



NOTE NO. 22 - July 14 1987

## SELECTIVITY AND REPRODUCIBILITY AS FUNCTIONS OF pH, WITH PINKERTON ISRP GFF-S5-80 HPLC COLUMNS

**Analytes:** Toluic Acid, Caffeine, Phenobarbital, Trimethoprim, Sulfapyrazole, Carbamazepine, Methyl Salicylate

**Sample Matrix:** Water

**Sample Size:** 200 microliters

**Column:** 5 micron GFF ISRP, 15 cm x 4.6 mm ID

**Regis Product Number:** 731451

**Mobile Phase:** 80% 0.1 M  $\text{KH}_2\text{PO}_4$  (pH 6.0, 6.8, 7.5), 20% Acetonitrile

**Discussion:** Glycine-phenylalanine-phenylalanine (GFF) is one of the more complex stationary phases in modern HPLC. It is not only a hydrophobic reversed phase but also a weak cationic exchanger. In consequence of its complexity, it shows extremely wide selectivity. In further consequence, its characterization demands a complex, multicomponent mixture.

In this Application Note, we describe and summarize part of a study of GFF retention for a set of selected physiologically active solutes as a function of pH within the 6.00-7.50 pH range of protein stability. We believe the reader will find this report and discussion as interesting and surprisingly instructive as the successively developed findings have been to us.

Among its other characteristics, the user of an ISRP GFF column desires it to show adequate selectivity and reproducibility. Selectivity, to make separations. Reproducibility, to guarantee performance. With the ISRP GFF column, however, selectivity and reproducibility are strong functions of mobile phase composition. For exact reproducibility in particular, the mobile phase must be replicated exactly; but this is up to the user.

With the ISRP GFF column, therefore, satisfactory selectivity and reproducibility depend more on the user than the user is likely to realize, and in any event quite as much as on the given column. During the last half year, as the study (part of which is reported here) has progressed, the truth of this has been brought home to us with increasing force.

A Probe

Components. Probe components were selected primarily to show retentions not to exceed 10 minutes with a 15-cm

GFF-S5-80 column. Within that limit, they were selected to be mutually dissimilar in retention and functionality.

In order of increasing retention, the solutes were toluic acid, caffeine, phenobarbital, trimethoprim, sulfinpyrazone, carbamazepine, and methyl salicylate. For the convenience of the reader, we have shown the structures of these substances.

### Effect of Mobile Phase pH on Retentions

Increasing retentions with decreasing pH. Except for caffeine, the retention of which is stable, each substance in this probe shows a retention that increases more or less substantially with decrease in mobile phase pH (from 7.50 to 6.80 to 6.00). This retention increase does not seem to reflect either the acidity or the basicity of the probe component. Trimethoprim, for instance, with two primary amine groups, is a base; phenobarbital, with two ionizable hydrogens, is an acid; and sulfinpyrazone is essentially neutral. Yet the retentions of trimethoprim, phenobarbital, and sulfinpyrazone can be seen to increase in that order with decreasing mobile phase pH.

### Obtaining Replicate Retentions

Equilibration. Obtaining good ISRP GFF retention measurements can be difficult. To get these, we had to adopt what we came to call "assured equilibration": Inject each probe sample repeatedly until its last 3 retentions replicate each other within the precision of measurement--perhaps 5 seconds. (The range of these last 3 retention measurements is indicated by the width of the block at the foot of each retention.)

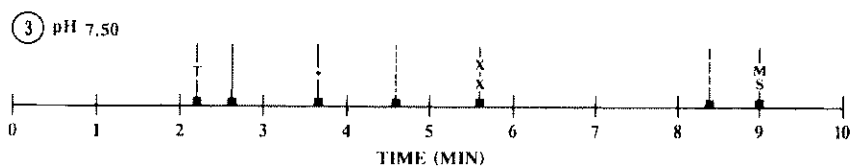
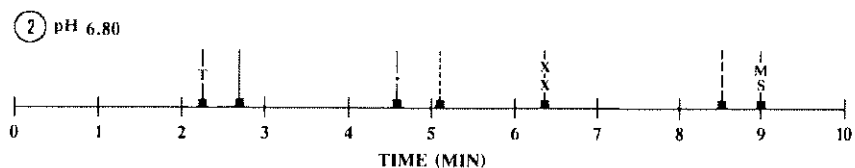
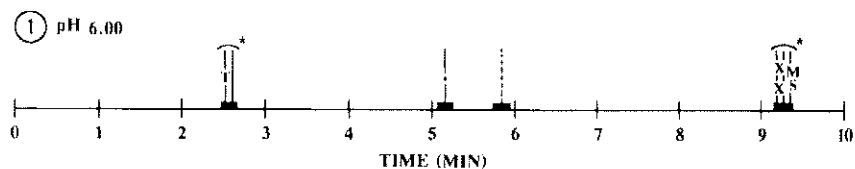
Given column, nominally replicate mobile phases. With ISRP columns, it is important that the user realize how profoundly reproducibility depends on his or her own efforts. Fairly late in this study and much to our surprise, we found that -even with a given column left in place-, obtaining precisely replicate retentions with nominally replicate mobile phases can be almost impossible. This was particularly surprising because the observation came well after we had learned the need for assured equilibration and had therefore repeated, with great deliberation, a long set of measurements. The GFF stationary phase, new to HPLC in more ways than one, requires that the user learn. As one way of beginning, let the user test himself or herself by using nominally replicate mobile phases on a given column.

Summary: Without at this time attempting to explain our observations, we have reported here, for several physiologically active substances injected in a water matrix, large changes in ISRP GFF retention with changes in mobile phase pH. In general, retention is seen to increase with decrease in pH from 7.50 to 6.00, without regard to the native acidity or basicity of the solute.

We also report unusual difficulty in reproducing given retentions, even with a given ISRP GFF column and nominally

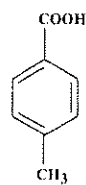
replicate mobile phases. These findings suggest that much greater attention than usual be given to mobile phase replication when exact retention reproducibility is desired.

### Illustrations:

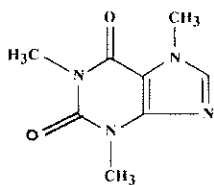


A. \*Did not separate

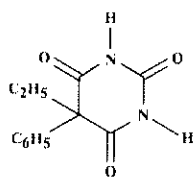
B. ■ Measurement range shown at base



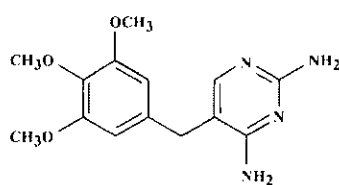
Toluic Acid



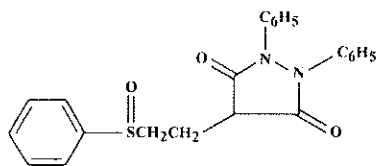
Caffeine



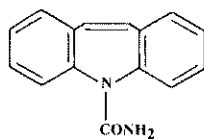
Phenobarbital



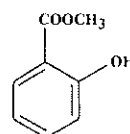
Trimethoprim



Sulfon Pyrazone



Carbamazepine



Methyl Salicylate