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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–FLUOROMETRIC DETERMINATION OF CINNAMALDEHYDE IN PERFUME, COLOGNE AND TOILET WATER

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SUMMARY

A high-performance liquid chromatographic (HPLC)–fluorometric method is described for the determination of *trans*-cinnamaldehyde in fragrances. The fragrance is added to isooctane and extracted with an aqueous solution of the sodium salt of 6-aminocaproic acid to isolate the aldehyde fraction. After dilution with water, an aliquot of the extract is added to a solution of 1,2-diaminonaphthalene monosulfate in dilute formic acid. The fluorescent derivative of cinnamaldehyde, 2-styrylnaphth[1,2-*d*]imidazole, is prepared by incubating and then cooling the solution and adding pyridine. Aliquots of the fluorophore solution are analyzed on a reversed-phase C_{18} HPLC column by using a buffered tetrahydrofuran–water eluent. Cinnamaldehyde is quantitated by comparing fluorescence emission intensity with that of a standard. Recoveries from samples of various commercial fragrances, spiked with cinnamaldehyde at the 0.01, 0.05 and 0.1% levels, ranged from 94 to 112% with a mean of 103% and a standard deviation of 5.3. The limit of detection is approximately 1 ng.

INTRODUCTION

Trans-cinnamaldehyde (*trans*-3-phenyl-2-propenal) occurs naturally in a wide range of concentrations in oils of cinnamon, cassia, hyacinth, myrrh and Bulgarian rose[1]. It is synthesized commercially by the alkaline catalyzed condensation of benzaldehyde with acetaldehyde[2]. The average level of use of cinnamaldehyde in perfumes is approximately 0.1%[3]. Cinnamon oil or cinnamaldehyde is also widely used to flavor foods, candy, dental products, medicinals and beverages. From his review of toxicological data, Opdyke[3] concluded that cinnamaldehyde is responsible for eliciting a significant number of skin sensitization responses in human subjects.

Cinnamaldehyde and other aldehydes have been determined by gas chromatography (GC)[4]. Isonicotinoyl hydrazones of cinnamaldehyde and other aldehydes were separated and semiquantitatively determined by circular thin-layer chromatography[5]. Cinnamaldehyde can also be determined fluorometrically by allowing it to react with either 4,5-dimethoxy-1,2-diaminobenzene[6] or 1,2-diaminonaphthalene monosulfate (DNS)[7,8] to form fluorescent derivatives.

In the method described here, the aldehyde fraction of the fragrance is isolated by extraction with a basic aqueous solution of 6-aminocaproic acid. An aliquot of the diluted extract is added to a solution of DNS in formic acid. The solution is incubated, then cooled and made slightly basic by the addition of pyridine. The solution of the fluorophore is analyzed by high-performance liquid chromatography (HPLC)–fluorometry to determine cinnamaldehyde content.

EXPERIMENTAL

Reagents

DNS–formic acid reagent. The sulfate of 1,2-diaminonaphthalene (Pfaltz & Bauer, Stamford, CT, U.S.A.) was recrystallized from water and dried in vacuum with phosphorus pentoxide, and 22 mg was dissolved in 1.5 ml 88% formic acid (ACS grade, Fisher Scientific, Pittsburgh, PA, U.S.A.) with stirring and heating. The solution was made up to 50 ml with degassed water. DNS–formic acid reagent can be stored overnight in the refrigerator.

6-Aminocaproic acid extraction solutions. Extraction solution 1 was prepared by adding 1.74 g of 6-aminocaproic acid (Pfaltz & Bauer) to 25 ml of degassed water, and adding 5 ml of 2.6 *M* sodium hydroxide solution (prepared from degassed water) with stirring until the acid dissolved. The pH was adjusted to 12.6–12.8. Extraction solution 2 was prepared as above, but 10 ml of 95% ethanol was added.

Cinnamaldehyde standard solutions. The following standard solutions were prepared on the day of use from a stock solution, which was kept in the refrigerator under nitrogen, of 10 mg cinnamaldehyde (99%; Aldrich, Milwaukee, WI, U.S.A.) per ml ethanol: 0.02 mg, 0.01 mg, and 0.002 mg/ml degassed water.

Cinnamaldehyde fluorophore standard solutions. Standard solutions were prepared in duplicate by adding 4 ml of freshly prepared DNS–formic acid reagent to 1-ml aliquots of cinnamaldehyde standard solutions, which were kept at 95–98°C for 35 min, then cooled in ice water for 20 min. After addition of 2 ml pyridine (ACS grade, J. T. Baker, Phillipsburg, NJ, U.S.A.) to each solution, they were diluted to 10 ml with tetrahydrofuran (THF) (distilled-in-glass, MC/B Manufacturing Chemists, Norwood, OH, U.S.A.). The fluorophore standard solutions can be stored overnight in the refrigerator.

A fluorophore blank was prepared by diluting 1 ml of ethanol, 3 ml of extraction solution 1 and 4 ml extraction solution 2 to 50 ml with degassed water. A 1-ml aliquot of this solution was added to 4 ml of DNS–formic acid reagent, and the procedure for preparation of the fluorophore standard solutions was followed, except that a duplicate was not prepared.

HPLC eluent. Triethanolamine (0.7 g, ACS grade, Eastman-Kodak, Rochester, NY, U.S.A.), laurylpyridinium chloride (0.17 g, ICN K&K Labs., Plainview, NY, U.S.A.) and THF (180 ml) were diluted to 300 ml with water. The pH of the eluent was adjusted to 7.8–7.9 with dilute acetic acid. The eluent was filtered through a 0.45-μm filter and degassed.

Apparatus

The HPLC system consisted of a Model 244 liquid chromatograph and a Model 6000A solvent delivery system; a Model 420 fluorescence detector, equipped

with a 365-nm narrow-band excitation filter, a 460-nm band-pass emission filter and source No. F4T5-BL, all from Waters Assoc. (Milford, MA, U.S.A.); an injector valve (Rheodyne, Cotati, CA, U.S.A.), equipped with a 20-μl loop; and a Zorbax ODS column, 250 × 4.6 mm I.D. (DuPont, Wilmington, DE, U.S.A.), with a column temperature of 40 ± 1°C. The column was pre-equilibrated with 50–60 ml of eluent, and the flow-rate was 1.0 ml/min. An A-25 strip-chart recorder with 10 mV input (Varian, Palo Alto, CA, U.S.A.) was used.

Sample preparation

A volume of 10 ml of isooctane (ACS grade), 3.0 ml of extraction solution 1 and 1.0 ml of the sample were agitated for 2 min in a separatory funnel. The aqueous extract was transferred to a 50-ml volumetric flask and the extraction was repeated with 4 ml of extraction solution 2. The aqueous extracts were combined and diluted to volume with degassed water. Duplicate fluorophore sample solutions were prepared according to the procedure described under *Cinnamaldehyde fluorophore standard solutions.*

Determination

Standard solutions were repeatedly injected until reproducible peak heights indicated that the column was equilibrated. Then standard solutions were analyzed in duplicate, peak heights were averaged, adjusted to common attenuation (sensitivity) and a calibration curve was established. Sample solutions and standards were injected alternately. The amount of cinnamaldehyde in the sample was estimated by comparison with the standard curve. Standard solutions falling within ±20% of the cinnamaldehyde estimated to be in the sample were used for the determination. The fluorophore blank value was subtracted from sample and standard peak heights. If the response slowly increased or decreased with time, injections were made at timed intervals to minimize this error.

RESULTS AND DISCUSSION

The method was validated by recovery studies on commercial fragrance compositions that were shown to contain no cinnamaldehyde or its acetals. The spiking solution consisted of cinnamaldehyde dimethyl acetal and cinnamaldehyde in a molar ratio of 2:1 in ethanol. This solution was added to 20% ethanolic solutions of the fragrance oils at levels corresponding to 0.01, 0.05 and 0.1% cinnamaldehyde. To inhibit the possible hydrolysis of the cinnamaldehyde dimethyl acetal by acidic fragrance oils, the ethanol used for dilution of fragrance oils, stock solutions, and the spiking mixture was saturated with sodium bicarbonate. Preliminary work indicated that aqueous solutions of cinnamaldehyde dimethyl acetal are slowly hydrolyzed at pH 6–7. Six spiked fragrance solutions were analyzed in triplicate by the method at each of the three levels. The mean recovery and standard deviation from these analyses were 103 and 5.3%, respectively.

Fragrance compositions are complex mixtures of natural and synthetic ingredients that typically contain over 100 compounds. The determination of cinnamaldehyde in these products requires an analytical system which combines good chromatographic resolution with relatively selective detection. An HPLC or GC method

based on non-specific detectors cannot be used for the determination of cinnamaldehyde in fragrances, because of the high probability of interference from other ingredients. The required selectivity of the proposed method was achieved by first isolating the aldehyde fraction from the fragrance and then converting the aldehyde to a fluorescent derivative.

The isolation of the aldehyde fraction from the other fragrance ingredients is necessary because fragrances frequently contain acetals of cinnamaldehyde and other aldehydes. Under the acidic conditions used to prepare the fluorescent derivative, cinnamaldehyde acetals are readily hydrolyzed to form cinnamaldehyde and, therefore, interfere.

The use of basic solutions of 6-aminocaproic acid for isolating the aldehyde fraction is based on the work of Ohta and Okamoto[9]. They reported quantitative aqueous extraction of cinnamaldehyde from diisopropyl ether with a 1.2 *M* aqueous solution of the sodium salt of 6-aminocaproic acid. The Schiff base from the amino acid and aldehyde was decomposed by acidification and the cinnamaldehyde was quantitatively recovered. In our hands, sodium 6-aminocaproate gave recoveries of less than 50%. We therefore investigated the effect of pH on the extraction efficiency of the aminocaproic acid reagent. Extraction solutions containing equal amounts of 6-aminocaproic acid and increasing levels of sodium hydroxide were prepared, and the pH of each extraction solution was measured. Isooctane solutions of cinnamaldehyde were extracted with aminocaproic acid at different pH values and the amount of unextracted cinnamaldehyde remaining in isooctane was determined by GC. A plot of pH *vs.* percent cinnamaldehyde recovered is shown in Fig. 1. From these data we selected a pH of 12.7 for the extraction solutions. At this pH, 96% of the cinnamaldehyde is removed in a single extraction. Two extractions should recover more than 99% of the cinnamaldehyde. The Ohta and Okamoto procedure[9] was modified not only by controlling the pH of the extraction reagent, but also by substituting isooctane for diisopropyl ether as the organic phase in order to avoid the possibility that peroxides would oxidize the cinnamaldehyde.

Aldehydes react with 1,2-diaminonaphthalene under acidic conditions to form substituted naphth[1,2-*d*]imidazole derivatives. When a solution of the derivatives is made basic, two fluorescent mesomeric tautomers are formed. Zaitsu and Ohkura[7,8] found that the cinnamaldehyde derivative, 2-styrylnaphth[1,2-*d*]imidazole, had excitation and emission maxima (370 and 460 nm, respectively) that were of longer wavelengths than those of most of the aldehydes studied. In addition, aromatic aldehydes that were nitro-, amino- or hydroxyl-substituted showed almost no fluorescence. These factors greatly reduce the possibility of interference by other aldehydes.

In order to determine the fluorescent cinnamaldehyde derivative by HPLC, several modifications of the Zaitsu and Ohkura procedure[7,8] were necessary. Reversed-phase HPLC columns which contain silica cannot be used above pH 8, because a significant amount of silica is then dissolved and the column is gradually destroyed. Thus, samples were adjusted to pH 7.8–8.0 by substituting pyridine for sodium hydroxide. Other organic amines such as triethanolamine, diethanolamine, and morpholine were also screened as substitutes for sodium hydroxide. Optimal fluorescence emission intensity was obtained when pyridine was used as the base. The plot of cinnamaldehyde concentration *vs.* fluorescence intensity, however, gave a curve of much smaller slope than would be expected. Because the change in fluores-

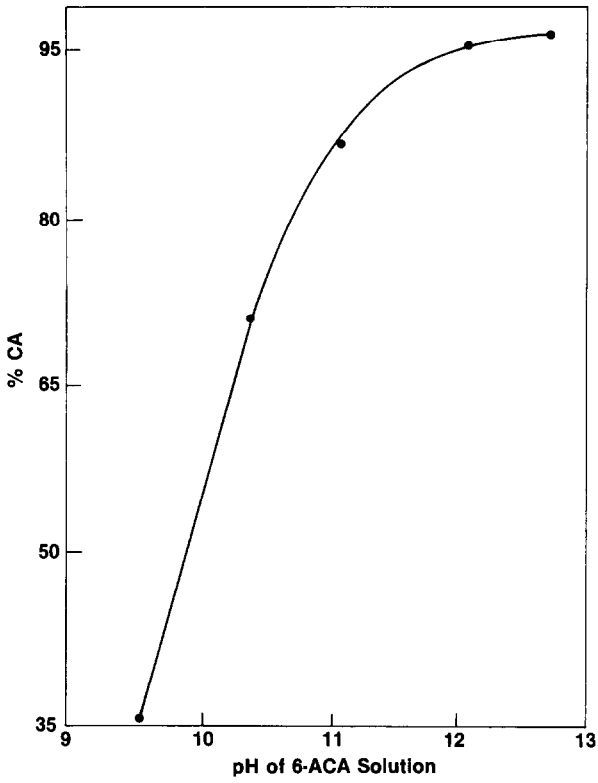

Fig. 1. Recovery of cinnamaldehyde (CA) *vs.* pH of 6-aminocaproic acid (6-ACA) solution.

cence intensity produced by a change in concentration was relatively small, the accurate determination of cinnamaldehyde would have been difficult. A study of reaction conditions demonstrated that formic acid was more suitable than sulfuric acid as the catalyst. Peak intensities were significantly higher and more reproducible for all solutions in which formic acid was used, and the standard curve had a much greater slope. A large excess of DNS was used in this procedure to allow for the presence of other aldehydes in the fragrance.

Cinnamaldehyde undergoes rapid oxidation in air to form cinnamic acid, benzoic acid and other products. For this reason it is very important to protect the cinnamaldehyde standard and standard solutions from atmospheric oxygen and oxidizing agents. A fresh, sealed bottle of cinnamaldehyde is approximately 99% pure and may be used without further purification. After a bottle is opened, the headspace should be filled with nitrogen, and the bottle should be tightly resealed and stored in the refrigerator. Older samples that have been previously opened must be distilled under vacuum; only the constant boiling fraction is collected. The distilled cinnamaldehyde should be kept in an amber bottle, which is filled to the top, sealed tightly and stored in the refrigerator. Oxidation of cinnamaldehyde in standard solutions is minimized by using freshly boiled HPLC-grade water. All cinnamaldehyde standard solutions must be freshly prepared on the day of analysis.

Problems were encountered in the selection of the eluent. Aqueous THF had much higher emission intensities than aqueous methanol or acetonitrile, but gave very poor peak height reproducibility. Because the intensity of fluorescence emission is pH-dependent, it was necessary to buffer the eluent at pH 7.8–7.9 to obtain reproducible data. A number of buffer systems consisting of phosphoric or acetic acid in combination with morpholine, triethanolamine, diethanolamine, or piperidine were evaluated. Acetic acid–triethanolamine was judged to be the most suitable buffer. A small amount of lauryl pyridinium chloride was added to the eluent to improve the reproducibility of the data. It has been reported[10] that when the eluent contains small amounts of quaternary ammonium salts, the active sites on the silica packings are masked, thereby minimizing tailing of aromatic amines.

The HPLC column is maintained in a 40°C water bath to reduce back pressure and to stabilize conditions. After the column is equilibrated with 50–60 ml of eluent, the peak heights of a given standard solution may gradually increase or decrease with time. Apparently, complete equilibrium is attained very slowly. For this reason, it is recommended that a standard be injected before and after the sample injection and that the two standard solution values be averaged. The standard curve is non-linear in the range of interest, 0.002–0.02 mg cinnamaldehyde/ml. For this reason, it is important that the standard solution used for the determination be approximately (±20%) of the same concentration as the sample. The minimum detectable amount of cinnamaldehyde is 1 ng. This corresponds to a cinnamaldehyde concentration of 0.0025% in the product.

Possible interference from other aldehydes used in fragrances was investigated. α-Methylcinnamaldehyde, α-*n*-amylcinnamaldehyde, α-hexylcinnamaldehyde, 3-phenylpropylaldehyde, phenylacetaldehyde, citral and 2-phenylpropanal were analyzed. With the specified excitation and emission filters, only α-methylcinnamaldehyde gave a significant fluorescence peak. However, it was well separated from the cinnamaldehyde peak.

The proposed method was developed for the determination of cinnamaldehyde in commercial fragrance products. It should also be applicable, possibly with minor modifications, to other products, such as food, candy, beverages, and toothpastes that are flavored or scented with complex mixtures of essential oils.

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