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WATER AS A STATIONARY PHASE MODIFIER IN PACKED-COLUMN SUPERCRITICAL FLUID CHROMATOGRAPHY

I. SEPARATION OF FREE FATTY ACIDS

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SUMMARY

A procedure is described for supercritical fluid chromatography (SFC) of free fatty acids (lauric, myristic, palmitic, stearic and arachidic acid) with either dry carbon dioxide or water-saturated carbon dioxide as the mobile phase and with flame-ionization detection. Seven stationary phases were evaluated with water-saturated and dry carbon dioxide under otherwise identical conditions. The retention time for lauric acid with water-saturated carbon dioxide varied from 5.6 min ($k' = 8.7$) for Deltabond™ Octyl to 23.6 min ($k' = 39.7$) for YMC-Gel PVA-Sil™. The range of stationary phase polarity reported in this study should enable an investigator to adjust SFC conditions to resolution requirements for various solute mixtures. Water-saturated carbon dioxide improved the chromatographic resolution and sensitivity of all seven stationary phases, particularly of covalently bonded phases.

INTRODUCTION

Saturation of supercritical carbon dioxide with water or with water–formic acid has been reported to improve significantly the resolution of polar solutes such as long-chain free fatty acids in packed-column supercritical fluid chromatography (SFC)[1]. This technique has been shown to be compatible with universal detection methods such as flame ionization detection (FID), thereby enabling the separation of free fatty acids and related natural products which are not easily separated by conventional liquid or gas chromatographic methods, especially at the low concentrations typically found in pharmaceutical formulations or in biological samples.

Earlier reports of equipment for saturating carbon dioxide have described the use of a precolumn (150 × 4.6 mm I.D.) packed with silica gel (100–200 mesh) and inserted between the pump and the sample injection port. The silica gel precolumn was saturated with about 40% (w/w) water. As carbon dioxide passed through the precolumn at 25°C, water was desorbed from silica gel, thereby saturating the carbon

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dioxide with an estimated 0.15–0.18% (w/w) water at 25°C at 1800–5500 p.s.i. carbon dioxide[1,2].

Although prior investigators have proposed a mechanism by which polar organic modifiers (*e.g.* methanol) in low concentration deactivates packed-column stationary phases[3–5], we are not aware of studies in which commercially available packed-column stationary phases were tested in separations with "dry" and water-saturated supercritical carbon dioxide in combination with FID. Such a technique should prove useful for the rapid screening and quality control of suitable packed column SFC and high-performance liquid chromatographic (HPLC) stationary phases.

The objectives of our investigation were: (1) to automate the water saturation of supercritical carbon dioxide so that SFC could be conducted easily with and without water, (2) to screen a variety of silica-based stationary phases using free fatty acids as model polar solutes, (3) to propose a mechanism for the observed chromatographic improvement with water-saturated carbon dioxide, and (4) to use this mechanistic model to propose "ideal" packed-column SFC stationary phases, and to examine qualitatively the relative polarity of a variety of commercially available packed-column stationary phases so that investigators could adjust SFC conditions to the resolution requirements of various sample mixtures.

In this study, saturated free fatty acids (lauric, myristic, palmitic, stearic, arachidic acid) were separated sequentially on seven silica-based stationary phases, using "dry" and water-saturated supercritical carbon dioxide as the mobile phase. The stationary phases selected for the study included: four stationary phases previously identified[6] as suitable for SFC (Deltabond Methyl, Deltabond Octyl, Deltabond Cyano, and Nucleosil Cyano), and three stationary phases for which SFC separations have not been reported in the literature (YMC-Gel phenyl, YMC-Gel silica, and YMC-Gel PVA-Sil). YMC-Gel PVA-Sil was of particular interest to us since it is reported to exhibit long term stability at pH values as high as 13.5 even though it consists of a silica gel support, coated with polymerized polyvinyl alcohol[7]. We are not aware of published reports in which this unique stationary phase has been used in a non-aqueous chromatographic system, such as in SFC with carbon dioxide.

EXPERIMENTAL

SFC system

The SFC system consisted of a CCS 5000 SFC and CCS 727 data system (Computer Chemical Systems, Avondale, PA, U.S.A.). The CCS 5000 SFC system was equipped with a 0.1-μl injection loop, a post-column restrictor, calibrated to give an expanded gas flow-rate of 22 ml/min at 1800 p.s.i. to 90 ml/min at 6000 p.s.i. (column oven temperature, 70°C), a remotely actuated 8-port valve, which could be switched conveniently back and forth to the water-saturated precolumn, and a flame ionization detector set at 350°C.

Reagents

The following free fatty acids were purchased from Chem Services (West Chester, PA, U.S.A.): lauric acid ($C_{12}H_{24}O_2$), myristic acid ($C_{14}H_{28}O_2$), palmitic acid ($C_{16}H_{32}O_2$), stearic acid ($C_{18}H_{36}O_2$), arachidic acid ($C_{20}H_{40}O_2$), oleic acid

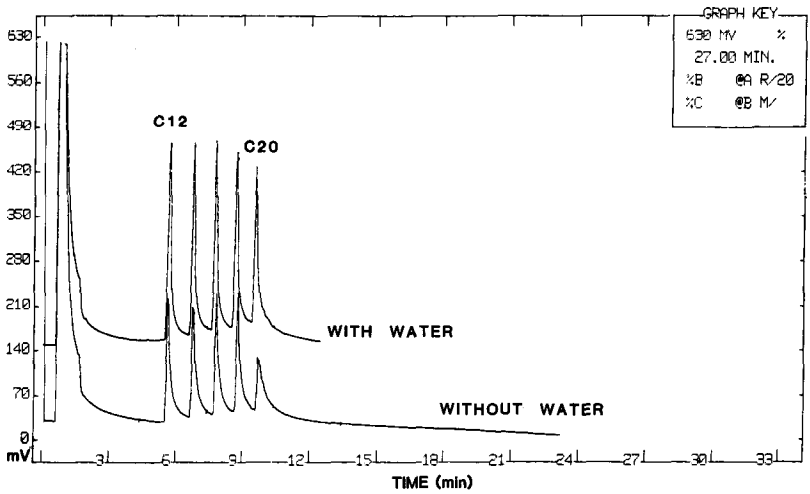

Fig. 1. SFC of C_{12}, C_{14}, C_{16}, C_{18}, C_{20} saturated free fatty acids. Carbon dioxide mobile phase at 70°C with and without water modifier. Column: 100 × 1 mm I.D. 5 μm Deltabond Octyl (300 Å) (C8). Conditions: 1800 p.s.i. for 2 min after injection; 1800 to 6000 p.s.i. at 100 p.s.i./min.

($C_{18}H_{34}O_2$), linoleic acid ($C_{18}H_{32}O_2$), and linolenic acid ($C_{18}H_{30}O_2$). Baker-analyzed HPLC-grade water was purchased form VWR Scientific (Philadelphia, PA, U.S.A.). SFC-grade liquid carbon dioxide was purchased from Scott Specialty Gases (Plumsteadville, PA, U.S.A.).

SFC columns

The water-saturation precolumn (100 × 6 mm I.D., nominal 5 μm, 100 Å) appropriately conditioned for SFC was supplied by Computer Chemical Systems. Three 100 × 1 mm I.D. columns packed with YMC-Gel PVA-Sil (silica gel encapsulated with polymeric polyvinyl alchohol), YMC-Gel phenyl (covalently-bonded diphenylmethyl-silyl), and YMC-Gel silica (non-bonded) stationary phases (all nominally 5 μm, 120 Å spherical) were supplied by YMC, Inc. (Morris Plains, NJ, U.S.A.). One 100 × 1 mm I.D. column packed with Nucleosil Cyano stationary phase (covalently-bonded cyanopropylsilyl, nominal 5 μm, 100 Å spherical), and three 100 × 1 mm I.D. columns packed with Deltabond Methyl (covalently bonded polymerically cross-linked methylsilyl), Deltabond Octyl (covalently bonded polymerically cross-linked octylsilyl), and Deltabond Cyano (covalently bonded polymerically cross-linked cyanopropylsilyl), stationary phases (all nominal 5 μm, 300 Å spherical) were purchased from Keystone Scientific (State College, PA, U.S.A.).

SFC column conditioning

All microbore columns (100 × 1 mm I.D.) were prepared for SFC by elution with 20 ml of methanol at a rate of 0.5 ml/min, followed by drying for at least 2 h at 150°C in the SFC oven with varying pressure increases from 1500 to 6000 p.s.i. carbon dioxide. YMC-Gel PVA-Sil was found not to tolerate exposure to 150°C and was therefore conditioned at 125°C for 12–16 h (overnight). When columns were

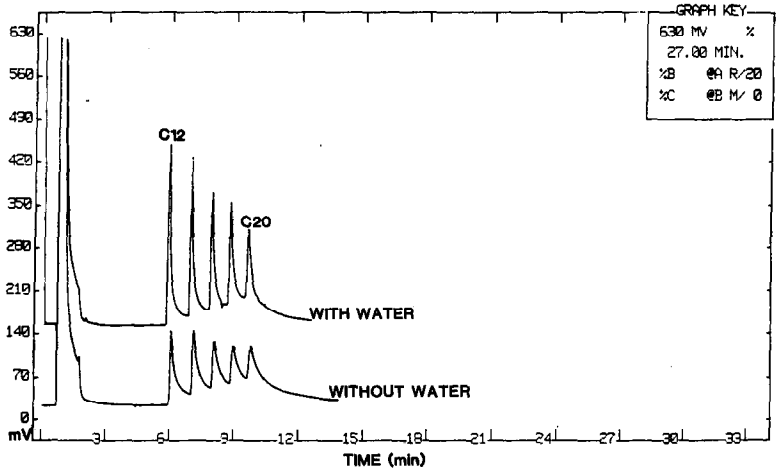

Fig. 2. SFC of C_{12}, C_{14}, C_{16}, C_{18}, C_{20} saturated free fatty acids. Conditions: as per Fig. 1. Carbon dioxide mobile phase at 70°C, with and without water modifier. Column: 100 × 1 mm I.D., 5 μm Deltabond Methyl (300 Å) (C1).

installed in the SFC system, the baseline FID response when pressurized to 6000 p.s.i. (at 90°C) did not exceed 100 mV.

Preparation of water saturation columns

The preconditioned water-saturation SFC column (100 × 6 mm I.D.) was connected to an 8-port valve, and 20 ml of HPLC-grade water was pumped into the column at a rate of 0.5 ml/min. The column was subsequently dried partially with gaseous carbon dioxide at 150 p.s.i. for 30 min. The valve was actuated in order to

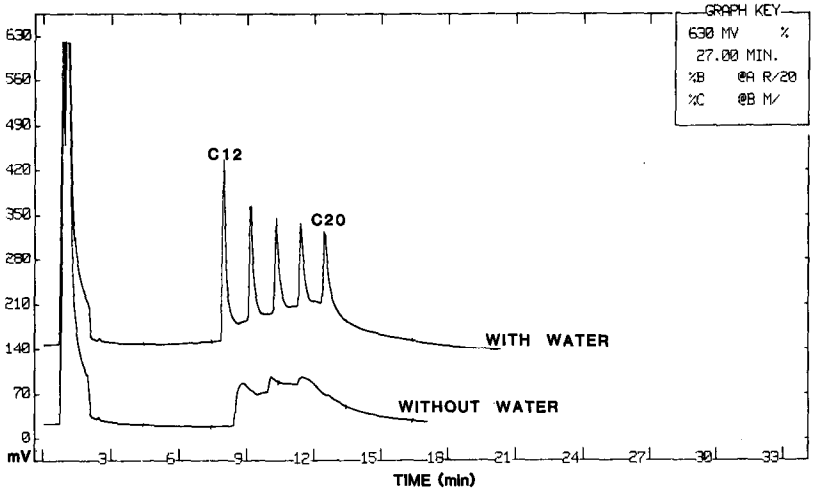

Fig. 3. SFC of C_{12}, C_{14}, C_{16}, C_{18}, C_{20} saturated free fatty acids. Conditions: as per Fig. 1. Carbon dioxide mobile phase at 70°C, with and without water modifier. Column: 100 × 1 mm I.D., 5 μm YMC Gel phenyl (120 Å) (PH).

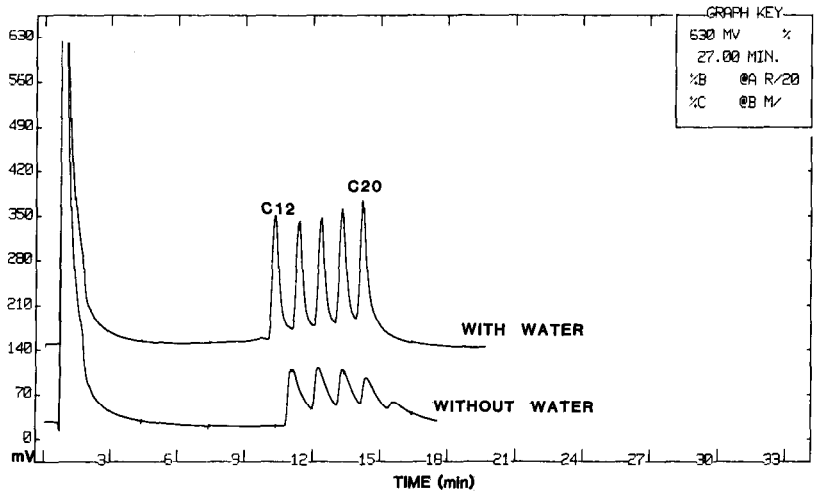

Fig. 4. SFC of C_{12}, C_{14}, C_{16}, C_{18}, C_{20} saturated free fatty acids. Conditions: as per Fig. 1. Carbon dioxide mobile phase at 70°C, with and without water modifier. Column: 100 × 1 mm I.D., 5 μm Deltabond Cyano (300 Å) (CN1).

allow carbon dioxide to flow through the water-saturated precolumn, and the pressure was raised from 1500 to 6000 p.s.i. at the rate 500 p.s.i./min and held at 6000 p.s.i. (at 90°C) until the FID baseline response did not exceed 100 mV.

RESULTS AND DISCUSSION

Screening of stationary phases: separation of saturated free fatty acids

The following chromatographic conditions were used for each stationary phase

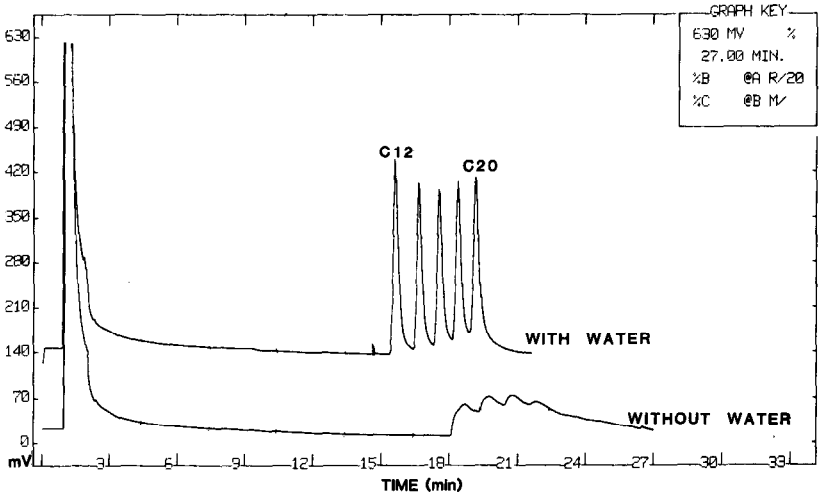

Fig. 5. SFC of C_{12}, C_{14}, C_{16}, C_{18}, C_{20} saturated free fatty acids. Conditions: as per Fig. 1. Carbon dioxide mobile phase at 70°C, with and without water modifier. Column: 100 × 1 mm I.D., 5 μm Nucleosil Cyano (100 Å) (CN2).

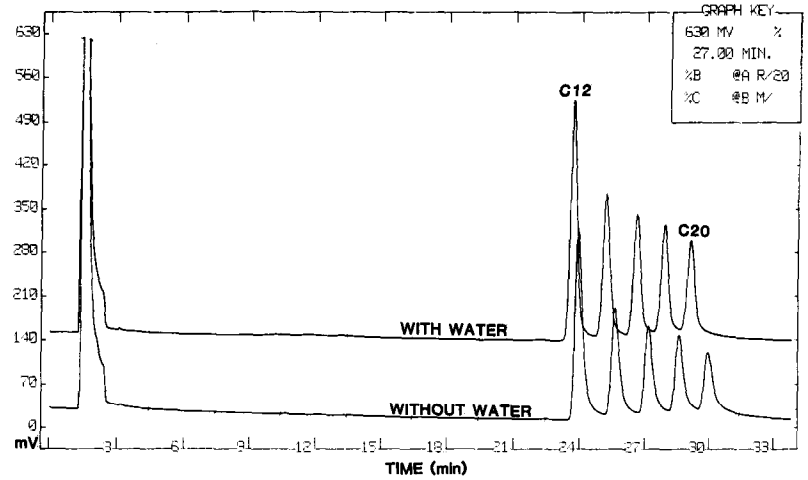

Fig. 6. SFC of C_{12}, C_{14}, C_{16}, C_{18}, C_{20} saturated free fatty acids. Conditions: as per Fig. 1. Carbon dioxide mobile phase at 70°C, with and without water modifier. Column: 100 × 1 mm I.D., YMC Gel PVA-Sil (120 Å) (PVA).

column: a solution of lauric, myristic, palmitic, stearic, and arachidic acids (*ca.* 5 mg/ml each in dichloromethane) was injected via the 0.1-μl injection loop. The column oven temperature was held at 70°C. After a 2-min hold at 1800 p.s.i., the carbon dioxide pressure was increased to 6000 p.s.i. at a rate of 100 p.s.i./min. Each column was tested in duplicate with dry carbon dioxide, and in duplicate with water-saturated carbon dioxide. Water was removed from the SFC columns by heating the column oven to 150°C for 15 min at 6000 p.s.i., then keeping them at 70°C for 10 min. Due to

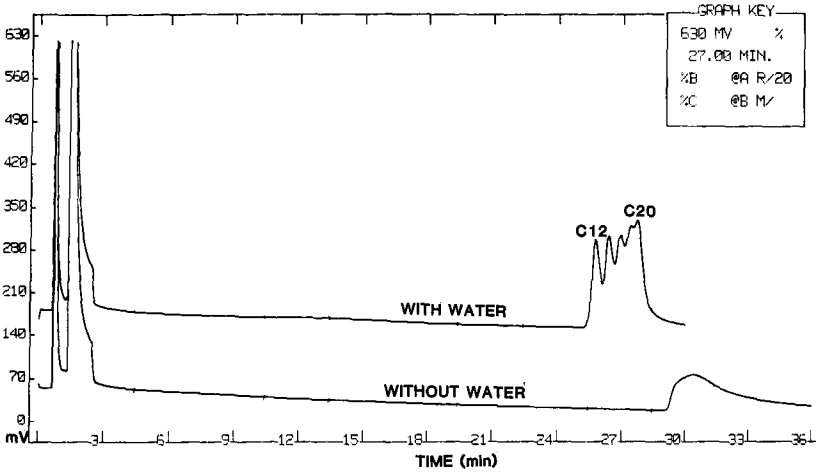

Fig. 7. SFC of C_{12}, C_{14}, C_{16}, C_{18}, C_{20} saturated free fatty acids. Conditions: as per Fig. 1. Carbon dioxide mobile phase at 70°C, with and without water modifier. Column: 100 × 1 mm I.D., YMC Gel Silica (120 Å) (SIL).

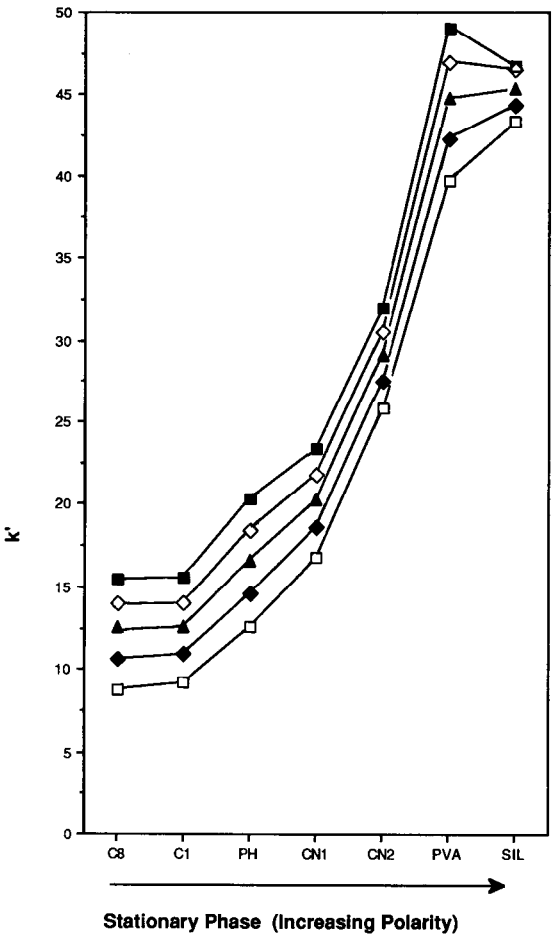

Fig. 8. Free fatty acid k' data *vs.* increasing stationary phase polarity for seven stationary phases. SFC conditions are the same as per Fig. 1, water-saturated carbon dioxide mobile phase, column oven 70°C. Stationary phase abbreviations are given in Figs. 1–7. Free fatty acid solutes were: (⊡) lauric (C_{12}), (◆) myristic (C_{14}), (▲) palmitic (C_{16}), (◇) stearic (C_{18}), (■) arachidic (C_{20}) acid.

the temperature limitations of YMC-Gel PVA-Sil, the drying conditions were modified to 125°C for 12–16 h (overnight).

Column performances with dry and with water-satuarted carbon dioxide are presented in Figs. 1–7 in the order of increasing retention time and stationary-phase polarity. To illustrate the polarity variations of the seven stationary phases futher, k' data for the five free fatty acids were plotted in Fig. 8. These results illustrate that an investigator could vary the retention of lauric acid from $k' = 8.7$ (5.6 min) for Deltabond Octyl to $k' = 39.7$ (23.6 min) for YMC-Gel PVA-Sil. The range of exhibited stationary phase polarities should enable an investigator to select a column based on the resolution requirements for a particular analysis.

Using the protocol presented, this technique should prove useful for rapid

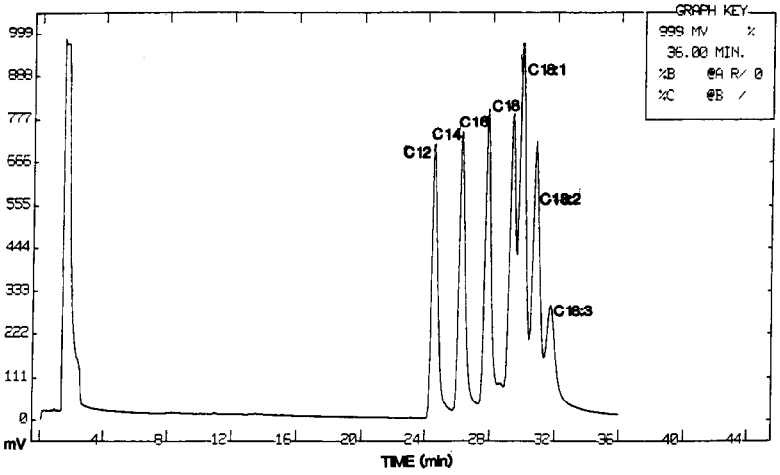

Fig. 9. SFC of saturated and unsaturated free fatty acids. Conditions: same as Figure 1. Water-saturated carbon dioxide mobile phase, column oven 70°C. Column: 100 × 1 mm I.D., 5 μm YMC Gel PVA-Sil. Unsaturated free fatty acids are oleic (C_{18}:1), linoleic (C_{18}:2) and linolenic (C_{18}:3) acid.

screening and selection of suitable packed column SFC stationary phases, and allow these phases to be compared with the performance of existing phases.

In a preliminary study on mixtures of saturated and unsaturated fatty acids, YMC-Gel PVA-Sil was selected for evaluation because of its superior resolving power. The results of the separation are given in Fig. 9 in which oleic, linoleic, and linolenic free fatty acids were separated from a mixture of lauric, myristic, palmitic, and stearic acids under the chromatographic conditions used in Figs. 1–7. Since the unsaturated free fatty acids are model compounds for potent natural products, such as prostanoids, leukotrienes, and thromboxanes, additional research will be directed to evaluating packed-column SFC stationary phases which would enable the FID detection of these important classes of compounds.

CONCLUSIONS

Water-saturated carbon dioxide improved the chromatographic performance of all seven packed-column stationary phases evaluated in the study: (A) peak shape and resolution were improved for covalently-bonded stationary phases (YMC-Gel Phenyl and Nucleosil Cyano) and for non-bonded silica gel (YMC-Gel Silica); (B) peak height and sensitivity were increased for polymer-coated stationary phases (Deltabond Methyl, Octyl, and Cyano, and YMC-Gel PVA-Sil).

A mechanism is proposed which attributes the primary benefit of water-saturated carbon dioxide to masking of undesirable sites (probably unreacted silanol) on the stationary phase. From these results we conclude that packed column SFC stationary phase research should be directed to the manufacture of stationary phases that minimize the requirement for masking of unwanted sites by modifiers. These stationary phases would be expected to exhibit the highest reproducibility and utility, especially in the separation of non-chromophoric solutes requiring the use of FID.

From the qualitative data presented in this study, we conclude that YMC-Gel PVA-Sil (Fig. 6) and Deltabond C8 (Fig. 1) are suitable models for packed-column SFC stationary phases. We recommend that conditions for packed-column SFC separation conditions should routinely include the use of water-saturated carbon dioxide, because of its clear enhancement of chromatographic resolution and sensitivity, especially with polar solutes.

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