Gas Chromatography/Principal Component Similarity System for Detection of *E. coli* and *S. aureus* Contaminating Salmon and Hamburger

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Coho, Atlantic, Spring, and Sockeye salmon and five commercial samples of hamburger patties were analyzed by processing gas chromatography (GC) data of volatile compounds using the principal component similarity (PCS) technique. PCS scattergrams of the samples inoculated with *Escherichia coli* and *Staphylococcus aureus* followed by incubation showed the pattern-shift lines moving away from the data point for uninoculated, unincubated reference samples in different directions with increasing incubation time. When the PCS scattergrams were drawn for samples incubated overnight, the samples inoculated with the two bacterial species and the uninoculated samples appeared as three separated groups. This GC/PCS approach has the potential to ensure quality of samples by discriminating good samples from potentially spoiled samples. The latter may require further microbial assays to identify the bacteria species potentially contaminating foods.

Keywords: *GC; principal component similarity; salmon; hamburger; E. coli; S. aureus*

INTRODUCTION

Gas chromatography (GC) has been applied to pure culture to identify bacterial species. Cellular fatty acids (Athalye et al., 1985), carbohydrates (Fox et al., 1993), and metabolites (Zechman et al., 1986) from pure cultures were analyzed by GC. The GC metabolite analysis was utilized in detecting spoilage of foods (Eyles and Adams, 1986). A review paper on rapid detection of food-borne pathogenic bacteria (Swaminathan and Feng, 1994) identified two commercially available GC systems, that is, the Microbial Identification System (MIS) and the Biological Identification System. All previous studies were intended to determine the causes of food spoilage by identifying bacterial species in the contaminated foods.

In this study, metabolites were analyzed directly from food samples without culturing in standard media. The intention of our study was to warrant the safety of the samples rather than to identify the causes of spoilage. The analysis should, therefore, ensure that the samples are free from volatile metabolites, which are produced as results of contamination of products with bacteria or by other causes such as chemical or enzymatic reactions. Therefore, it was not our intention to accurately identify species of pathogenic bacteria contaminating spoiled foods. Furthermore, use of the principal component similarity (PCS) program was attempted, instead of cluster analysis or principal component analysis (PCA), which had been utilized for bacteria species differentiation, as exemplified in the above MIS system.

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Principal component similarity (PCS) developed by Vodovotz et al. (1993) computes a pattern similarity coefficient using principal component scores instead of the original chromatographic data directly. Because of no requirement of a priori knowledge on sample grouping, the PCS is an unsupervised classification technique (Aishima and Nakai, 1991). Despite the fact that artificial neural networks (ANN) may presently be the most accurate algorithm for nonlinear classification and prediction, PCS is simpler and more comprehensive, with an easier interpretation of the classification results than ANN because no hidden variables are used in the computation. Furthermore, the unsupervised technique is sometimes advantageous because it includes no influence of preconceived image of sample grouping, which may be occasionally incorrect. An example is the finding of an unexpected new type of beany flavor in soy milk, which a sensory panel could not detect (Wang et al., 1998). Another advantage of the PCS is the suitability of evaluating changes, such as cheese aging (Furtula et al., 1994a,b).

The PCS was applied to GC data of sterilized milk and successfully distinguished samples inoculated with *Pseudomonas fragi*, *P. fluorescences*, *Lactococcus lactis*, *Enterobactor aerogenes*, and their mixed culture from uninoculated samples (Horimoto et al., 1997). Because GC patterns as a whole but not individual volatile compounds are required for this classification, expensive GCs for precise analysis may not be needed for routine inspection purposes when the costs of analysis are most critical.

The objective of this study was to confirm the feasibility of applying the GC/PCS system for purposes of quality assurance of salmon and hamburger. *Escherichia coli* and *Staphylococcus aureus* were chosen as important infecting bacteria as they were the main

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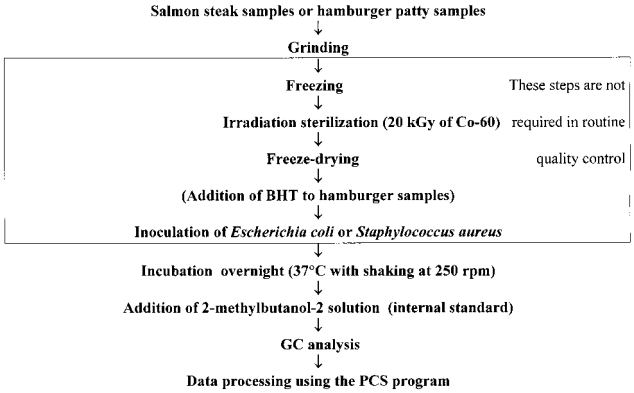

Figure 1. Flowchart of the assay method.

concern of local food inspectors. Because this study is the first of its kind, the covering of all pathogenic bacteria important in food poisoning, such as *Salmonella* and *Pseudomonas*, was not intended. Confirmation of the feasibility of the new approach was our major interest.

EXPERIMENTAL METHODS

Samples. Salmon steak samples were the products of local manufacturers. Hamburger patty samples were purchased from local supermarkets.

Bacteria Strain and Culture Conditions. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as test organisms. These strains were subcultured and finally grown in L-broth (10 g of bactotrypton, 5 g of bactoyeast extract, 5 g of NaCl, 1 g of glucose, and water to 1 L) at 37 °C overnight. The fresh cells were diluted with L-broth and microscopically adjusted to give the concentration of 10^3 cells/mL by using a hematometer. One hundred microliters of the cell suspension was added to the test sampe in 5 mL of saline solution. Bacterial number was determined with MacConkey agar and mannitol salt agar (Difco, Detroit, MI) for *E. coli* and *S. aureus*, respectively.

Sterilization and Inoculation. Samples to be inoculated were sterilized by irradiation as follows (Figure 1): Burger patty samples (10 cm diameter × 2 cm) and salmon cut samples (20 × 4 × 2 cm) were stored overnight at −35 °C. The sliced samples were then irradiated with 20 kGy of ^{60}Co (IOTRON, Port Coquitlam, BC) in frozen state and then freeze-dried. The dried samples were ground into fine powder, packed, and sealed in sterile plastic bags and then stored frozen until analyzed. The freeze-drying was preferred to simple freezing to carry out uniform, homogeneous sampling from the sliced samples. To 0.1 g of the powdered samples in test tubes was added 25 μg of BHT in 5 μL of ethanol to the hamburger patty samples only (20 mg/L BHT). The samples with and without BHT dissolved in 5 mL saline solutions were inoculated with 20 bacterial cells/mL. After vortexing, the inoculated samples were incubated at 37 °C while rotating at 25 rpm.

GC Analysis. After incubation for the preset duration, 25 μL of a 30 ppm 2-methyl-2-butanol solution was added to the incubated samples as an internal standard. The internal standard was used to standardize peak areas as well as elution times as described below. The mixtures were applied to a dynamic headspace SRI 8610 GC with FID (SRI Instrument, Torrance, CA) installed with a DB-624 megabore 75 m × 0.53 mm capillary column coated with 3.0 μm thick stationary phase (J&W Scientific, Rancho Cordova, CA). Chromatographic conditions were as follows: purge temperature, 52−53 °C; preheat time, 5 min; purge time, 15 min; trap temperature, 20 °C; desorbing temperature, 185 °C; temperature program, hold at 40 °C for 30 min, ramp to 80 °C at 10 °C/min, ramp to 220 °C at 4 °C/min, hold for 20 min; detector temperature, 250 °C.

Data Processing. The GC data of peak areas and elution times were transformed using a tailored program for entering into the PCS program (Vodovotz et al., 1993; Horimoto et al., 1997) written in Visual Basic 4 for Windows 95. To identify each peak, a widow in the form of $t_p/(t_l - t_i)$ was used. The quantities t_p, t_l, and t_i are elution times of a peak, a distinct peak at the last portion of the GC profile, and the peak of the internal standard. The principle of PCS is to compute a similarity coefficient, which is similar to a correlation coefficient (r) in the formula (Datta and Nakai, 1992) using principal component (PC) scores obtained from principal component analysis of the original peak areas of GC patterns.

To make classification easier, instead of using the similarity coefficient alone (one dimension), linear regression analysis of [deviation of PC scores of unknown sample from those of reference sample] on [variability accounted for by PC scores] was carried out. The number of PC scores used is dependent on their eigenvalues, which are ≥1.0; therefore, usually four scores were used for the linear regression. Slope versus coefficient of determination (r^2) of the linear regression analysis was plotted as a PCS scattergram (Vodovotz et al., 1993). Therefore, when a sample GC profile is perfectly identical to the reference profile, the slope and the coefficient of determination are both 1.0. The PCS scattergram is a 2D illustration, which is more effective and useful for classification of samples than the 1D expression using similarity coefficient alone.

RESULTS AND DISCUSSION

Effects of Salmon Species Differences. The peak development is slow until 9h of incubation, but characteristic patterns have appeared after 12 h in inoculated salmon samples. In general, the pattern changes are more pronounced for samples inoculated with *S. aureus* than for those inoculated with *E. coli*, with marked differences in the early one-third of GC patterns (Figure 2). When 0 time patterns are compared, Atlantic salmon appears different from other species. It is unclear if this difference is due to the fact that this species was shipped from a fish farm on Vancouver Island and other species were caught from the Pacific Ocean.

Figure 3 show the PCS scattergrams of four salmon species. Data points of *S. aureus* and *E. coli* samples shift in different directions from the reference point at coefficient of determination and slope both being 1.0. The slopes of the *S. aureus* samples always increase more quickly than those of the *E. coli* samples. Whereas the uninoculated Coho and Spring salmon move only slightly, Atlantic slamon moves substantially, with Sockeye slamon being intermediate. A reason for data point shifts of uninoculated samples seems to be lack of freshness and possible postcatch contamination of the samples leaving enzymes in muscle which are difficult to destroy by irradiation. The Atlantic species was a fish-farm product shipped to Vancouver after preparation of steak. Therefore, it is uncertain if the data point shift of this species is due to the species difference.

When the scattergram was drawn using only samples incubated for 12 h, with E3 (*E. coli*, Spring salmon) as a reference, three separate groups of *E. coli*- and *S. aureus*-incubated and uninoculated samples appeared on the scattergram (Figure 4). E1 is an outlier for reasons unknown. The only explanation is that 12 h is not adequately long for clear distinction with a great chance of introducing error into the 12 h data (Figure 3). At any rate, these results show that the contamination of salmon with *E. coli* and *S. aureus* can be detected with minimum influence of species difference of salmon.

Effects of Different Sources of Hamburger Patties. Figure 5 shows the GC patterns of uninoculated

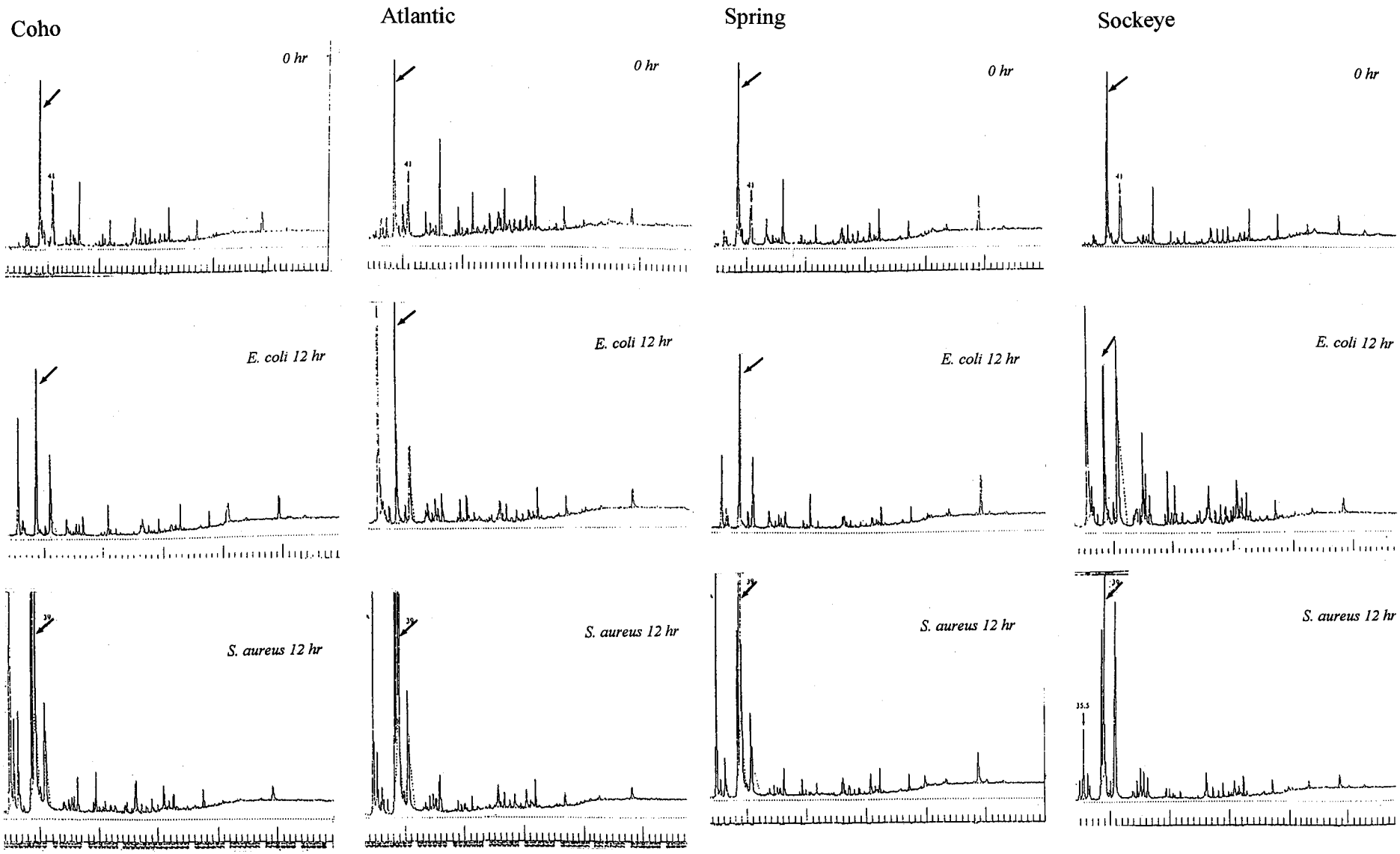

Figure 2. GC patterns of four species of salmon: Coho; Atlantic; Spring; Sockeye. The peak at ~39 min is the internal standard (the locations are marked by arrows).

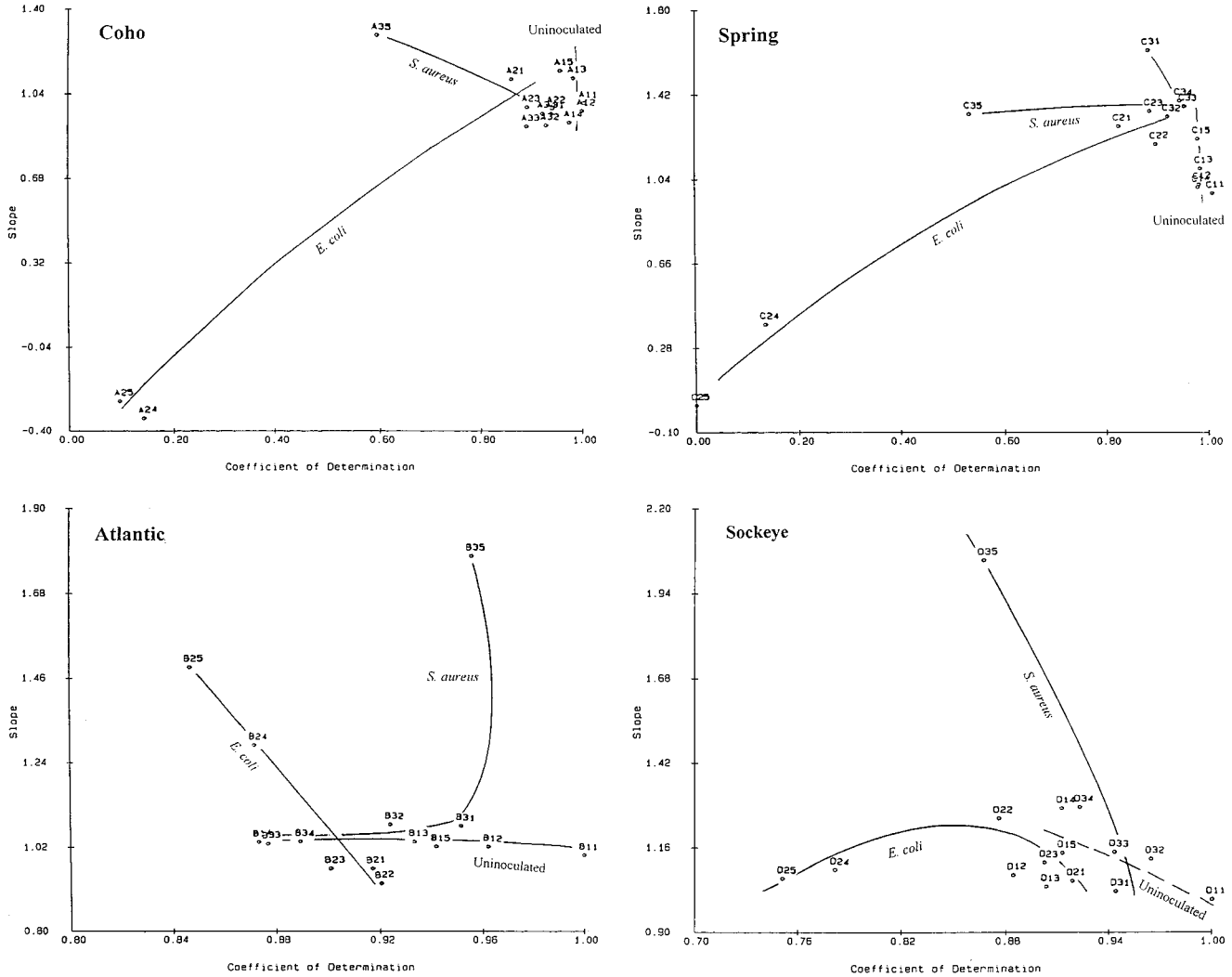

Figure 3. PCS scattergrams of four species of salmon: Coho; Atlantic; Spring; Sockeye. Digits following A−D are the incubation times (in hours) of samples prior to application to GC.

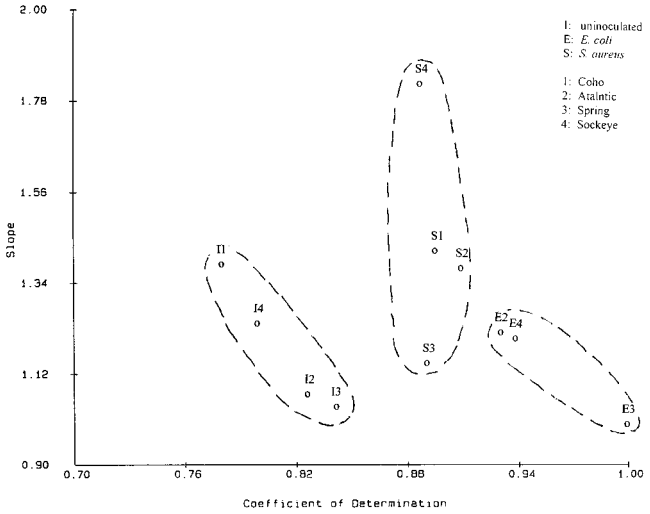

Figure 4. PCS scattergram of four species of salmon incubated for 12 h.

hamburger samples. Zero-hour samples of burgers A−E are slightly different from each other, especially burger E, which has many more peaks at the middle of the pattern. However, there are no appreciable changes until 15 h of incubation, except for burger B, which has a remarkable increase in the peak as marked with an asterisk. Hamburger samples are different from salmon in terms of freshness. It was difficult to obtain samples of fresh beef from where the hamburger patty samples were prepared.

Figure 6 is the GC patterns of inoculated samples. Whereas no substantial changes were observed until 12 h (data not shown), considerable changes can be detected after 15 h of incubation. Similar to the case of salmon, many more peaks appear from *S. aureus*-inoculated samples than from *E. coli*-inoculated samples, which appear at the early one-third portion of GC patterns. However, the patterns of *E. coli*- and *S. aureus*-infected hamburgers are not the same as those of salmon at the portion earlier than the internal standard marked with an arrow and the last portion of GC patterns.

Figure 7 shows the PCS scattergrams of hamburger patty samples. Contrary to salmon, hamburger shows steeper upward shifts for *E. coli* than for *S. aureus*. The substantial shift of uninoculated burger B in Figure 7 is unusual. Because these samples of burger B were sterilized by irradiation, this shift should not be due to bacterial growth. Possibly, it is caused by enzyme actions, and there may be quite different enzyme systems in different hamburger meat due to variable environments during post-mortem storage. It should be emphasized that the PCS is measuring a pattern

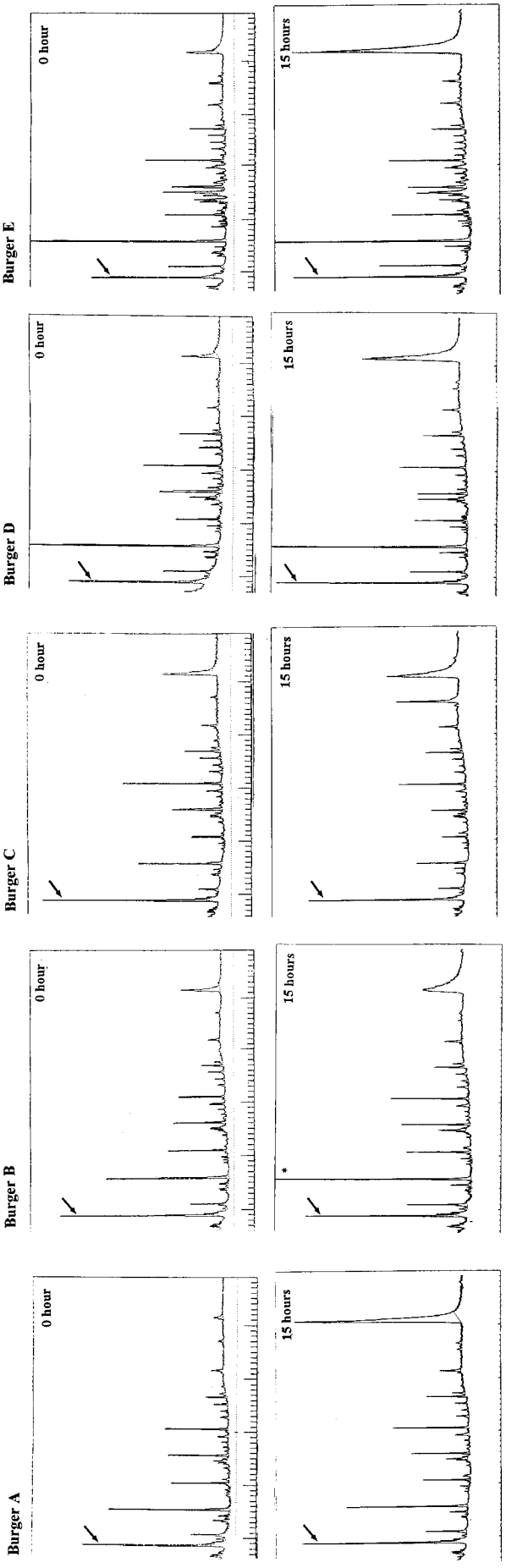

Figure 5. GC patterns of five hamburger patty samples, uninoculated. Early peaks (prior to elution time of 35 min) were not included in the PCS computation because they were volatiles characteristic of beef samples without relation to spoilage.

similarity and not the changes in specific volatile compounds. As seen in Figure 7, *Staphylococcus* shifts farther than *Escherichia* except for burger B. This must be a reflection of more extensive changes in the GC pattern for *S. aureus* than for *E. coli* as shown in Figure 6.

Probably because of considerable nonsimilarity among volatiles in hamburger patties at 0 time due to difference in post-mortem conditions, classification using incubated sample alone, which was similar to data in Figure 4, was unsuccessful. However, contamination with bacteria can be detected using a PCS scattergram (Figure 7) despite the difference in samples before incubation (Figure 5). Therefore, microbial contamination of hamburger samples may be detectable because the GC patterns would not show changes similar to inoculated samples as shown in Figure 7. Shift of the patterns in the direction toward uninoculated samples does not mean contamination with at least two bacterial species tested in this study. Although irradiation sterilization was used in this study for inoculation experiments, comparison with a reference sample made from meat as fresh as possible with a minimum shift on the PCS scattergram such as burgers A, D, and E should be adequate for routine quality control (Figure 1). For conducting inoculation experiments, an ethanol washing of beef patty samples was tried for sterilization in our laboratory with partial success.

BHT was added to hamburger patties after irradiation in this study to prevent oxidation of lipids in the samples, thereby eliminating the effects of volatile production due to lipid oxidation but not bacterial growth. However, BHT simultaneously retarded bacterial growth (Fung et al., 1985); therefore, longer incubation was needed (Figure 8). The overnight incubation reached a plateau for almost all cases. For general food inspection purposes, it is recommended not to add BHT; thus, the assay process may be simplified with an additional advantage of detecting objectionable odors, such as rancidity (Horimoto, 1996), not only bacterial infection. This may confuse the classification; however, the speeds of developing volatiles during incubation between microbial and chemical effects should be different.

Advantages of the GC/PCS Approach. Swaminathan and Feng (1994) cautioned readers about the barriers to implementation as "a food may be simultaneously tested for the presence of all pathogens by simple modification of a procedure to include several types of selective media in the analysis. Such generality is not feasible with current rapid methods, because most systems target only a single pathogen. If several rapid assay kits are needed to do multipathogen analyses on one food sample, rapid analysis becomes prohibitively expensive". A rapid method for trouble-free samples, such as our approach as described in this study, may then be quite useful for multipathogen analysis. After clearing safe samples by using our GC/PCS method, the above rapid detection of food-borne pathogenic bacteria can be applied to the only potentially spoiled samples screened by the GC/PCS assay.

Advantages of our approach that have been introduced in this study are as follows: (1) There is no need for sophisticated, precise GC systems; it is possible that electronic noses (Mermelstein, 1997) can replace GC used in this study. (2) The GC/PCS system does not require a large database, such as used in the MIS,

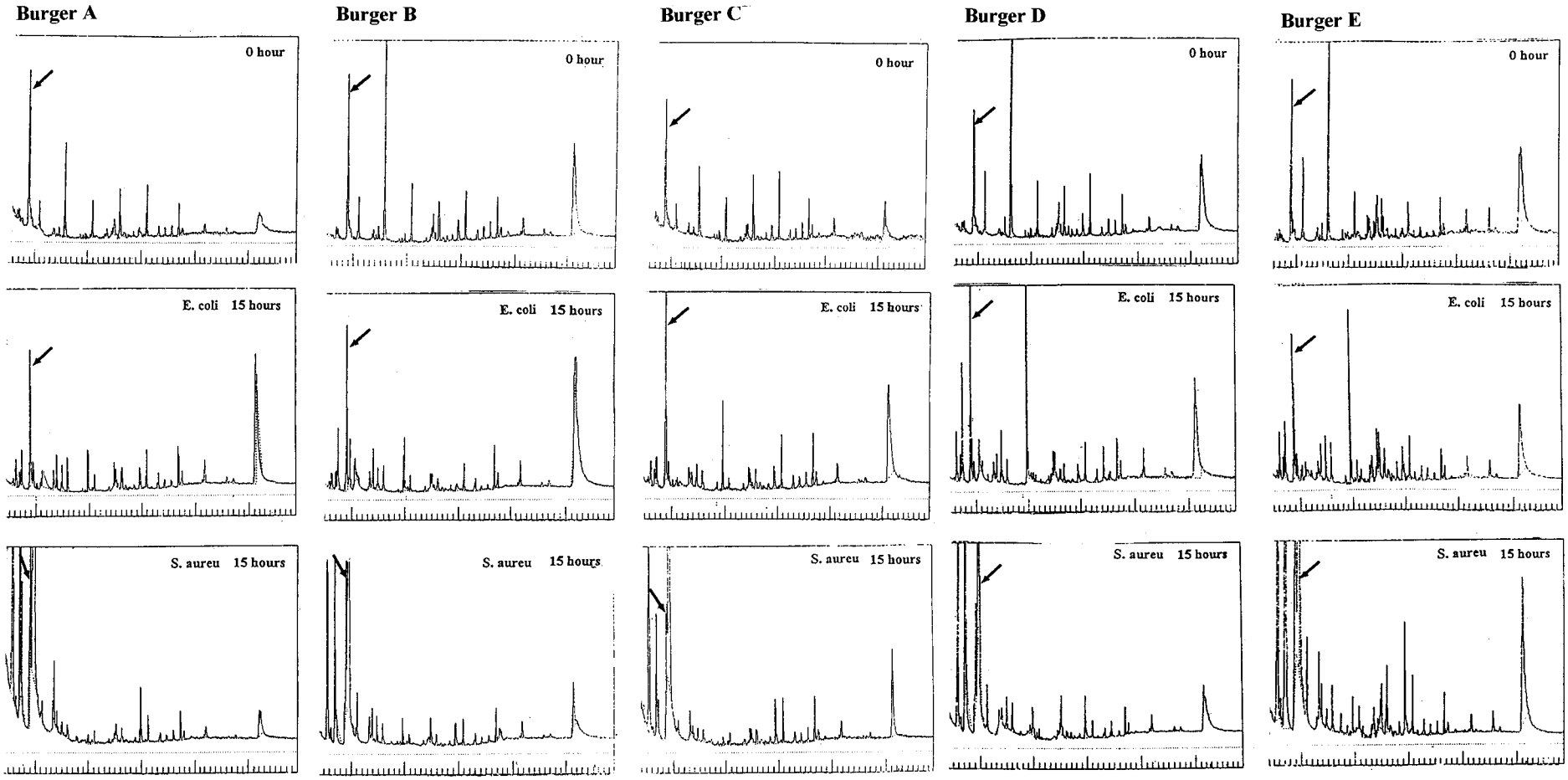

Figure 6. GC patterns of five hamburger patty samples inoculated with *E. coli* and *S. aureus*. Early peaks up to elution time 35 min were not included in the PCS computation.

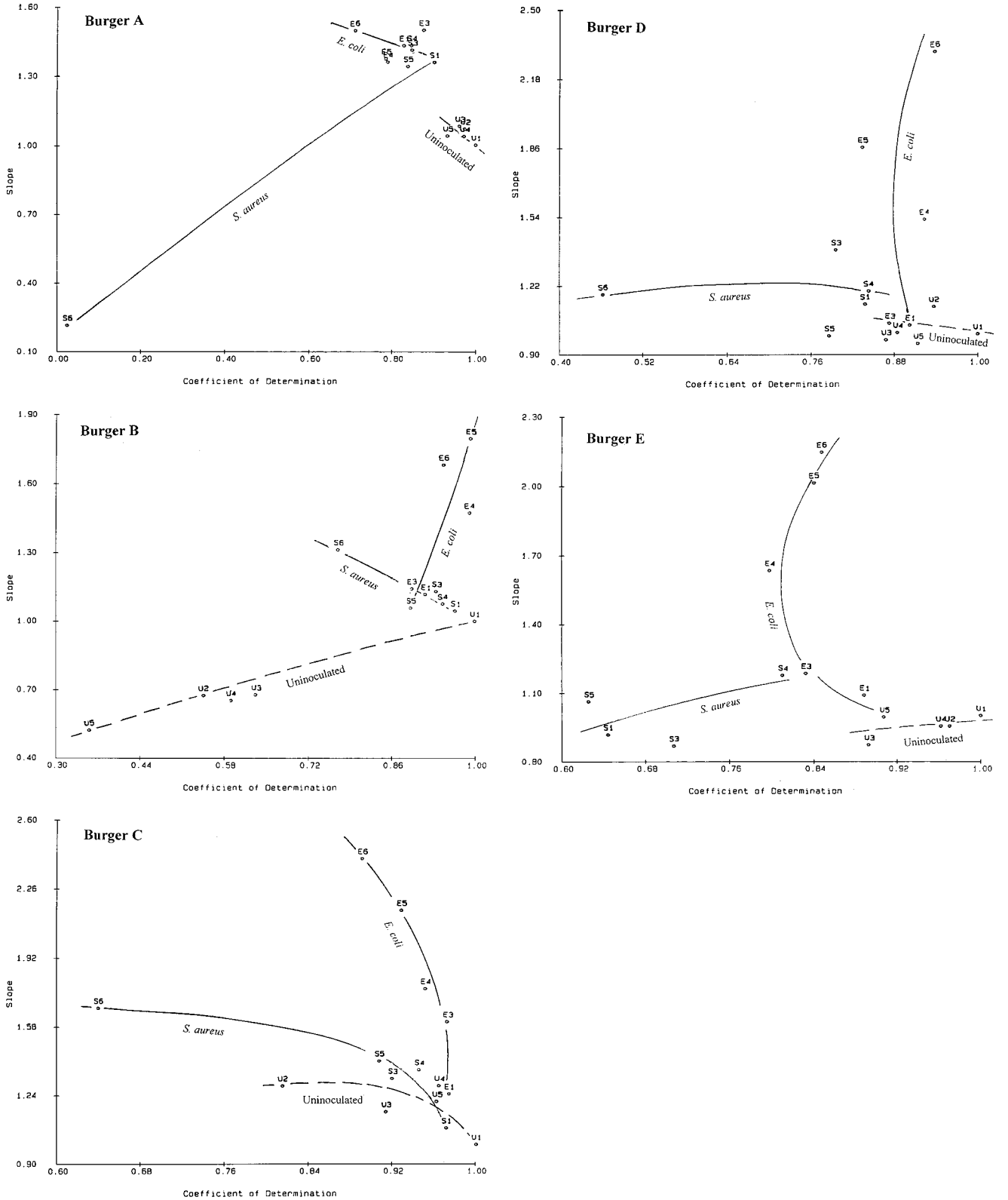

Figure 7. PCS scattergrams of five hamburger patty samples: U, uninoculated; E, *E. coli*; S, *S. aureus.* Digits 1−6 following letters U, E, and S show incubation times of 0, 3, 6, 9, 12, and 15 h.

because the data for a large number of pathogenic bacteria are not required. The GC/PCS system can start building a personal database by accumulating data from daily quality control operations. (3) For a routine check of safety of products, this new approach should be simpler than other methods. This is because the GC/PCS approach is not for detection of pathogenic contamination; rather, it is for detection of no contamination. The direction and shape of the curve on the PCS scattergram are the major criteria for distinction of infecting bacteria; however, if the distinction between pathogenic bacteria A and B is important, instead of using uninfected reference, either sample A or B can be used as reference, thus magnifying the difference between them. Even though some pathogenic bacteria A and B cannot be discriminated, as long as their

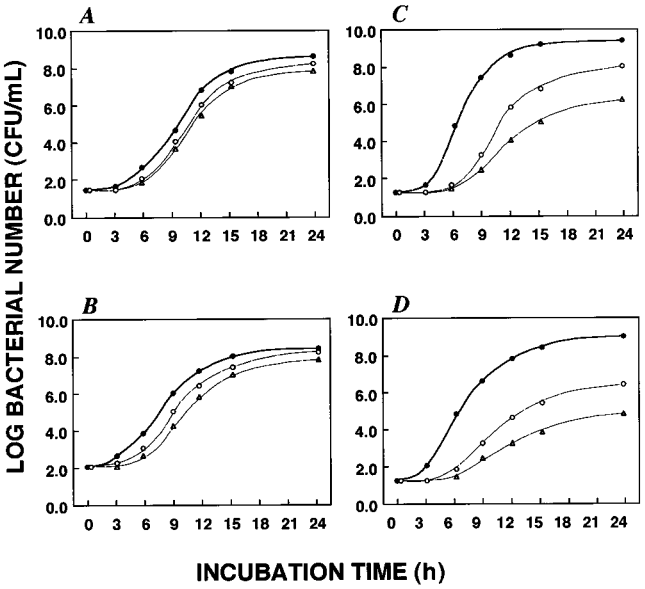

Figure 8. Bacterial growth in 2% homogenate of hamburger (A and C) and salmon (B and D) with and without BHT: (A) *S. aureus* in hamburger; (B) *S. aureus* in salmon; (C) *E. coli* in hamburger; (D) *E. coli* in salmon; (●) without BHT; (○) with 10 mg/L BHT; (△) with 20 mg/L BHT. The hamburger samples used for PCS analysis contained 20 mg/L BHT.

growth curves are different from that of uninoculated reference, the safety of the product can be warranted; thus, the GC/PCS system may be able to function as a warning mechanism.

The final decision on acceptability of samples for marketing without off-flavors due to either microbial or chemical causes may have to be made on the basis of criteria set by the industry or even by individual processing plants on the basis of a past record of safe samples. These standards to ensure safety may be different by many factors, such as products, analytical facility, or even processing plants. This is why each processing plant may start constructing its own database for safety maintenance for its products.

Electronic noses have great potential to be used for quality control in the future. It would be simple, objective, and thus practical when their costs become reasonably economical and the number of data available per sample becomes adequate for the PCS data processing for the required accuracy in classifying samples on the bases of quality standard. Except for the initial investment to install a gas chromatograph equipped with a personal computer and to train its operator with a potential of full automation, the maintenance costs are minimal because there is no need for chemical reagents.

A simpler technique for collecting samples for volatile compounds to analyze is under investigation in our laboratory. If successful, it will eliminate headspace concentrators from the current GC system in use. According to the results from our preliminary experiments, after samples are taken in small vials and then incubated overnight, volatile components are collected in a simple extracting device and then applied to GC by heating. The lengthy operation of headspace concentration that is difficult to automate can be eliminated. As a result, a completely automated system for food safety assurance may be constructed.

Conclusion. By using a low-cost headspace GC to analyze metabolites produced in microbially contaminated salmon and hamburger patties, the PCS computer program could differentiate GC patterns of samples inoculated with *E. coli* and *S. aureus* from the uninoculated reference sample, which is regarded as safe. Although the study should be continued for detecting the growth of many other important pathogenic bacteria, the new approach may be feasible for ensuring safety by warning for the potential spoilage of food samples.

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