A New Approach to the Phenolic Components in Beer. Application to the Determination of Sinapic, Ferulic, and *p*-Coumaric Acids

A new approach is described for the determination of trace quantities of some phenolic compounds in beer. Specific procedures for simple phenolic acids have been developed and applied to nine commercial beers. The range of sinapic acid (0.056–1.04 μg/mL), ferulic acid (0.159–0.615 μg/mL), and *p*-coumaric acid (0.063–0.274 μg/mL) was determined for these products with a relative standard deviation of ±2.8%. The detection limit of these three acids was found to be about 20 pg using liquid chromatography with electrochemical detection. Many other compounds can be quantitated with equivalent sensitivity and precision using the general approach developed for phenolic acids.

Phenolic compounds play an important role in the determination of beer stability and flavor characteristics (Dadic, 1974a,b). Simple monocyclic phenolic acids apparently result from the degradation of higher molecular weight anthocyanogens (Harris and Ricketts, 1958, 1959; Dadic et al., 1970, 1971; Dadic and Belleau, 1973; Dadic 1974b). Subsequent free radical coupling of the simple phenolic acids may in turn result in a variety of complex products.

The existing methods used for the determination of beer phenolics are not entirely satisfactory, relying on multidimensional paper or thin-layer separations utilizing nonspecific visualization reagents, UV–visible absorption, and fluorescence for identification (Dadic and Van Ghelume, 1971; Gramshaw, 1973; Dadic, 1976). High-performance liquid chromatography (HPLC) has entered the field in a study of "model" systems of beer phenolics (Charalambous et al., 1973) and has recently been used with high-vacuum sublimatography for the assay of beer phenolics (Dadic and Belleau, 1975). High-vacuum sublimatography has also been used with a gas chromatograph (GLC) for the assay of these compounds (Dadic and Belleau, 1975). As in all GLC systems the nonvolatile phenolics require derivatization (Dallas et al., 1967).

Assay procedures developed in our laboratory for the quantitative identification of tyrosine metabolites using liquid chromatography with electrochemical detection (LCEC) have been found to be widely applicable to other compounds in clinical samples and food products (Kissinger et al., 1974, 1975; Riggin et al., 1975; Felice and Kissinger, 1976; Felice et al., 1976; Pachla and Kissinger, 1976). The present paper describes the application of this new technique to the determination of some phenolic constituents in commercial beers. The approach described is generally applicable to all nonpolymeric acidic, neutral, and basic phenols in plants and products derived therefrom.

EXPERIMENTAL SECTION

Reagents. The following reagents were used: (1) hydrochloric acid, 6 M; (2) mobile phase (pH 3.8), combine one part 1.0 M acetate buffer (pH 4.7), one part 0.25 M citric acid (pH 1.8), and eight parts doubly distilled water; (3) Folin and Ciocalteaus's reagent (Sigma Chemical Co.); (4) bottled beers, purchased from commercial distributors.

Apparatus. The liquid chromatographic system was similar to that previously described (Riggin et al., 1975) using an electrochemical detector (Bioanalytical Systems, Model LC-40). Zipax SAX (Du pont) pellicular anion-exchange resin was dry packed in a 50 cm × 2.1 mm glass column (Altex Scientific, Model 251-02). Separations were carried out under ambient conditions (25 ± 1 °C). The mobile phase was pumped at a rate of 0.49 mL/min. The amperometric detector was set at an applied potential of +0.80 V vs. SCE.

Procedure. Beer samples were acidified to pH 2 with 6 M HCl and stored frozen at −35 °C prior to analysis. Two milliliters of beer (pH 2) in a 12-mL glass centrifuge tube was saturated with NaCl and extracted with 2 mL of ethyl acetate. After shaking the tube for 10 min on a reciprocal shaker and centrifuging briefly, the ethyl acetate layer was transferred to a 6-in. culture tube using a disposable Pasteur pipet. The extraction was repeated with three additional 2-mL volumes of ethyl acetate. The combined ethyl acetate layers were dried over anhydrous sodium sulfate (ca. 1.0 g) and then transferred to an acid-washed 12-mL centrifuge tube. The residual sodium sulfate was washed with approximately 1 mL of ethyl acetate which was then combined with the original extract. The solvent was evaporated to dryness under a stream of nitrogen at 20 °C. (The use of elevated temperatures causes a significant loss of the easily air-oxidized *o*-dihydroxycinnamic acids.) Identification of phenolic compounds was accomplished using a thin-layer chromatography isolation step, followed by HPLC separation according to literature procedures (Felice and Kissinger, 1976).

Quantitative results for well-resolved compounds were obtained by dissolving the residue of the original ethyl acetate extraction in 2 mL mobile phase and injecting 25 μL of this solution directly into the LCEC analyzer. Quantitation of selected phenolic components was accomplished by comparing peak heights in selected beers against calibration curves prepared from standard solutions. The standard addition technique gave results which were indistinguishable from those obtained using calibration curves for external standards. Due to the complexity of the phenolic mixtures often encountered in food products, it is not wise to assume that every HPLC peak represents a single compound. In cases where coretention of two or more components is encountered, quantitative work may necessitate the use of a preliminary TLC step to effect greater overall selectivity. The advantages of the TLC–LCEC approach have been noted in earlier publications from this laboratory (Felice and Kissinger, 1976; Felice et al., 1976).

RESULTS

Sinapic, ferulic, and *p*-coumaric acid levels were determined as an example for nine commercial beers. Relative amounts of these well-resolved phenolic acids in beer are presented in Table I. Samples were also run after acid hydrolysis of the beers at pH 1 and 95 °C for 10 min. As a result of the acid hydrolysis the levels of certain phenolic acids were increased due to the cleavage of conjugates. Significant changes also occurred when beer samples were subjected to alkaline hydrolysis (pH 10) at room temperature for 10 min. Comparable findings have been reported for the analysis of nonbiological hazes in beer (Harris, 1965), where the breakdown of antho-

Table I. Concentration of Selected Phenolic Acids in Commercial Beers[a]

Sample	Sinapic acid, μg/mL	Ferulic acid, μg/mL	p-Coumaric acid, μg/mL
Coors	0.071	0.468	0.226
Budweiser	0.056	0.615	0.234
Schlitz	0.516	0.250	0.147
Stroh's	0.357	0.464	0.274
Old Milwaukee	0.516	0.159	0.063
Pabst Blue Ribbon	0.266	0.373	0.226
Braumeister	1.04	0.543	0.274
Miller's Lite	0.286	0.321	0.159
Miller's High Life	0.579	0.413	0.127

[a] The concentrations reported represent averages obtained from no fewer than four samples with a relative standard deviation of ± 2.8%.

cyanogens results in the formation of simple phenolic compounds which may undergo oxidative coupling reactions to form polyphenolic molecules.

The absolute recoveries of sinapic, ferulic, and *p*-coumaric acids of 88 ± 3% and 91 ± 3% (relative standard deviation) were found to be constant over a concentration range from 0.024 to 2.4 μg/mL in beer. The overall precision of the method was ±2.8% relative standard deviation. A linear detector response was obtained over the range 6.0–60 ng injected. The detection limit of the three phenolic acids in aqueous standard solutions was about 20 pg. Chromatograms representative of the various extraction steps are shown in Figure 1.

DISCUSSION

Phenolic compounds play an important role in determining a beer's stability and flavor characteristics. Any technique which allows for ease in quantitation of these flavor-stability components may be valuable for routinely monitoring the quality of the brew. High-performance liquid chromatography with electrochemical detection (LCEC) has been demonstrated to be a powerful new technique for study of phenolic compounds. Numerous compounds previously investigated (Felice and Kissinger, 1976) were reexamined under the present chromatographic conditions to quantitatively monitor potentially significant beer phenolics. A liquid chromatographic separation of beer phenolics is presented in Figure 1. The separation for a whole beer sample is illustated in Figure 1A. The separation of phenolics extracted from beer at pH 2 with ethyl acetate is shown in Figure 1B. The residual beer phenolics following ethyl acetate extraction resulted in chromatogram 1C. A comparison of the figures indicates that acidic and neutral compounds are quantitatively extracted into ethyl acetate.

Though adequate resolution of *some* beer phenolics is possible with the isocratic conditions described here, increased separation is required. This can be obtained by the use of gradients, microparticle columns, and/or the combined TLC–LCEC approach. Sinapic, ferulic, and *p*-coumaric acids are well resolved under present conditions, and levels were quantitated in a variety of commercial beers (Table I). Though these simple phenolic compounds may result from the degradation of complex anthocyanogenic species during the brewing process, it is interesting to note that these common phenolic acids are also formed in sequence via the shikimic acid pathway. Sinapic (3,5-dimethoxy-4-hydroxycinnamic) acid is formed from ferulic (3-methoxy-4-hydroxycinnamic) acid which results from *p*-coumaric (4-hydroxycinnamic) acid. The phenolic acid levels may reflect the composition of the brewing hops. Regardless of their origin, the simple

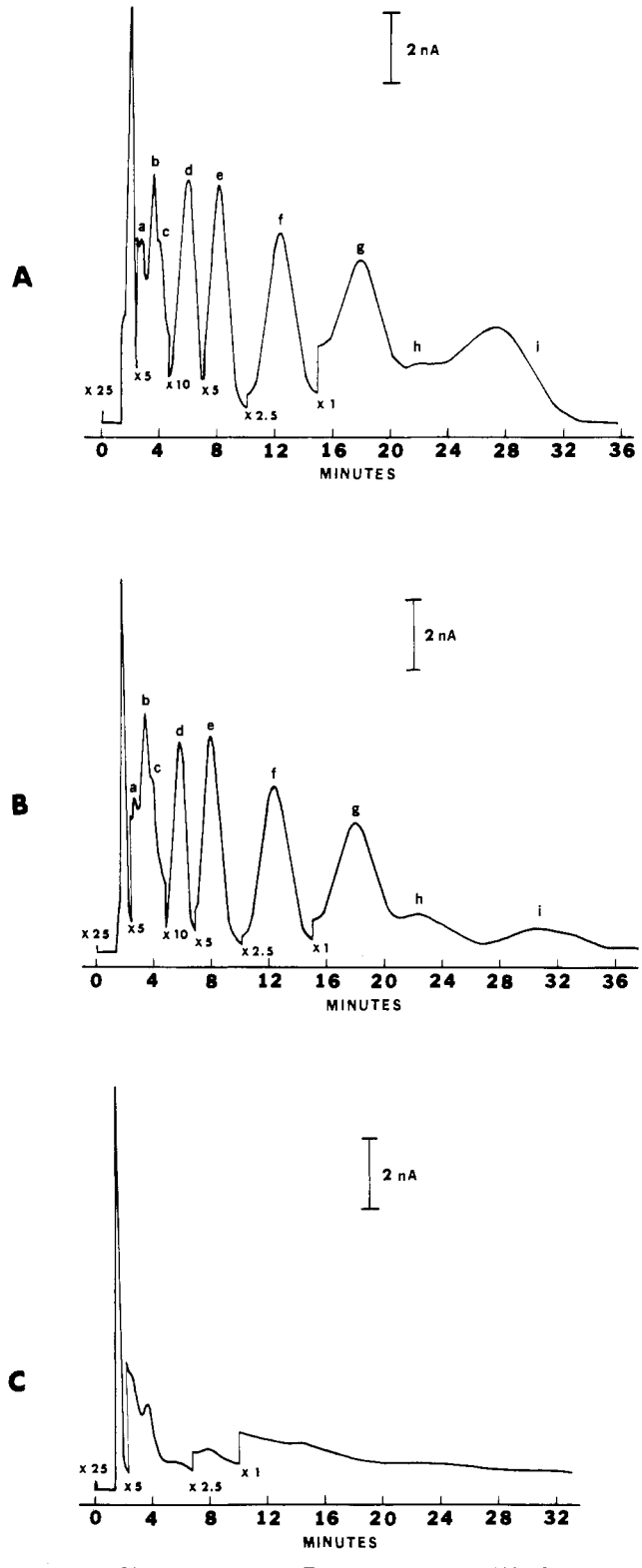

Figure 1. Chromatograms of Braumeister beer: (A) whole beer, (B) whole beer extract, (C) residuals following extraction. Identification of components using TLC–LCEC: (a) syringic acid, (b) vanillic acid, (c) gallic acid, (d) sinapic acid, (e) ferulic acid, (f) *p*-coumaric acid, (g) caffeic acid, (h) D-catechin, (i) gentisic acid.

phenolic acids apparently constitute a flavor-stability center of beer, and improved techniques for routine monitoring of their concentration should lead to a better understanding of the brewing process. The methodology described here can be adapted to selective determination of neutral phenols (e.g., flavonoid compounds) and basic

phenols (e.g., alkaloids derived from tyrosine) by utilization of microparticle reverse phase columns. This has been recently demonstrated in a study of phenolic constituents in *Theobroma cacao* (Kenyhercz and Kissinger, 1977).

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Thomas M. Kenyhercz
Peter T. Kissinger*

Department of Chemistry
Purdue University
West Lafayette, Indiana 47907

Received for review November 10, 1976. Accepted February 28, 1977. Support for this research from the National Science Foundation (GP-42452X), the National Institute of General Medical Sciences (GM-22713-02), and the Showalter Trust Fund is gratefully acknowledged.

A Convenient Method for Multiple Extraction of Volatile Flavor Components from Food Slurries and Pulps Using a Two-Chambered Glass Bomb Extractor and Dichlorodifluoromethane (Freon 12) Solvent

A two-chambered glass "bomb" apparatus has been designed for multiple extraction of volatile flavor components from food slurries and pulps. The solvent, dichlorodifluoromethane (Freon 12) is low boiling (−29 °C) and relatively inert and at no time during extraction or concentration process is the food, solvent, or extract warmed above room temperature. Using this method, extracts of good reproducibility and high organoleptic quality from various fruit pulps have been obtained.

Among the most important tasks facing a researcher or technologist in a flavor study is obtaining a representative and organoleptically true sample of volatile components for investigation. A poor initial flavor extract usually leads to difficult interpretations or erroneous results and conclusions at subsequent stages of the work. The task is made more difficult by the varying nature of food products. Such factors as high or low water content, lipid content or solids content, and stability or lack of it with heat or other external conditions have brought about numerous procedures for obtaining flavor extracts. The usual methods involve some combination of distillation or stripping and extraction by solvent. One must always consider the possibility of contamination or artifact formation in the presence of a reagent or external factors such as heat.

Fluorocarbons have been used by many workers as extraction solvents, primarily for alcoholic beverages, fruit essences, and vegetables (for examples see Stanley et al., 1963; Schiede and Bauer, 1967; Schultz et al., 1967; Hardy, 1969; Stevens et al., 1969). These solvents have been shown to be very good for this purpose because they are relatively inert, nontoxic, nonexplosive, noninflammable, and in most cases, give an extract of very high organoleptic quality. The most commonly used fluorocarbon has been Freon 11 (bp 23.7 °C), primarily because of ease of handling at room temperature. Schultz et al. (1967) has indicated that Freon 114 (bp 4 °C) is a preferable solvent since the lower boiling point allows easier solvent stripping. In our laboratories, partially freeze-dried fruit puree and fruit skins standing at −30 °C in Freon 12 (bp −29 °C) have yielded good extracts (Ballschmieter and Torline, 1973; Torline and Ballschmieter, 1973).

We would like to report here a simple, easily constructed, and inexpensive glass bomb apparatus which allows convenient and relatively rapid extraction of volatile components from aqueous food slurries and pulps using the low-boiling Freon 12 as solvent.

EXPERIMENTAL SECTION

Extractor Design. A diagram of the extraction bomb appears in Figure 1 and is self-explanatory. The construction is entirely of heavy-walled Pyrex glass and the upper tube is sealed with a pressure-tight screw down Teflon plug.

Extraction Procedure. The aqueous food pulp or slurry (300 mL) is placed in the lower chamber by means of a long-stem funnel. The entire bomb is then cooled at −30 °C in a cold room and 300 mL of Freon 12 is added. The system is sealed, placed in a protective steel-mesh