welchade (less than 0.05 μg/mL) to 8.8 μg/mL in the Welch grape soda.

Note the early eluting components found in certain of the beverages. Since these fluoresce very strongly, they appear to be vitamins that have been deliberately added to the beverages.

QUALITATIVE ASPECTS

An effort was made to identify some of the other compounds detected in the grape soda by the fluorescence
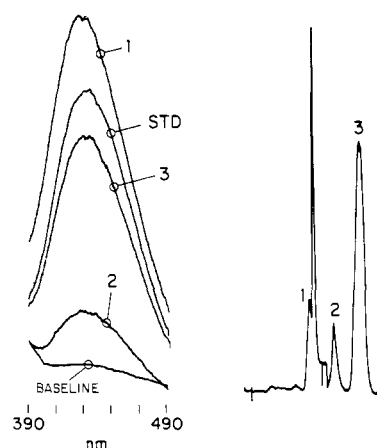

Figure 6. Emission scans from a typical grape beverage product. The chromatogram on the right of the Welch grape soda was stopped at each peak and the fluorescence emission curve was recorded.

COMPOUND	STRUCTURE	PEAK WAVELENGTH (nm)
p-AMINO BENZOIC ACID		416
ANTHRANILIC ACID		416
METHYL ANTHRANILATE		432
METHYL SALICYLATE		452

Figure 7. Fluorescence emission peak wavelength is shown with respect to the position of various substituent groups. Excitation was at 330 nm.

analysis. Figure 6 shows emission scans for the methyl anthranilate standard and for the three peaks. As each peak entered the fluorescence cell the flow was stopped and the emission monochromator was scanned. The two unidentified peaks have their emission maxima at essentially the same wavelength as the methyl anthranilate, about 430 nm. The excitation wavelengths are also essentially the same, as tested by using several excitation filters.

The Model 1000 was used as a conventional fluorescence spectrophotometer to study the fluorescence emission of compounds similar to methyl anthranilate to see what effect various substituents would have on the wavelength of the emission band. Figure 7 shows the structure of p-aminobenzoic acid, anthranilic acid, methyl anthranilate, and methyl salicylate. Changing the position of the NH_2 band does not alter the wavelength peak. However, addition of other substituents produces considerable shift in the wavelength peak. Since the emission peaks for the other compounds in the grape juice of Figure 6 are essentially at the same wavelength as the methyl anthra-

nilate, we would speculate that they are isomers of the methyl anthranilate.

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Aroma Quality Evaluation of Tomatoes, Apples, and Strawberries

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For aroma quality evaluation of fresh fruits and vegetables the procedure used for isolation of the volatiles from the food matrix is of the highest importance. Representative isolation of tomato, apple, and strawberry volatiles is performed by adsorption of the volatiles, liberated while macerating the material, on Tenax. Injection via reconcentration in a cooling trap and gas chromatographic–mass spectrometric analysis showed chromatograms, which when compared with other isolation procedures had a simpler composition with higher amount of organoleptic important compounds. However, a major problem of the described procedure was the contamination of the chromatogram of aroma compounds by organic air pollutants.

Until recently the major concern in quality of fresh fruits and vegetables has been focused on external appearance attributes such as color, size, shape, and external defects. However, due to several reasons (such as excessive production; selection of varieties for optimal external quality, optimal production, and resistance to infections, without concern for flavor quality; longer storage times, etc.) problems have arisen concerning the flavor of several economically important fruits and vegetables and a decreasing flavor quality has been observed. The first step in solving this problem is the establishment of a method to judge the organoleptic quality of fresh fruits and vegetables on an objective basis. Indeed, in dialogue with cultivators one could, e.g., select varieties for optimal flavor characteristics in order to come to an improved flavor of economically important fruits and vegetables.

Flavor quality of fruits and vegetables is a combination of an impression on the tongue (taste), mainly determined by the acid–sugar ratio and an impression on the nose (aroma), due to the volatile organic compounds. From these two analyses the aroma analysis is the most difficult to perform and flavor quality evaluation should concentrate on the study of the volatile organic compounds, which determine aroma. Complex mixtures of volatile organic compounds can be analyzed by high-resolution capillary gas chromatography. However, an important aspect is the isolation of the volatile organic compounds from the food matrix. For flavor quality evaluation it is important to isolate a mixture of volatiles, which is representative for the mixture, that we unconsciously send to the nose while eating. Isolation procedures such as steam distillation and solvent extraction are useless for that purpose. Classical head space analysis (10–20 mL sample injection) is not compatible with high-resolution capillary gas chroma-

tography and preconcentration of the volatiles is necessary before analysis. We wish to report on a method for head-space analysis by collection of the volatiles (liberated while macerating the material) on Tenax, injection via reconcentration in a cooling trap and a capillary gas chromatographic–mass spectrometric analysis. The selection of Tenax as adsorbing agent has been inspired by pollution chemistry (Bertsch et al., 1974) and the injection system is an adaption of the system described by Flath et al. (1969a).

Up to now investigations have been concentrated on tomatoes, apples, and strawberries. Fundamental studies with emphasis on component identification have been performed by several laboratories: apples (Schultz et al., 1967; Flath et al., 1967; Drawert et al., 1969; Flath et al., 1969b), tomatoes (Schormüller et al., 1969; Viane et al., 1969; Kazeniac and Hall, 1970; Buttery et al., 1971), strawberries (Winter and Willhalm, 1964; McFadden et al., 1965; Tressl et al., 1969; Black et al., 1971). According to Flath the components directly associated with the characteristic apple-like aroma are: ethyl 2-methylbutyrate, *n*-hexanal, and *trans*-2-hexenal (Flath et al., 1967). Also strawberry flavor has been associated with several volatile organic esters (Black et al., 1971). Several compounds have been suggested to be important to fresh tomato flavor: *trans*-2-hexenal (Kazeniac and Hall, 1970), *n*-hexanal (Kazeniac and Hall, 1970), *cis*-3-hexen-1-ol (Kazeniac and Hall, 1970), β-ionone (Buttery et al., 1971), *trans,trans*-2,4-decadienal (Buttery et al., 1971), and especially 2-isobutylthiazole (Viane et al., 1969; Kazeniac and Hall, 1970; Buttery et al., 1971), and *cis*-3-hexenal (Kazeniac and Hall, 1970; Buttery et al., 1971; Guadagni et al., 1972).

The occurrence and concentration of these organoleptic important compounds in a chromatogram of a representatively isolated flavor mixture is a suitable method for flavor quality evaluation (Dirinck et al., 1975, 1976). In the present study the new isolation procedure is illustrated for apples, tomatoes, and strawberries and its advantages

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