Sequential multimodal elution for pseudomultidimensional liquid chromatography on a single column

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Japan also was made by the present RPHPLC method using spectrophotometric detection at 390 nm. Table IV lists the analytical results for Al(III), Fe(III), and Mn(III), together with those obtained on sample solutions spiked with metal ion standards. Comparison of both sets of data revealed that the sea water sample could be determined successfully for the three metal ions and that there was no interference from matrices such as alkali and alkaline-earth metals. The sensitivity of this method could be lowered by using a preconcentration technique, i.e., a liquid–liquid extraction or a liquid–solid extraction using a C_{18}-bonded silica cartridge column.

LITERATURE CITED

(1) O'Laughlin, J. W. *J. Liq. Chromatogr.* **1984**, *7*, 127-204.
(2) Willeford, B. R.; Veening, H. *J. Chromatogr.* **1982**, *251*, 61-88.
(3) Nickless, G. *J. Chromatogr.* **1985**, *313*, 129-159.
(4) Uden, P. C.; Bigley, I. E. *Anal. Chim. Acta* **1977**, *94*, 29-34.
(5) Schwedt, G. *Chromatographia* **1979**, *12*, 613.
(6) Schwedt, G. *Chromatographia* **1978**, *11*, 145.
(7) Smith, R. M.; Yankey, L. E. *Analyst (London)* **1982**, *107*, 744-748.
(8) Smith, R. M.; Butt, A. M.; Thakur, A. *Analyst (London)* **1982**, *110*, 35-37.
(9) Ichinoki, S.; Morita, T.; Yamazaki, M. *J. Liq. Chromatogr.* **1983**, *6*, 2079-2093.
(10) Ichinoki, S.; Morita, T.; Yamazaki, M. *J. Liq. Chromatogr.* **1984**, *7*, 2467-2482.
(11) Ichinoki, S.; Yamazaki, M. *Anal. Chem.* **1985**, *57*, 2219-2222.
(12) Bond, A. M.; Wallace, G. G. *Anal. Chem.* **1981**, *53*, 1209-1213.
(13) Bond, A. M.; Wallace, G. G. *Anal. Chem.* **1982**, *54*, 1706-1712.
(14) Bond, A. M.; Wallace, G. G. *Anal. Chem.* **1983**, *55*, 718-723.
(15) Bond, A. M.; Wallace, G. G. *Anal. Chem.* **1984**, *56*, 2085-2090.
(16) Bond, A. M.; Wallace, G. G. *Anal. Chim. Acta* **1984**, *164*, 223-232.
(17) Mueller, B. J.; Lovett, R. J. *Anal. Chem.* **1985**, *57*, 2693-2699.
(18) Mueller, B. J.; Lovett, R. J. *Anal. Chem.* **1987**, *59*, 1405-1409.
(19) King, J. N.; Fritz, J. S. *Anal. Chem.* **1987**, *59*, 703-708.
(20) King, J. N.; Fritz, J. S. *Anal. Chem.* **1987**, *59*, 1016-1020.
(21) Shih, Y. T.; Carr, P. W. *Anal. Chim. Acta* **1982**, *142*, 55.
(22) Berthod, A.; Kolosky, M.; Rocca, J. L.; Vittori, O. *Analysis* **1979**, *7*, 395.
(23) Hoshino, H.; Yotsuyanagi, T. *Bunseki Kagaku* **1980**, *29*, 807-808.
(24) Bond, A. M.; Nagaosa, Y. *Anal. Chim. Acta* **1985**, *178*, 197-208.
(25) Gurira, R. C.; Carr, P. W. *J. Chromatogr.* **1982**, *20*, 461.
(26) Tabata, M.; Tanaka, M. *Anal. Lett.* **1980**, *13* (A6), 427.
(27) Roston, D. A. *Anal. Chem.* **1984**, *56*, 241-244.
(28) Hoshino, H.; Yotsuyanagi, T.; Aomura, K. *Bunseki Kagaku* **1978**, *27*, 315.
(29) Hoshino, H.; Yotsuyanagi, T. *Talanta* **1984**, *31*, 525.
(30) Hoshino, H.; Yotsuyanagi, T. *Anal. Chem.* **1985**, *57*, 625-628.
(31) Noffisinger, J. B.; Danielson, N. D. *J. Liq. Chromatogr.* **1986**, *9*, 2165-2183.
(32) Zhang, X. S.; Zhu, X. P.; Lin, C. S. *Talanta* **1986**, *33*, 838.
(33) DiNunzio, J. E.; Yost, R. W.; Hutchison, E. K. *Talanta* **1985**, *32*, 803.
(34) Sandell, E. B.; Onishi, H. *Photometric Determination of Traces of Metals*, 4th ed.; John Wiley and Sons: New York; p 432; Part I.
(35) Palmieri, M. D.; Fritz, J. S. *Anal. Chem.* **1987**, *59*, 2226-2231.
(36) Hearn, M. T.; Grego, B. *J. Chromatogr.* **1983**, *266*, 75-87.
(37) Umland, F. *Z. Anal. Chem.* **1962**, *190*, 186.
(38) Johnson, D. C.; Weber, S. G.; Bond, A. M.; Wightman, R. M.; Shoup, R. E.; Krull, I. S. *Anal. Chim. Acta* **1986**, *180*, 187-250.
(39) Kissinger, P. T., Heineman, W. R., Eds. *Laboratory Techniques in Electroanalytical Chemistry*; Dekker: New York, 1984.
(40) Kitagawa, T.; Ichimura, A. *Bunseki Kagaku* **1973**, *22*, 1042-1045.
(41) Nagaosa, Y.; Kobayashi, K. *Talanta* **1984**, *31*, 593-596.

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Sequential Multimodal Elution for Pseudomultidimensional Liquid Chromatography on a Single Column

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A method of elution is described that yields significantly higher (2–3 times) peak capacities and facilitates the separation of compounds by class (e.g., acids versus neutrals), thereby increasing the informing power and the selectivity. The method is based on the sequential application of two or more elution modes, all of which, except the last one, are selective, i.e., designed to elute only a certain class of compounds. The total peak capacity is given approximately by $r\phi_i$, where r is the number of elution modes and ϕ_i is the peak capacity of an individual elution mode, roughly equal to that obtained in a conventional one-dimensional separation. The increase in information content is proportional to log r but is also dependent on the relative proportion of sample components eluted during individual separation modes. Several reversed-phase examples utilizing sequential pH and solvent gradients are presented, including separations of benzoic acids from neutral aromatic compounds, peptides from neutral species, and phenols from polyaromatic hydrocarbons. Solute bandwidths, retention time, and area reproducibility are comparable to that of conventional reversed-phase separations. Although the resolving power of this multimodal, single-column elution method is somewhat less than that of a true two-dimensional (multiple column) method, it can be an effective and simple alternative for the analysis of moderately complex samples.

INTRODUCTION

Liquid chromatography has emerged as the separation method of choice for numerous complex solute mixtures. The introduction of high-performance liquid chromatography (HPLC) has further increased its usefulness due to greater column efficiency and overall reproducibility. Reversed-phase high-performance liquid chromatography (RPLC) has received more attention than any other separation mode due to its broad applicability. Other advantages of RPLC include greater column stability than in conventional liquid–liquid chromatography (LLC); the variety, economy, and low toxicity of common RPLC solvents; and the ease of gradient elution.

As samples become more complex, the ability of a particular separation method to resolve all components decreases. Several factors may lead to insufficient resolution for complex samples, including inadequate column efficiency and/or gradient optimization. Even in cases where these factors are optimized, however, the separation may still be unsuitable due to limitations of selectivity and/or peak capacity. With regard to the latter, a statistical study of component overlap has shown that "a chromatogram must be approximately 95% vacant to provide a 90% probability that a given compound of interest will appear as an isolated peak" (*1*). In instances where this condition is not met, the additional resolution of components within a complex sample would typically require supplemental separation steps, thereby reducing the speed

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and convenience of using HPLC. Due to this problem, separation methods with greater selectivity and peak capacity but otherwise similar attributes are desirable.

Multidimensional separations have become popular due to the dramatic improvement in resolution they usually provide. Although the improved resolution is usually attributed to the much greater peak capacity, it is also a result of selectivity-related improvements (vide infra). Numerous examples of multidimensional separations have been reported, particularly those utilizing thin-layer chromatography (TLC), paper chromatography, or electrophoretic methods. Isoelectric focusing/gel electrophoresis perhaps best exemplifies the advantages of multidimensional separations by providing peak capacities in the thousands (*2*). More recently, multidimensional separations have been applied to column chromatography, i.e., GC/GC (*3, 4*), HPLC/HPLC (*5–7*), and HPLC/GC (*8–10*), with a reasonable amount of success. However, there has been some degree of difficulty in interfacing column chromatographic dimensions (*11*), and the additional instrumentation or instrumental modifications can be costly. These problems may discourage one from considering coupled-column multidimensional separations as the technique of choice for complex samples.

In light of the above shortcomings, we have investigated the feasibility of performing multidimensional-like separations on a single column, made possible by sequential multimodal elution using secondary chemical equilibria and organic solvents. Our efforts have resulted in a reversed-phase gradient technique capable of separating compounds by chemical class as well as resolving sample components within each class. This technique significantly increases the attainable peak capacity and between-class selectivity for a given column.

THEORY

Basics of Multidimensional Separations. The basic criteria described by Giddings for true multidimensional separations are as follows: (i) (all) components of a sample are subjected to two or more independent separation modes, and (ii) the resolution of components from one displacement is not reduced by subsequent separations (*12, 13*). These criteria are satisfied by a number of techniques including those mentioned in the Introduction. To briefly summarize in terms of TLC, suppose the components of a sample, introduced in a corner, undergo a displacement along one axis of the plate. The degree of migration of each component is independent of all others, yet there may be insufficient resolution due to inadequate selectivity or peak capacity. A subsequent separation along the other axis using a solvent of different strength and/or selectivity increases the overall area of separation with a concurrent increase in the resolution of sample components.

Giddings has explained the overall increase in resolving power of multidimensional separations in terms of increased peak capacity (*14*). By constructing a gridwork of areas corresponding to resolution units within the plane of separation, the total (or maximum) peak capacity can be given by the summation of the number of area increments or, in simpler terms, the product of individual peak capacities, ϕ_y and ϕ_z:

$$\phi_{2\text{-D}} \approx \phi_y \phi_z \qquad (1)$$

Although the general superiority of multidimensional separations over one-dimensional separations can be attributed to the dramatically increased peak capacity, it can also be viewed from the perspective of increased selectivity. More precisely, it is the increased probability of obtaining sufficient selectivity to achieve a separation. Since in the ideal case of a two-dimensional separation the two separation modes are independent, the probability that one mode *or* the other will provide sufficient selectivity to separate a given pair of compounds is the sum of the individual probabilities. Because

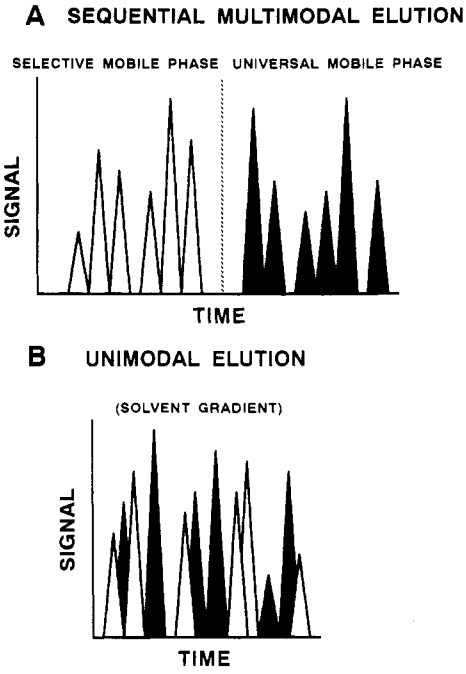

Figure 1. Illustration of a typical sequential multimodal elution separation (A) and a conventional reversed-phase separation (B). Mobile phases are described in the text. The dotted line in A represents the time at which the second elution mode begins.

the *sum* of two (positive) probabilities, however, is always greater than either individual probability, the odds of achieving sufficient selectivity with multidimensional approaches are increased. In summary, it is both the increased peak capacity and selectivity of multidimensional separations that results in increased resolution and makes multidimensional approaches so beneficial for complex samples.

Sequential Multimodal Elution (SME). The basis of our elution scheme is the *sequential* use of one or more *selectively strong* mobile phase(s) followed by a *universally strong* mobile phase, as illustrated in Figure 1A for the case where only one selective mobile phase is employed. Shown for comparison in Figure 1B is the conventional RPLC elution scheme.

In the multimodal elution scheme of Figure 1A, a specific class or classes of compounds are first eluted and resolved by a *selective* mobile phase; the remaining classes of compounds are then eluted and resolved by the *universal* mobile phase. Selective mobile phases are designed so that any compounds to be separated in later elution modes are highly retained (see eq 15 and related text), negligibly broadened, and thus unaffected during the selective mobile-phase elution. The *sequential* use of these elution modes is the key to increasing peak capacity, selectivity, resolution, and decreasing the randomness (disorder) of the separation. The *simultaneous* use of these elution modes is inadequate, as will be illustrated later.

Glajch and Kirkland have provided (*15*) a systematic classification of mobile-phase elution programs based on changes (or lack thereof) in mobile-phase strength and/or selectivity: simple isocratic (SI), isocratic multisolvent programming (IMP), isoselective multisolvent gradient elution (IMGE), and selective multisolvent gradient elution (SMGE—note that their use of "selective" differs from ours). Upon inspection, it can be shown that SME does not fit into any of the four categories mentioned above. First, *whereas SI, IMP, IMGE, and SMGE employ only one independent elution mode (isocratic or gradient), SME couples two or more.* Second, *regardless of how solvent strength and/or selectivity changes with time during SI, IMP, IMGE, and*

SMGE, the changes are felt more or less equally by all solutes. In contrast, during the selective elution mode(s) of SME, the mobile-phase strength changes for only a portion (class) of the sample while remaining fixed (i.e., very weak) for the remainder. This is why it is possible to separate compounds by class by using SME (vide infra). Finally, it is also important to realize that SME is not a form of multisegmented mobile-phase programming. The latter can be employed as necessary in IMP, IMGE, and SMGE or *within* one or more elution modes of SME, but it is not the same as SME.

Does the sequential multimodal elution scheme of Figure 1A result in a multidimensional separation as defined earlier? As we show later in this paper, the separation of sample components achieved during a given elution mode need not be significantly affected by earlier or later elution modes. Thus, SME fulfills the second criterion of multidimensionality discussed above. In addition, sample components eluted during the second and later separation modes are subjected to more than one independent displacement. For these components, the first criterion of multidimensionality is also fulfilled. On the other hand, sample components eluted during the first separative mode are *not* subjected to more than one independent displacement. Thus, by design, a portion of the sample does not fulfill the first criterion, and therefore, from a rigorous viewpoint, SME does not represent a true multidimensional approach. Nevertheless, *the sequential application of two or more elution modes on a single column does result in significantly increased peak capacity and selectivity, two characteristic features of multidimensional separations.* In view of these improvements over one-dimensional separations and the fulfillment of most of the criteria for multidimensionality, we believe it is appropriate to refer to SME-based separations as "pseudomultidimensional" or "multidimensional-like" to reflect the common features of increased peak capacity and selectivity that they share with true multidimensional separations.

In general, there are four selective mobile-phase systems (predominantly aqueous) that can be utilized in SME in the reversed-phase mode (SME-RPLC): (i) pH buffers, (ii) ion-pairing agents, (iii) chelating agents, and (iv) metal ions. These selective mobile phases can be used to elute and separate ionizable compounds (acids and bases), permanent ions, metal cations, and/or analyte ligands in the presence of neutral compounds or other species unaffected by these mobile-phase systems. Obviously, all of these mobile phases are based on the exploitation of secondary chemical equilibria (SCE) in which the selected analytes can participate (*16*). Analytes not participating in SCE can be eluted last by either of two possible universal mobile-phase systems: organic solvents or micellar solutions.

The primary requirement for the sequential use of selective and universal mobile phases is their compatibility (miscibility, etc.). In most cases, the concentration of the SCE reagent required for the selective elution mode(s) is relatively low so that precipitation of the reagent does not occur under low organic conditions. In the present research, our efforts were centered around the (bimodal) combination of pH gradients and organic solvents due to the ease of mobile-phase preparation and its suitability to a vast number of chemical systems. An ion-pairing agent/micellar solution scheme is also an interesting bimodal combination since, in principle, only water and a surfactant, serving first as the ion-pair reagent and then as the monomer component of the micelle, would be required for this approach.

Note that more than one selective elution mode may be used consecutively prior to the general (solvent or micellar) elution mode. The only requirement is that the analytes to be eluted in a given elution mode be relatively unaffected by any prior elution mode. Thus, trimodal and perhaps even tetramodal mobile-phase elution schemes may be feasible. One promising trimodal system would be the combination of an ion-pairing agent, pH buffer, and organic solvent or micellar solution for the separation of permanent ions, ionizable compounds, and neutral compounds, respectively. Although the gradients of trimodal and tetramodal systems would be more difficult to generate sequentially (probably necessitating the use of a quaternary LC pumping system), the scope of the separation could encompass virtually all possible chemical classes of a diverse, complex sample.

Peak Capacity. Although significant increases in peak capacity are achieved with SME-LC, the peak capacity is less than with an optimized multidimensional system. It is therefore appropriate to consider peak capacity from both a theoretical (*14, 17*) and practical viewpoint (*18*).

For an individual elution mode, peak capacity can be expressed as

$$\phi_i = 1 + \frac{\sqrt{N}}{m} \ln (1 + k'_z) \tag{2}$$

where N is the number of theoretical plates, k'_z is the capacity factor of the last eluting peak, and m represents a factor pertaining to the peak width for minimum separation ($m\sigma$), where m is usually equal to 4. Since *each* sequential elution mode results in the elution and separation of only a subset (class) of the analytes, peak capacities are not multiplicative as in true two-dimensional (orthogonal) separations, but instead are (assumed to be) additive in nature:

$$\phi_{total} = \phi_1 + \phi_2 + \ldots + \phi_n \tag{3}$$

In order for eq 3 to be applicable in all cases, it is imperative that each gradient within the sequential framework be highly selective for a particular class of compounds. Also, *those components that are not being selectively eluted during a given gradient should be highly, if not infinitely retained at the head of the column* (*see eq 15* and *related text*). With this assumption, combining eqs 2 and 3 along with the identity $1 + k'_z = V_r^*/V_0$, where V_r^* is the retention volume of the last eluting peak and V_0 is the column void volume, gives an equation to describe the total peak capacity for the sequential separation, where n is the number of sequential elution modes:

$$\phi_{total} = n + \frac{1}{m}\left(\sqrt{N_1} \ln \frac{V_{r_1}^*}{V_{0,1}} + \sqrt{N_2} \ln \frac{V_{r_2}^*}{V_{0,2}} + \ldots + \sqrt{N_n} \ln \frac{V_{r_n}^*}{V_{0,n}}\right) \tag{4}$$

Equation 4 is a very general expression for the total peak capacity, allowing for differences, however minor, in void volume, efficiency (plate count), and retention volume of the last peak among the various elution modes employed sequentially on a given column. Minor differences in V_0 might be expected due to differences in the stationary-phase wetting capabilities of the mobile phases used for the specific elution modes; if necessary, these differences could be virtually eliminated by adding to the mobile phase of each elution mode a small amount of solvent that preferentially solvates the stationary phase relative to the other mobile-phase components (e.g., 3–6% 1-propanol for typical reversed-phase situations). Somewhat greater differences in N might be expected since it is affected by both stationary-phase mass-transfer effects due to the wetting differences discussed above as well as differences in mobile-phase mass transfer also arising from compositional differences. In practice, we believe that the potentially less efficient elution mode(s) of a given column

can be made nearly equal in efficiency to the more (most) efficient elution mode via judicious control over various mobile-phase properties, as we have demonstrated for predominantly aqueous buffers in RPLC (*19*).

In its present form, the peak capacity expression (eq 4) is somewhat difficult to interpret. Recognizing or assuming that differences in V_0 and N are negligible, eq 4 can be simplified to

$$\phi_{\text{total}} = n + \frac{\sqrt{N}}{m \ln V_0}(\ln V_{r_1}^* + \ln V_{r_2}^* + \ldots + \ln V_{r_n}^*) = n + \frac{\sqrt{N}}{m \ln V_0}\sum_{i=1}^{n} \ln V_{r_i}^* \quad (4a)$$

In addition, if the retention volume of the last peak is the same among the various elution modes, i.e., $V_{r_1}^* = V_{r_2}^* = \ldots = V_{r_n}^*$, the $n\sum \ln V_{r_i}^* \approx n \ln V_r^*$, and eq 4a can be further simplified to

$$\phi_{\text{total}} \approx n + \frac{n\sqrt{N}}{m} \ln \frac{V_r^*}{V_0} \quad (5)$$

At this point, the number of sequential elution modes, n, can be factored from each term and the retention volume ratio can be redefined in terms of k' to yield

$$\phi_{\text{total}} \approx n\left\{1 + \frac{\sqrt{N}}{m} \ln (1 + k'_2)\right\} \quad (6)$$

Equation 6 is directly related to eq 2 and thus shows that the total peak capacity in SME-LC is given approximately by

$$\phi_{\text{total}} \approx n\phi_{\text{i}} \quad (7)$$

where ϕ_{i} is the peak capacity of an individual elution mode (eq 2) and n is the number of elution modes sequentially applied.

Although the peak capacity described by eq 7 is much less than what would be expected for multidimensional separations in which the separation modes are orthogonal, it is still a great improvement over one-dimensional separations (eq 2). In practice, the observed peak capacity may differ from that predicted by eq 7. In cases where solute band broadening may be less than expected ($\sigma_{\text{obs}} < \sigma_{\text{pred}}$) due to the focusing effects of a gradient, the observed peak capacity may be higher and vice versa. As a general rule, however, the peak capacity of the elution modes should not differ significantly from each other unless one or more of the mobile phases has a detrimental effect on column efficiency.

Information Content. As we will presently show, SME-LC provides more information than conventional unimodal elution chromatography. To describe this mathematically, the inverse relationship between informing power and entropy can be exploited. That is, in order to show that SME-LC provides an increase in information content, we will show that SME-LC results in a lower entropy for a given separation.

The entropy of a system can be simply described by

$$S = k \ln W \quad (8)$$

where k is Boltzmann's constant and W is interpreted as a probability (*20*). This equation can be applied to chromatographic separations by letting W represent the number of possible elution orders for a given sample and chromatographic technique.

Figure 2A illustrates the possible elution orders for a sample containing eight components. For the unimodal elution case, the number of possible elution orders is $W = 8!$ or 40 320. For the sequential bimodal elution case, if there are two different chemical classes containing 4 components each, as shown in Figure 2B, then the number of elution orders for SME-LC will be the product of the number of elution orders within each separative mode, $W = (4!)(4!)$ or 576.

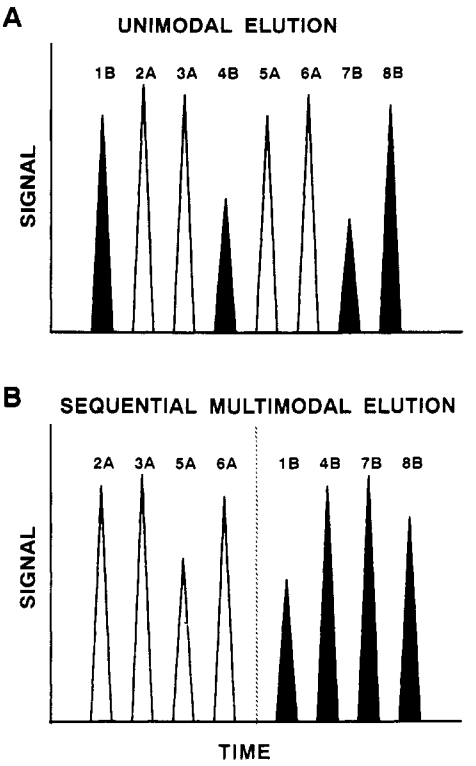

Figure 2. Examples of possible elution orders for a one-dimensional separation (A) and a sequential multimodal elution separation (B). Dotted line as in Figure 1.

The above results can be generalized for a sample with n components as

$$W_{\text{1-D}} = n! \quad (9a)$$

for unimodal elution and

$$W_{\text{SME}} = n_1!n_2!\ldots n_r! = \prod_{i=1}^{r} n_i! \quad (9b)$$

for sequential multimodal elution, where n_i is the number of components eluted during the ith elution mode, r is the total number of elution modes, and $n_1 + n_2 + \ldots + n_r = n$. Assuming that the number of components eluted by each mode is approximately the same (i.e., $n_1 \approx n_2 \approx \ldots \approx n_r$), eq 9b simplifies to

$$W_{\text{SME}} \approx \left\{\frac{n}{r}!\right\}^r \quad (9c)$$

where n/r is understood to be rounded to the nearest whole number. From eqs 8–9c, it is evident that the entropy of the separation is much lower for SME-LC than for a one-dimensional separation. Thus, more information is obtained from SME-LC because the elution orders are less random.

The difference in entropy between SME-LC and one-dimensional chromatographic techniques can be described by

$$\Delta S = S_{\text{SME}} - S_{\text{1-D}} = k \ln \frac{W_{\text{SME}}}{W_{\text{1-D}}} \quad (10)$$

where S_{SME} and $S_{\text{1-D}}$ are the individual entropies and W_{SME} and $W_{\text{1-D}}$ are the number of possible elution orders for a SME-LC and a one-dimensional separation, respectively. Substitution of eq 9a into eq 10 yields after rearrangement

$$\frac{\Delta S}{k} = \ln \left(\frac{n}{r}!\right)^r - \ln n! = r \ln \left(\frac{n}{r}!\right) - \ln n! \quad (11)$$

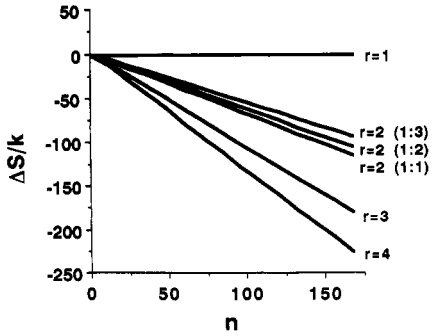

Figure 3. Comparison of the disorder of separation (randomness of elution order) for a one-dimensional elution technique (r = 1) and sequential multimodal chromatography (r = 2–4). n is the number of sample components, and r is the number of sequential elution modes. Numbers in parenthesis for r = 2 indicate relative proportion of components that elute in the first and second separation modes. See eqs 9–12 and related text.

By use of Stirling's approximation ($\ln x! \approx x \ln x - x$), eq 11 can be transformed to

$$\frac{\Delta S}{k} = r\left\{\frac{n}{r}\ln\frac{n}{r} - \frac{n}{r}\right\} - (n \ln n - n) = -n \ln r \quad (12)$$

A plot of $\Delta S/k$ versus n (calculated via eq 11) is shown in Figure 3 for several multimodal possibilities. Note that the relative disorder of separation (relative number of possible elution orders) decreases as the number of elution modes, r, increases. This is predicted by eq 11 but is more obvious in eq 12. Since informing power and entropy are inversely proportional, this is equivalent to stating that, for a given number of sample components, as the number of separative modes increases, the information content increases. This result is logical since fewer components will be eluted per separation mode and, from a statistical viewpoint, the overall elution order will be more predictable. Figure 3 and eq 12 also show that the reduction in disorder achieved by using SME-LC increases as the number of sample components increases, a result also consistent with intuitive notions.

In cases where an unequal number of components elute in different modes, the entropy increases steadily with increasing disparity in the relative proportion of components as indicated in Figure 3 for the bimodal sequential elution mode (r = 2; 1:1, 1:2, and 1:3). If all of the sample components should elute within a single mode during sequential multimodal elution, then SME-RPLC would be only slightly advantageous in terms of information content over conventional RPLC (r = 1). One can then assume that either (i) only one class of compounds is present or (ii) the mobile phase being used in the given elution mode is inappropriate for the sample. If assumption (i) is true, then the results provided by SME-LC enables one to eliminate the possibility of one or more specific classes of compounds being present in the sample. For example, if no components elute during a well-designed pH gradient, then none of the components are weak acids or bases over the range of the pH gradient. On the other hand, SME-LC would be somewhat disadvantageous in terms of analysis time if the sample components all eluted within a single separation mode since all of the SME-LC modes except one would be wasted. As for assumption (ii), the potential selection of an inappropriate elution mode is usually of little concern since typically one already suspects the presence of different chemical classes within the sample and will accordingly choose reasonable mobile-phase conditions for the selective elution mode(s).

Selectivity. SME-LC can provide significantly better selectivity than conventional HPLC, where selectivity is defined as the ratio of the retention factors (isocratic elution) or adjusted retention times (gradient elution) of a pair of solutes,

$$\alpha = \frac{k'_2}{k'_1} = \frac{t_{R'2}}{t_{R'1}} = \frac{t_{R2} - t_m}{t_{R1} - t_m} \quad (13)$$

where t_m is the retention time of an unretained peak.

To compare SME-LC with conventional (unimodal) separations, it is useful to differentiate between two types of selectivity: *within-class* selectivity, α_{WC}, and *between-class* selectivity, α_{bc}. For α_{WC}, both $t_{R'1}$ and $t_{R'2}$ in eq 13 refer to compounds of the same class, whereas for α_{bc}, $t_{R'1}$ and $t_{R'2}$ in eq 13 correspond to compounds from different classes. Unfortunately, the present definition of α_{bc} frequently results in too many combinations to be useful. An appropriate and useful simplification is to use average values in eq 13 for either $t_{R'1}$, $t_{R'2}$, or both. When averages for both are used, the result is

$$\bar{\alpha}_{bc} = t_{R'\text{avg,class2}}/t_{R'\text{avg,class1}} \quad (14)$$

Although we will postpone our detailed discussion of within-class and between-class selectivities until later (for specific separations), it is easy to appreciate qualitatively the dramatic increase in the average between-class selectivity, $\bar{\alpha}_{bc}$, provided by sequential multimodal elution. By simple inspection of Figure 1, $\bar{\alpha}_{bc} \approx 3$ for SME compares to $\bar{\alpha}_{bc} \approx 1$ for a conventional unimodal separation. It should be noted that, in theory, if there is sufficient control of solute retention and band broadening in the later elution mode, $\bar{\alpha}_{bc}$ could be made extremely large by purposely delaying the onset of the later elution mode. Such an artificial improvement in between-class selectivity would generally be undesirable since it would result in a very large gap (delay) between elution modes and hence an excessive analysis time.

Before concluding the discussion on selectivity, we offer two precautions. First, in comparing the selectivity provided during gradient runs among various elution strategies, it is best if the initial mobile-phase strength and gradient steepness for the modes to be compared are similar (*21*). Otherwise, the retention times of one run or both runs may need to be adjusted to reflect these differences. Second, when evaluating the within-class selectivity in SME-LC, one should use the retention time of the analytes relative to the start of the separation mode in which they eluted. That is, the time prior to the beginning of the pertinent elution mode should be substracted from the analytes' retention time since the duration of the prior elution mode(s) is ideally independent of the pertinent separation mode.

Retention Requirements. As mentioned earlier, it is important that compounds to be separated in later elution modes be highly retained during an earlier separation mode(s). For the bimodal case, it is possible to derive an expression for the minimum retention factor (during the first elution mode) of an analyte to be eluted in the second mode, k'_{min}, by using only simple, well-known identities such as $d = vt$ (distance = velocity × time) and $u_s = u_{mp}/(1 + k')$, where u_s and u_{mp} are the linear velocity of the solute and mobile phase, respectively. The result is

$$k'_{min} = \left(\frac{1}{f_1} - 1\right)\frac{V_1}{V_0} \quad (15)$$

where f_1 is the distance migrated down the column by the solute relative to column length (x/L) during the first elution mode, V_1 is the total volume of the first elution mode ($\approx V_{r_1}$* of eq 4), and V_0 is the column void volume. Assuming the first elution mode is complete after 15 column volumes (V_1/V_0 = 15) and that analytes to be eluted during the second mode are to migrate less than 20% during the first mode (f_1 = 0.2),

eq 16 indicates that the minimum retention factor for these analytes in the first mode is 60. Although the constraint of 20% migration might be viewed as too liberal, our experience has shown that up to 30% migration can be tolerated without significant adverse effects on the second elution mode. Even with a more conservative approach, however, in which V_1/V_0 = 30 and only 5% migration is allowed (f_m = 0.05), the minimum retention factor is still only 570. Although 570 may seem to be a prohibitively high degree of retention, this condition can easily be met in RPLC by using only a small amount of organic solvent (e.g., <10%) in the first elution mode. In fact, even larger retention factors are routinely observed in RPLC under these conditions.

Equation 15 can also be used to estimate the necessary retention for late-eluting solutes in the trimodal and tetramodal cases. For solutes that elute during the second mode, eq 15 can be used directly without any assumptions. For solutes that elute during later modes, V_1 in eq 15 should be replaced by the total volume of the early elution modes. It is also necessary to assume that solute retention is exactly the same during all of the early elution modes; some changes in retention could conceivably occur due to secondary effects such as changes in ionic strength, etc.

Summary. SME-LC is more informative (eqs 8 and 12) than conventional HPLC (a one-dimensional, unimodal technique) due to the greater predictability of elution order and the ability to eliminate specific chemical classes from the list of possible classes within the sample. As shown by eq 7, it can provide a peak capacity *significantly* higher than conventional HPLC. It also provides a dramatic increase in between-class selectivity and, as we will later show, may also provide improved within-class selectivity in the selective elution modes over that furnished by conventional HPLC.

EXPERIMENTAL SECTION

LC System. A ternary liquid chromatograph (Rainin Instruments, Woburn, MA) was used, along with a Model 7125 injection valve (Rheodyne, Cotati, CA) with a 20-μL loop, a Model V[4] UV–visible absorbance detector (Isco, Lincoln, NE), and a Model LC-4B thin-layer electrochemical detector (Bioanalytical Systems, Lafayette, IN). Silica-based reversed-phase columns (C_1, C_8, and C_{18}; 150 × 4.6 mm; d_p = 5 μm; Rainin Instruments, Woburn, MA) were used for analyses of samples containing benzoic acid derivatives or peptides. A 150 × 4.1 mm PRP-1 column (Hamilton Co., Reno, NV) was used for samples containing phenols and polycyclic aromatic hydrocarbons (PAHs). All columns were washed periodically with 100% organic solvent to remove any buildup of highly retained compounds that might have been present in the samples. The columns were thermostated at 25.0 ± 0.1 °C by using a glass water jacket and a Model RMS-6 circulating bath (Brinkmann Instruments, Westbury, NY). All mobile-phase solutions were filtered through 0.45-μm Nylon-66 filters (Rainin Instruments, Woburn, MA) before use. Subsequent degassing of the mobile phase was accomplished by placing the solutions in a heated ultrasonic bath and then applying a partial vacuum for a few minutes.

Reagents/Standards. Mobile-phase components methanol, acetonitrile, and water were HPLC grade, as were sodium carbonate, sodium acetate, and sodium perchlorate. Sodium bicarbonate, formic acid, sodium formate, and acetic acid were ACS reagent grade. All chromatographic solutes (peptides, benzoic acids, neutral compounds, etc.) were reagent grade or better and used without further purification. They were dissolved either in the aqueous buffer or in a minimal amount of methanol or acetonitrile and then diluted with the aqueous buffer. The creosote sludge sample was acquired from LSU's Institute of Environmental Studies. The presence of phenols and polyaromatic hydrocarbons in the creosote sludge was confirmed by gas chromatography/mass spectrometry (GC/MS) prior to the SME-LC analysis.

Mobile-Phase Preparation. *Peptide/Neutral Samples.* The mobile-phase system consisted of 100 mM formic acid/sodium formate buffer and methanol. Sodium perchlorate was added to the acidic component of the buffer in order to ensure a constant ionic strength (100 mM) throughout the pH gradient. Detection of peptides and neutral compounds was accomplished at 254 nm. The flow rate was 1.0 mL/min. Measurement of pH gradients was accomplished either manually (fraction collection, $V_{frac} \approx 100$ μL) or with a Model FTPH-2 Micro Flow Through pH system (Lazar Research, Los Angeles, CA). In both cases, a Model 920 research grade pH meter (Orion, Cambridge, MA) was calibrated with pH 4 and 7 buffers so that the pH could be accurately determined at any given time during the gradient.

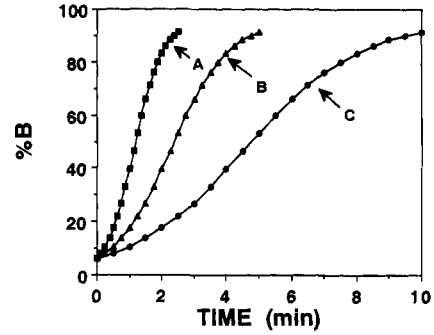

Figure 4. Examples of mobile-phase composition gradients necessary for linear pH gradients. The ordinate represents the percentage of conjugate base. Gradient identification: curve A, ΔpH/min = 1; curve B, ΔpH/min = 0.5; curve C, ΔpH/min = 0.25. Curves represent a pH gradient from 3.50 to 6.00 and were calculated for a 96 mM formic acid/sodium formate buffer with a constant ionic strength and 5% organic solvent.

Benzoic Acid/Neutral Samples. The mobile-phase system consisted of 43.5 mM acetic acid/sodium acetate buffer with either methanol or acetonitrile as the organic solvent. The pH was measured as described for the peptide samples. Three different hydrocarbonaceous silica columns were used for this sample mixture: C_1, C_8, and C_{18}. Methanol was used as the organic solvent for separations employing the C_1 and C_8 columns, whereas acetonitrile was used with the C_{18} column to avoid exceeding an arbitrary pressure limit for the column. The ionic strength of the mobile phase was not adjusted for these separations. Benzoic acid derivatives were detected at 280 nm followed by detection of the neutral compounds at 260 nm at 0.1 AUFS (AUFS = absorbance units full scale). The flow rate was 1.5 mL/min.

Creosote Sample. The selective mobile phase consisted of an aqueous sodium carbonate/sodium bicarbonate buffer and 30% acetonitrile. The sodium bicarbonate solution also contained 50 mM sodium perchlorate so that the ionic strength would remain constant as the pH was varied (ca. 35 mM after the buffer was mixed with acetonitrile). Continuous degassing (e.g., by sparging with helium) was avoided in order to minimize loss of the carbonate buffer. The pH of the aqueous buffer was measured after calibrating the pH meter with pH 7 and 10 standard buffers. Separation of phenolic substituents was performed at a constant pH of 9.11 to alleviate base-line drift during electrochemical detection. A solvent gradient from 30% to 100% acetonitrile was then used to elute and separate the polyaromatic hydrocarbons.

Generation of Linear pH Gradient. The conjugate acid and base of a particular buffer system were segregated so that the pH of the mobile phase could be changed by varying the proportions of the two solutions. Due to the logarithmic relationship between acid/base ratios and pH, linear pH gradient programming was accomplished by the use of "sigmoidal" composition gradients as illustrated in Figure 4. The required changes in the relative proportions of the buffer were deduced from pH calculations facilitated by an in-house MS-BASIC computer program. Calculations were performed in 1% increments of conjugate base, taking into account ionic strength and the constant percentage of organic solvent present in the mobile phase (during the pH gradient of a given run).

Bimodal Gradient Programming. Bimodal gradients were performed by linking selective and universal gradients. In all cases, a step gradient from the final conditions of the (selective) pH gradient to the initial conditions of the (universal) solvent gradient was necessary. Slight base-line disturbances were typically observed as a result of the dramatic change in solvent composition

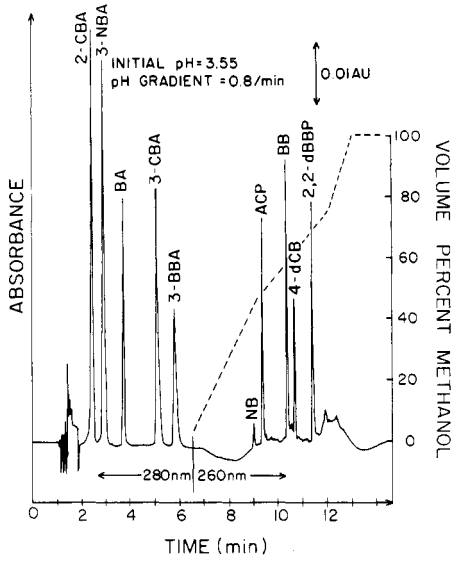

Figure 5. Sequential multimodal reversed-phase separation of a sample of acids and neutrals on a C_1 column with UV detection at 280 nm (acids) and 260 nm (neutrals). Analyte identification: 2-CBA, 2-chlorobenzoic acid; 3-NBA, 3-nitrobenzoic acid: BA, benzoic acid; 3-CBA, 3-chlorobenzoic acid; 3-BBA, 3-bromobenzoic acid; NB, nitrobenzene; ACP, acetophenone; BB, bromobenzene; 4-dCB, 1,4-dichlorobenzene; 2,2′-dBBP, 2,2′-dibromobiphenyl. Mobile-phase components: reservoir A = 43.5 mM acetic acid, B = 43.5 mM sodium acetate, and C = methanol. The pH gradient, initiated at the time of sample injection, was linear from pH 3.55 to 5.5 at 0.8 pH units/min and held constant at the final pH until all benzoic acid derivatives had eluted. The methanol gradient, shown in the figure, was then applied.

between gradients, but these disturbances did not interfere with any separation. For the peptide separations, chromatograms were cosmetically altered by subtracting a blank SME run from the actual SME separation.

RESULTS AND DISCUSSION

Chromatographic Expectations and Experimental Verification.

Hypothetical SME-LC and one-dimensional separations of a complex sample are shown in Figure 1. A one-dimensional separation (Figure 1B) is typically unable to provide separations by class and, due to insufficient peak capacity, might not be able to resolve all components of a complex sample. SME-LC (Figure 1A), with its increased peak capacity and selectivity, can separate and resolve two (or more) particular classes of compounds via selective and nonselective mobile phases. The region between chemical classes in Figure 1A can be minimized in most cases by starting the second elution mode prior to the completion of the first mode, but this may impair the resolution of late eluting components of the first separation mode. In general, this spacing should be considered an advantage rather than a disadvantage since a clearer definition of different chemical classes is facilitated with such gaps.

An experimental demonstration of SME-LC is illustrated in Figure 5 using a reversed-phase C_1 column. The "complex sample" in this case consists of benzoic acid derivatives and neutral aromatics. The benzoic acids were eluted by a pH gradient, whereas the neutral compounds loaded onto the top of the column and were not eluted until a methanol gradient was applied. The separation of different chemical classes (acids and neutrals) is clearly demonstrated. The time required for analysis could easily be shortened by starting the solvent gradient earlier or by increasing its initial strength. The sharpness of the neutral bands indicates that, at least for this bimodal pH/solvent elution scheme, excessive broadening of the later-eluting neutral compounds during the first elution mode (pH gradient) does not occur. Finally, to illustrate that SME-LC is possible with a broad range of reversed-phase columns and not just a C_1 column, the sample of Figure 5 was separated on C_8 and C_{18} columns in Figure 6 with only slight modifications to the bimodal elution conditions.

One of the primary concerns in using SME-LC is solvent and reagent purity. The extraneous peaks present in the pH and solvent gradients of Figure 5 (and Figure 6) are due,

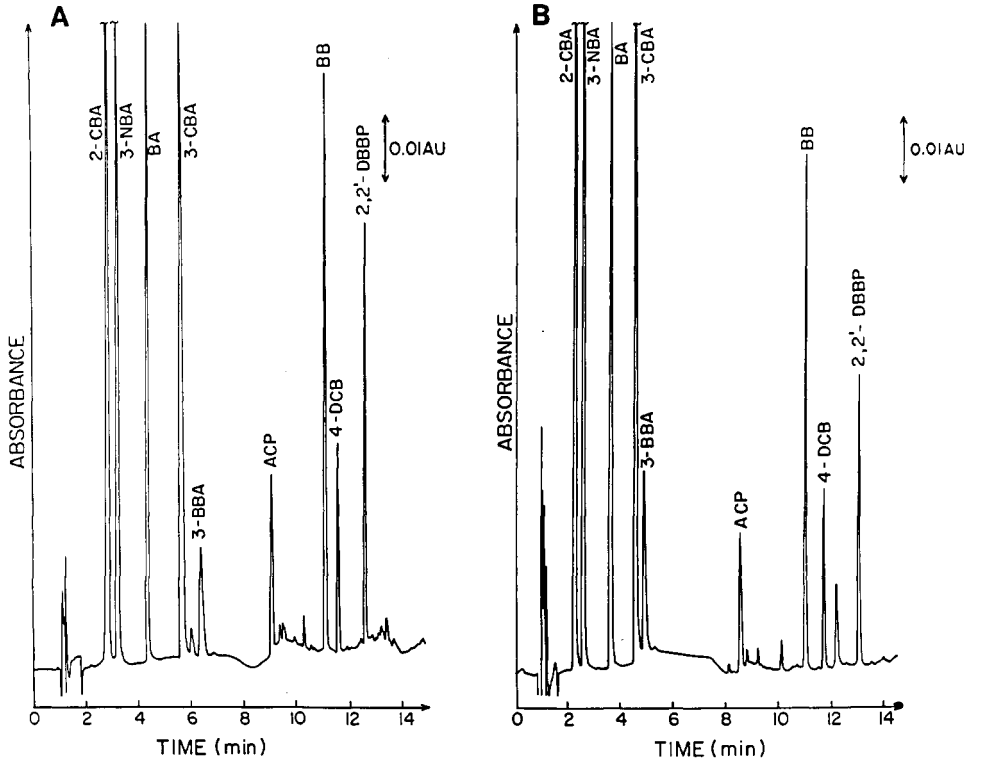

Figure 6. Sequential multimodal elution for RPLC separations using a C_8 (A) and a C_{18} (B) column. Detection and sample components as in Figure 5, except that nitrobenzene was omitted.

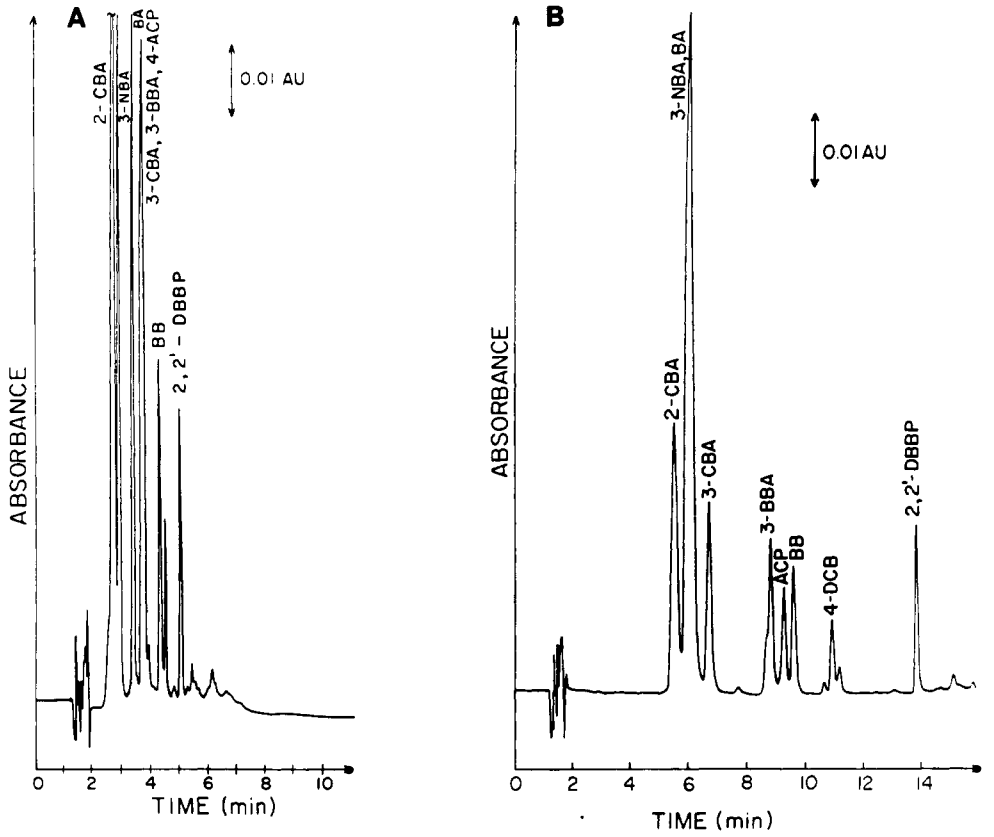

Figure 7. Alternative reversed-phase separations (cf. Figure 5) using (A) simultaneous bimodal elution (pH/methanol gradients) and (B) unimodal elution (methanol gradient at 5%/min) (B). Detection at 260 nm. Sample components as in Figure 6. pH and methanol gradients of A as in Figure 5, except that both were started at the time of injection.

respectively, to relatively polar and nonpolar impurities in the water or buffer reagents. The level of nonpolar impurities present in the water is probably somewhat exaggerated since they accumulate continuously on the column during the first separation mode. Nevertheless, if one takes precautions to use high-purity mobile phases, such impurity peaks can be minimized. Note also that the multimodal separation terminates naturally with a 100% methanol wash, thus eluting any highly retained compounds from the column at that time and avoiding their elution in subsequent runs.

To examine the necessity of using a *sequential* bimodal approach, the separation of Figure 5 was attempted with a *simultaneous* bimodal gradient and also a *unimodal* solvent gradient for comparison. As shown in Figure 7, neither the use of (A) a simultaneous bimodal gradient (pH and organic solvent) nor the use of (B) a unimodal organic solvent gradient results in satisfactory resolution. This was anticipated since the peak capacity is less for these separation modes than for the SME-LC separations of Figures 5 and 6. SME-LC clearly represents an effective approach for increasing the peak capacity of a given column.

In addition to the obvious increase in peak capacity for the SME-LC separation of Figures 5 and 6, an increase in both within-class and between-class selectivities (eqs 13 and 14 and related text) was also observed. In the present example, the weak acids comprise the first class of compounds and the neutral solutes comprise the second class. Table I shows the between-class selectivity, α_{bc}, for each of the acidic and neutral solutes achieved by the unimodal (Figure 7B) and bimodal (Figure 5) separations, as well as the average value, $\bar{\alpha}_{bc}$, defined according to eq 14. For both the individual solutes and the average value, α_{bc} is dramatically greater for the bimodal separation. This increase in α_{bc} is directly attributable to the class separation achieved by stepwise modification of mobile-phase selectivity using SME-LC.

Table I. Comparison of Between-Class Selectivity, α_{bc}, for Unimodal (Conventional) Elution and Sequential Multimodal Elution in RPLC[a]

compd	α_{bc} (unimodal)	α_{bc} (bimodal)	% change
weak acids			
2-chlorobenzoic acid	1.95	10.16	421
3-nitrobenzoic acid	1.74	6.42	269
benzoic acid	1.74	3.84	120
3-chlorobenzoic acid	1.57	2.48	58
3-bromobenzoic acid	1.08	2.13	97
neutrals			
acetophenone	1.57	2.48	58
bromobenzene	1.63	2.90	78
4-dichlorobenzene	1.84	3.02	64
2,2'-dibromobiphenyl	2.43	3.29	35
average[b]	1.85	4.26	131

[a] Selectivity reported as relative retention, defined as $t_{R'ACP}/t_{R'acid}$ for acidic solutes and as $t_{R'neutral}/t_{R'3CBA}$ for neutral solutes, where ACP = acetophenone and 3CBA = 3-chlorobenzoic acid. Data correspond to those of Figures 5 (bimodal) and 7B (unimodal). Since nitrobenzene is not present in the sample of Figure 7, it is necessarily excluded from these calculations. [b] Calculated according to eq 14.

In light of the differences in the between-class selectivity provided by conventional (unimodal) RPLC and SME-LC, it is appropriate to compare the within-class selectivity, α_{wc}, for these approaches. As shown in Table II, $\alpha_{wc,bimodal}$ is significantly larger than $\alpha_{wc,unimodal}$ for the acidic compounds. Perhaps the most dramatic change occurs with 3-nitrobenzoic acid and benzoic acid. Whereas they co-elute in ca. 6 min in conventional unimodal RPLC (Figure 7B), they are base-line resolved in less than 4 min by SME-LC (Figure 5). The differences in α_{wc} were anticipated since the retention mechanism for the ionizable solutes was changed from one

Table II. Comparison of Within-Class Selectivity, α_{wc}, for Unimodal (Conventional) Elution and Sequential Multimodal Elution in RPLC[a]

	α_{wc} (unimodal)	α_{wc} (bimodal)	% change
weak acids[b]			
2-chlorobenzoic acid	1.00	1.00	NA
3-nitrobenzoic acid	1.12	1.58	41
benzoic acid	1.12	2.64	136
3-chlorobenzoic acid	1.24	4.10	229
3-bromobenzoic acid	1.80	4.76	164
neutrals[c]			
acetophenone	1.55	2.10	36
bromobenzene	1.49	1.34	−10
4-dichlorobenzene	1.32	1.21	−8
2,2′-dibromobiphenyl	1.00	1.00	NA

[a] Data correspond to those of Figures 5 (bimodal) and 7B (unimodal). [b] Selectivity reported as relative retention ($t_{R'acid}/t_{R'ref}$), with 2-chlorobenzoic acid as the reference compound. [c] Selectivity reported as relative retention ($t_{R'ref}/t_{R'neutral}$), with 2,2′-dibromobiphenyl as the reference compound. Since nitrobenzene is not present in the sample of Figure 7, it is necessarily excluded from these calculations.

that depended primarily on distribution coefficients to one in which the ionization constants played a greater role (RPLC versus SCE-LC, ref 16). Moreover, these differences in α_{wc} are consistent with the results of Buck and Tomellini (*22*), who performed a systematic study of the unique selectivity provided by pH gradients in RPLC.

In contrast to the different retention mechanisms experienced by the acids in the unimodal and the bimodal elution, the neutral compounds experience essentially the same mechanism in both cases (a methanol gradient), since they are relatively unaffected by the pH gradient of the bimodal elution scheme. It is not surprising, therefore, that the within-class selectivity of the neutral compounds is essentially the same; the minor differences apparent in Table II are due to the somewhat different gradient conditions employed and, possibly for acetophenone, nonnegligible migration during the pH gradient.

In summary, the anticipated increases in both peak capacity and selectivity provided by SME-LC appear to have been realized. Although our results are limited to the samples of the present study, we believe that SME-LC could provide improved results for a variety of samples.

Feasibility of Biological Sample Analyses. Figure 8 illustrates the capability of SME-LC to separate and resolve two- and three-residue peptides from neutral aromatics (or other potential neutral interferents). Although these peptides contain both carboxyl and amino terminal groups, separation is based primarily upon the deprotonation of the carboxyl terminal groups since, under the present conditions, all of the amino terminal groups remain fully protonated over the pH gradient employed. The effect of this constant amino terminal group protonation at low pH is simply a more rapid elution of the peptides than one would find for analogous compounds without the amino group. The hydrophobicity of the side chains, however, is more than adequate for reasonable retention of the peptides. Somewhat longer peptides and even small proteins could possibly be separated by similar SME-LC elution schemes; in these instances, the reduced risk of denaturation due to the aqueous nature of the selective mobile phase(s) could be an important advantage.

Control of Retention. One prerequisite for the separation of different classes of compounds via SME-LC is sufficient control of retention of solutes during their elution in the desired elution mode and an almost infinite retention (eq 15) of later eluting compounds during earlier elution modes.

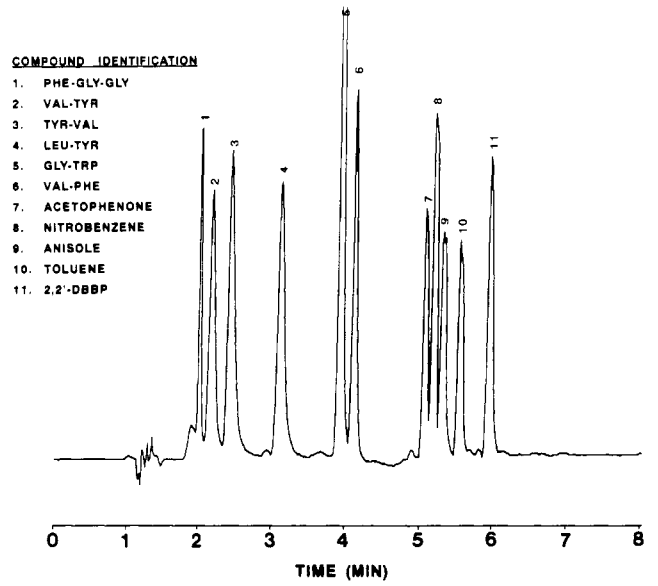

Figure 8. Sequential multimodal elution RPLC separation of a complex sample containing di- and tripeptides and neutral compounds. Detection conditions: λ = 254 nm, sensitivity = 0.2 AUFS. Mobile-phase program: reservoir A = 100 mM formic acid + $NaClO_4$, B = 100 mM sodium formate, and C = methanol. Solutions A and B contained 6% 1-propanol to improve column efficiency. pH gradient profile: initial pH held at 3.20 for 0.5 min, followed by a linear pH gradient of 1.1 pH units/min to pH 4.30 and held for 1.5 min. MeOH gradient profile: a step gradient from 0 to 65% MeOH was performed after 3 min and then maintained at 65% for 1 min, followed by a linear gradient at 10%/min to 95% MeOH and then held at 95% for 5 additional min.

Figure 9 illustrates the control of retention possible for the neutral compounds of the peptide sample of Figure 8. The second elution mode (solvent gradient) was started at the usual time in the top chromatogram but was delayed by 1 and 2 min in the middle and bottom chromatograms, respectively. As shown in parts B and C of Figure 9, the retention times of the neutral compounds were increased almost exactly by these delays. Except for this predictable increase in the retention times, the separation of the neutral compounds was otherwise relatively unaffected; i.e., efficiency, selectivity, and resolution were preserved. This indicates that no significant migration or band spreading of the neutrals occurs until after the universal elution mode (solvent gradient) is applied. Because a small amount of very strong reversed-phase solvent (6% 1-propanol) was used in the selective elution mode to promote good column efficiency (*19*), it was originally anticipated that some migration and band spreading of neutrals might occur. Our results show that these effects are minimal, however, at least on the time scale of the present separations.

One other point needs noting. The final two peaks (peptides) in the first half of Figure 9A have smaller band widths and are less retained than the same peaks of parts B and C. This apparent discrepancy is explained as follows: In Figure 9A, the organic solvent gradient elution mode was purposely begun just *before* the pH gradient was finished and the last two peptides had been eluted, whereas in parts B and C the solvent gradient was not started until *well after* the pH gradient/peptide elution was completed. Thus, for a small fraction of the time that the two peptides were on-column in Figure 9A, they felt the additional influence of the early portion of the organic solvent gradient, which caused them to elute more rapidly but with decreased selectivity. The overlap of the pH and organic solvent gradients in Figure 9A was done to minimize analysis time and is of little concern in the present example, but it does bring to attention a precaution that should generally be observed; i.e., it is best to avoid overlap of the elution modes in SME-LC; such overlap

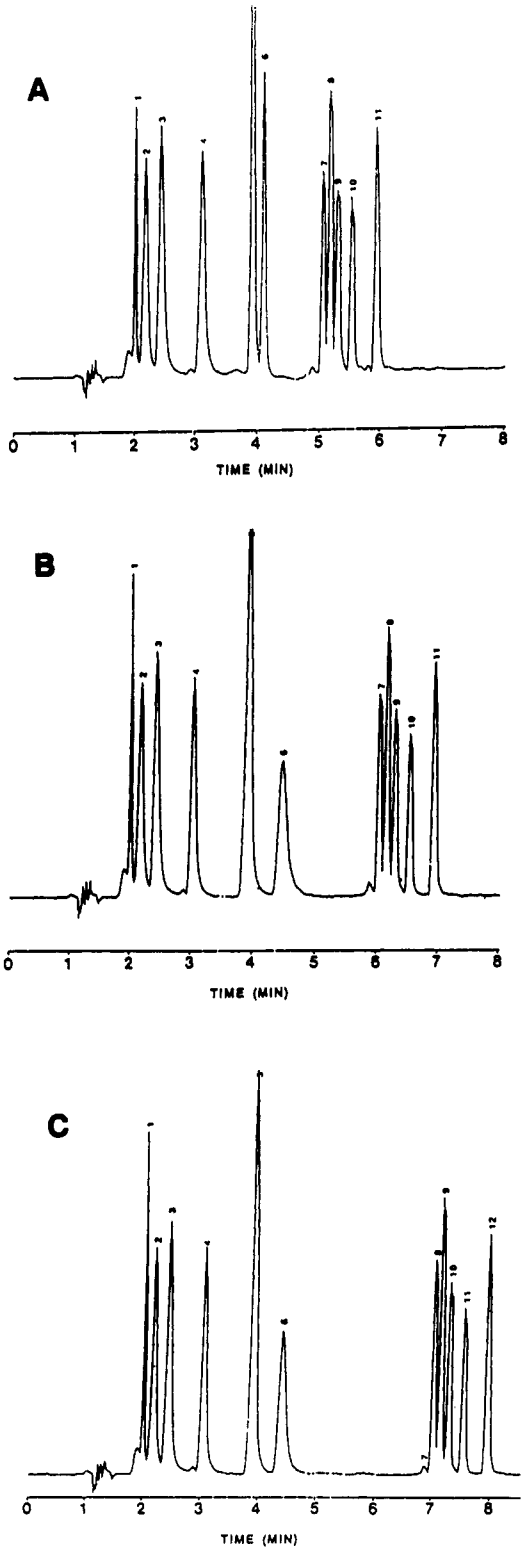

Figure 9. Example of control of solute retention in sequential multimodal elution RPLC. The elution of a second class of compounds during a second separation mode can be delayed (or accelerated) simply by delaying (or accelerating) the start of the second elution mode. Delay of second elution mode (methanol gradient): (A) no delay; (B) 1 min; and (C) 2 min. Sample and other conditions as in Figure 8.

may decrease both within-class and between-class selectivities.

Enhancement of Detection. Another advantage of SME-LC is the enhanced detection of different classes of compounds. In the case of the separation shown in Figure 5, a wavelength of 280 nm was used to detect the benzoic acids. Following their elution, the wavelength was adjusted to 260 nm for better detection of the neutral aromatics. Conventional one-dimensional RPLC does not allow such an enhancement since there is no guarantee that specific chemical classes will elute in a specific region of the chromatogram. The separation-by-class feature of SME-LC can also be an advantage when more than one detector is necessary. An excellent example is shown in Figure 10 for the separation of phenols from PAHs in creosote sludge. Detection of the low levels of phenols (ppm to ppb) was performed by using electrochemical detection while UV detection was used for the PAHs. The presence of phenols and PAHs in the sludge was verified prior to HPLC analysis by GC/MS; their elution during the pH and solvent modes, respectively, was confirmed by comparison of the retention times of the unknown peaks of Figure 10 with those of representative standards of each class of compounds (e.g., phenol, *p*-cresol, *p*-xylenol; benzene; naphthalene). The sequential detection of the phenols and PAHs made possible by SME-LC is quite advantageous; a simultaneous detection scheme for these compounds in one-dimensional RPLC would be difficult if not impossible to devise since (i) the gradient necessary to elute all components in a reasonable period of time would cause extreme base-line drift in the electrochemical detection and (ii) the absorbance of the phenols could potentially interfere with the detection of the PAHs and vice versa.

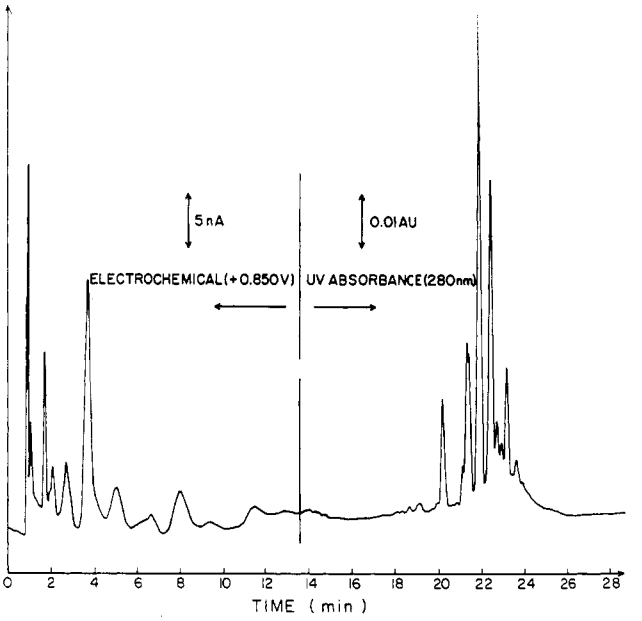

Figure 10. Separation of phenolic priority pollutants and polyaromatic hydrocarbons (PAHs) in creosote sludge using sequential multimodal elution RPLC. Mobile-phase program: reservoir A = 50 mM sodium carbonate, B = 50 mM sodium bicarbonate + $NaClO_4$, and C = acetonitrile. The pH was held constant at a pH_{opt} = 9.11 with 30% acetonitrile to accommodate electrochemical detection of phenols (alleviate baseline drift). The ACN gradient profile, initiated at 13 min, was 30–95% ACN at a rate of 5%/min with an isocratic hold at 95% ACN for 2 min.

Repeatability. The precision of SME-LC was measured by performing triplicate analyses of a sample identical with the one of Figure 5, except that nitrobenzene was omitted. The relative standard deviation (RSD) for the retention times and peak areas (without internal standardization) was less than 0.3% and 2.6%, respectively, for all sample components and is certainly comparable to that which can be achieved by conventional RPLC. Average RSDs for the retention times were 0.24% for the acids and 0.08% for the neutrals, whereas for peak areas the averages were 1.7% and 1.5%, respectively. Both the qualitative and quantitative precision are excellent since some systematic and random error is certainly present due to the imprecision of manual injection, HPLC flow rate

anomalies (23), and the complex gradient programming required to achieve a linear pH gradient, e.g., the use of a "sigmoidal" conjugate acid/conjugate base gradient as shown in Figure 4 to provide a linear change in pH with time. Even better retention and area reproducibility may be possible for separations involving selective (SCE) elution modes other than pH gradients, since the sigmoidal mobile-phase programming described above may be unnecessary. Note that the amount of sample required (injected on-column) is the same for SME-LC as for conventional RPLC; all comparisons between SME-LC and RPLC in this report were made with identical sample volumes.

Additional Considerations. A couple of points about SME-LC need to be emphasized. First, with regard to the pH/solvent bimodal elution system, the retention mechanism of the initial elution mode is *not* the same as ionization suppression. Elution of the ionogenic compounds is due to the gradual ionization of their functional groups and their subsequent lower affinity for the nonpolar stationary phase. In order to have control over the elution of ionizables, initial conditions of the gradient program should take into account the ionization constants of these components. For instance, if one wishes to separate components on the basis of acidity and/or basicity, their ionization constants should fall well within the range of the pH program being performed. Those components whose pK_a's or pK_b's fall outside this range may be eluted too rapidly and be poorly separated or may be elected too slowly and be confused with neutral species during the universal solvent gradient.

Second, an ideal feature of the pH/solvent bimodal elution scheme would be the capability to separate by class any group of ionizable species from any group of neutral compounds, regardless of systematic disparities in other chemical and physical properties such as molecular weight. Whether or not this ideal can be approached is difficult to predict at this stage. So far, however, we have demonstrated that the pH/solvent elution scheme works well for different classes of compounds of comparable molecular weight.

Comparison with Stationary-Phase-Generated Multimodal HPLC. Another interesting aspect of SME-LC is its relationship to separations performed with multimodal (mixed-mode) stationary phases. Many mixed-mode stationary phases have been studied recently (*24–28*), each having its own particular applications and advantages over other separation techniques. In the comparison that follows, we shall refer broadly to these mixed-mode separations as "stationary-phase-generated multimodal LC (SPGM-LC)".

Although SME-LC and SPGM-LC are both multimodal and require mobile phases of comparable complexity, significant differences between these separation methods are apparent almost immediately upon inspection. First, whereas SME-LC is multimodal with respect to the *mobile* phase, SPGM-LC is multimodal with respect to the *stationary* phase. Moreover, SME-LC is a *sequential* multimodal separation scheme whereas SPGM-LC is a *simultaneous* multimodal separation scheme and is the stationary-phase analogue to the simultaneous multimodal mobile-phase elution scheme of Figure 7A. This difference is important in terms of the total peak capacity, the between-class selectivity (α_{bc}), and the randomness of the separation. Whereas SME-LC provides considerable improvement in all three parameters over conventional one-dimensional HPLC or simultaneous multimodal *mobile-phase* schemes, SPGM-LC does not. Another difference between SME-LC and SPGM-LC is the type of column required. SME-LC merely requires a conventional RPLC column that is relatively inexpensive and can be found in almost any HPLC laboratory. SPGM-LC requires a specialized column that at present is more expensive, less versatile, and frequently less efficient. In summary, although mixed-mode stationary-phase separations are certainly very useful in many situations, in many instances a properly designed SME-LC separation is a less expensive, superior alternative.

Final Comparisons. For samples consisting of more than one class of compounds, SME-LC is much better than conventional (unimodal) or simultaneous multimodal reversed-phase separations in terms of peak capacity, selectivity between classes, and resolution. Little or no sacrifice in analysis time, solute band widths, or qualitative and quantitative reproducibility is required. In some cases, SME-LC is actually faster because of the compression of the elution modes made possible by the greater predictability of the separation. Limitations of SME-LC include (i) the additive instead of multiplicative nature of the peak capacities of the individual separation modes, resulting in a distinctly lower peak capacity than true two-dimensional techniques with orthogonal separation modes; (ii) the possibility that various classes of compounds may elute during the "wrong" separation mode if the sequential mobile-phase conditions are improperly selected; and (iii) the fact that SME-LC technique is not superior to (but somewhat more complex than) conventional one-dimensional RPLC if the sample contains only one class of compounds.

CONCLUSION

The analysis of complex samples often requires the use of multidimensional chromatographic techniques. The effectiveness of a technique, however, is not only based on the peak capacity and separating power that can be attained, but also on whether the technique is laborious and/or economically feasible. In this paper, we have presented a theory and some representative examples of a sequential multimodal elution technique that is applicable to the separation of simple to moderately complex samples. This technique has many features of multidimensional separations and requires little or no hardware modficiation to conventional, one-dimensional HPLC equipment. The advantages and disadvantages of this technique over alternative separation modes such as conventional reversed-phase chromatography have been noted throughout. In general, we feel that the benefits of sequential multimodal elution greatly outweight any shortcomings.

LITERATURE CITED

(1) Davis, J. M.; Giddings, J. C. *Anal. Chem.* **1983**, *55*, 418–424.
(2) Anderson, N. G.; Anderson, N. L. *Anal. Biochem.* **1978**, *85*, 331–340.
(3) Curvers, J.; Van der Sluys, P. *J. Chromatogr. Sci.* **1988**, *26*, 267–270.
(4) Duinker, J. C.; Schulz, D. E.; Petrick, G. *Anal. Chem.* **1988**, *60*, 478–482.
(5) Majors, R. E. *J. Chromatogr. Sci.* **1980**, *18*, 571–579.
(6) Alfredson, T. V. *J. Chromatogr.* **1981**, *218*, 715–728.
(7) Roston, D. A.; Wijayaratne, R. *Anal. Chem.* **1988**, *60*, 948–950.
(8) DeSanzo, F.; Uden, P.; Siggia, S. *Anal. Chem.* **1980**, *52*, 906–909.
(9) Apffel, J. A.; McNair, H. *J. Chromatogr.* **1983**, *279*, 139–144.
(10) Cortes, H. J.; Pfeiffer, C. D.; Richter, B. E. *HRC&CC J. High Resolut. Chromatogr. Chromatogr. Commun.* **1985**, *8*, 469–474.
(11) Davies, I. L.; Raynor, M. W.; Kithinji, J. P.; Bartle, K. D.; Williams, P. T.; Andrews, G. E. *Anal. Chem.* **1988**, *60*, 683A–702A.
(12) Giddings, J. C. *Anal. Chem.* **1984**, *56*, 1258A–1270A.
(13) Giddings, J. C. *HRC&CC J. High Resolut. Chromatogr. Chromatogr. Commun.* **1987**, *10*, 319–323.
(14) Giddings, J. C. *Anal. Chem.* **1967**, *39*, 1027–1028.
(15) Glajch, J. L.; Kirkland, J. J. *Anal. Chem.* **1982**, *54*, 2593–2596.
(16) Foley, J. P.; May, W. E. *Anal. Chem.* **1987**, *59*, 102–109.
(17) Westerlund, D. *Chromatographia* **1987**, *24*, 155–164.
(18) Samain, D. F. In *Advances in Chromatography*; Giddings, J. C., Grushka, E., Brown, P. R., Eds.; Marcel Dekker: New York, 1989; Vol. 29, pp 108–139.
(19) Foley, J. P.; May, W. E. *Anal. Chem.* **1987**, *59*, 110–115.
(20) Barrow, G. M. *Physical Chemistry*; McGraw-Hill: New York, 1979; Chapter 7.
(21) Snyder, L. R. In *High Performance Liquid Chromatography: Advances and Perspectives*; Horvâth, C., Ed.; Academic Press: New York, 1980; Vol. 1, pp 208–316.
(22) Buck, C. F.; Tomellini, S. A. *J. Chromatogr. Sci.* **1989**, *27* (4), 166–71.
(23) Foley, J. P.; Crow, J. A.; Thomas, B. A.; Zamora, M. *J. Chromatogr.* **1989**, *478*, 287–309.

(24) Yoshida, H.; Morita, I.; Tamai, G.; Masujima, T.; Tsuru, T.; Takai, N.; Imia, H. *Chromatographia* **1984**, *19*, 466-472.
(25) Hagestam, I. H.; Pinkerton, T. C. *Anal. Chem.* **1985**, *57*, 1757-1763.
(26) Gish, D. J.; Hunter, B. T.; Feibush, B. *J. Chromatogr.* **1988**, *433*, 264-268.
(27) Haginaka, J.; Yasuda, N.; Wakai, J.; Matsunaga, H.; Yasuda, H.; Kimura, Y. *Anal. Chem.* **1989**, *61*, 2445-2448.
(28) Floyd, T. R.; Hartwick, R. A. In *High Performance Liquid Chromatography: Advances and Perspectives*; Horváth, C., Ed.; Academic Press: New York, 1986; Vol. 4, pp 45-90.

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Electrodesorption from Single-Crystal Electrodes: Analysis by Differential Electrochemical Mass Spectrometry

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A newly designed thin-layer cell allows the use of differential electrochemical mass spectrometry (DEMS) for the identification of volatile electrochemical desorption products from single-crystal electrodes. The sensitivity is in the range of a percent of a monolayer. This is demonstrated for the case of preadsorbed benzene on a Pt(111) electrode. Contrary to the case of a polycrystalline electrode, benzene is mainly desorbed as such without H/D exchange; only a minor part is hydrogenated to cyclohexane, which is the main desorption product from a polycrystalline electrode. Oxidation of the adsorbate occurs in two well-separated steps: in the double-layer region a partially oxidized adsorbed intermediate is formed, which is oxidized to CO_2 in the oxygen adsorption region.

INTRODUCTION

In heterogeneous electrocatalysis, an understanding of the interaction of molecules with the electrode surface is of primary importance. In recent years, a number of methods have been developed that allow the identification of adsorbates not only ex situ by means of typical ultrahigh vacuum (UHV) techniques (*1-3*) but also in situ, e.g., various IR techniques (*4*, *5*), radio tracer techniques (*6*), the extended X-ray absorption fine structure technique (EXAFS) (*7*), and differential electrochemical mass spectrometry (DEMS) (*8*).

It is clear that different crystallographic orientations of surface atoms have an influence on the catalytic and double-layer properties of single-crystal electrodes. Since the pioneering work of Hamelin et al. (*9*) and Clavilier (*10*), the importance of using single-crystal electrodes for fundamental electrochemistry has been recognized in many electrochemical groups. For the study of adsorbates, the applicability of IR methods (*11*) to well-defined single-crystal electrodes and the first in situ characterization of an adsorbate layer by scanning tunneling microscopy (STM) (*12*) were major recent achievements.

Here we will report the extension of DEMS to single-crystal electrodes. Hitherto, a porous gas diffusion electrode was used as the interface between the electrolyte and the vacuum. Volatile reaction products (or desorption products) evaporate into the mass spectrometer where they are detected on-line with a time constant of less than a second. If the ion current is recorded in parallel to a usual cyclic voltammogram, so-called mass spectrometric cyclic voltammograms (MSCVs) are obtained (*8*, *13*). In the case of desorption experiments, these MSCVs can be regarded as "electrodesorption spectra" in analogy to thermodesorption spectra.

We recently reported the development of a new thin-layer cell that for the first time allowed the use of smooth electrodes (*14*). Here we will describe the use of a single-crystal electrode in such a cell and the analysis of electrodesorption products, using the anodic and cathodic desorptions of preadsorbed benzene from Pt(111) as a model system.

The adsorption and electrooxidation of benzene and its homologues on polycrystalline platinum have been examined in a number of papers. Gileadi and Bockris measured the adsorption isotherms by using electrochemical and radiotracer methods (*15*). Hubbard et al. (*16*) determined the packing densities of various hydroquinones by the thin-layer technique, and by comparison with the oxidation charge, they concluded that other oxidation products beside CO_2 are formed if adsorption was performed from solutions of higher concentrations. The oxidation of preadsorbed toluene on a porous Pt electrode was studied by DEMS. During the first oxidation cycle, adsorbed intermediates are formed that are in a higher oxidation state. From the number of electrons released per formed CO_2 molecule, one can conclude that CO_2 is the only oxidation product (*17*).

Far less attention has been paid to the cathodic desorption of unsaturated organic compounds from Pt electrodes. Using a long optical path thin-layer cell, Gui and Kuwana studied the hydrogenative desorption of various phenols from polycrystalline platinum (*18*). The desorption and hydrogenation of toluene, benzene, and acetone were studied in this laboratory (*13*, *14*, *17*). For both benzene and acetone, an extensive H/D exchange was found. The adsorption of difluorobenzene was studied by IR spectroscopy (*19*). To our knowledge, no such in situ characterization of adsorbed unsaturated compounds has been reported for the case of single-crystal electrodes.

The adsorption of benzene on Pt single-crystal surfaces in UHV has been studied by various authors. On Pt(111) surfaces, adsorbed benzene only forms ordered layers if coadsorbed with CO (*20*). A complete H/D exchange has been found for the reaction of C_6H_6 adsorbed on the Pt(110) face with gaseous D_2 (*21*).

EXPERIMENTAL SECTION

A thin-layer cell, which allows the use of smooth electrodes for DEMS, was already described in ref 14 and was slightly modified for single-crystal electrodes.

The thin-layer volume is formed by the electrode itself and a porous Teflon membrane, which are separated by a Teflon spacer

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