

A Study of the Use of Unconventional Solvents As Mobile Phase Additives with a Carbon-Clad Zirconia Stationary Phase: Effects on Retention, Peak Shape, Plate Count and Selectivity for Steroidal Compounds

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Abstract

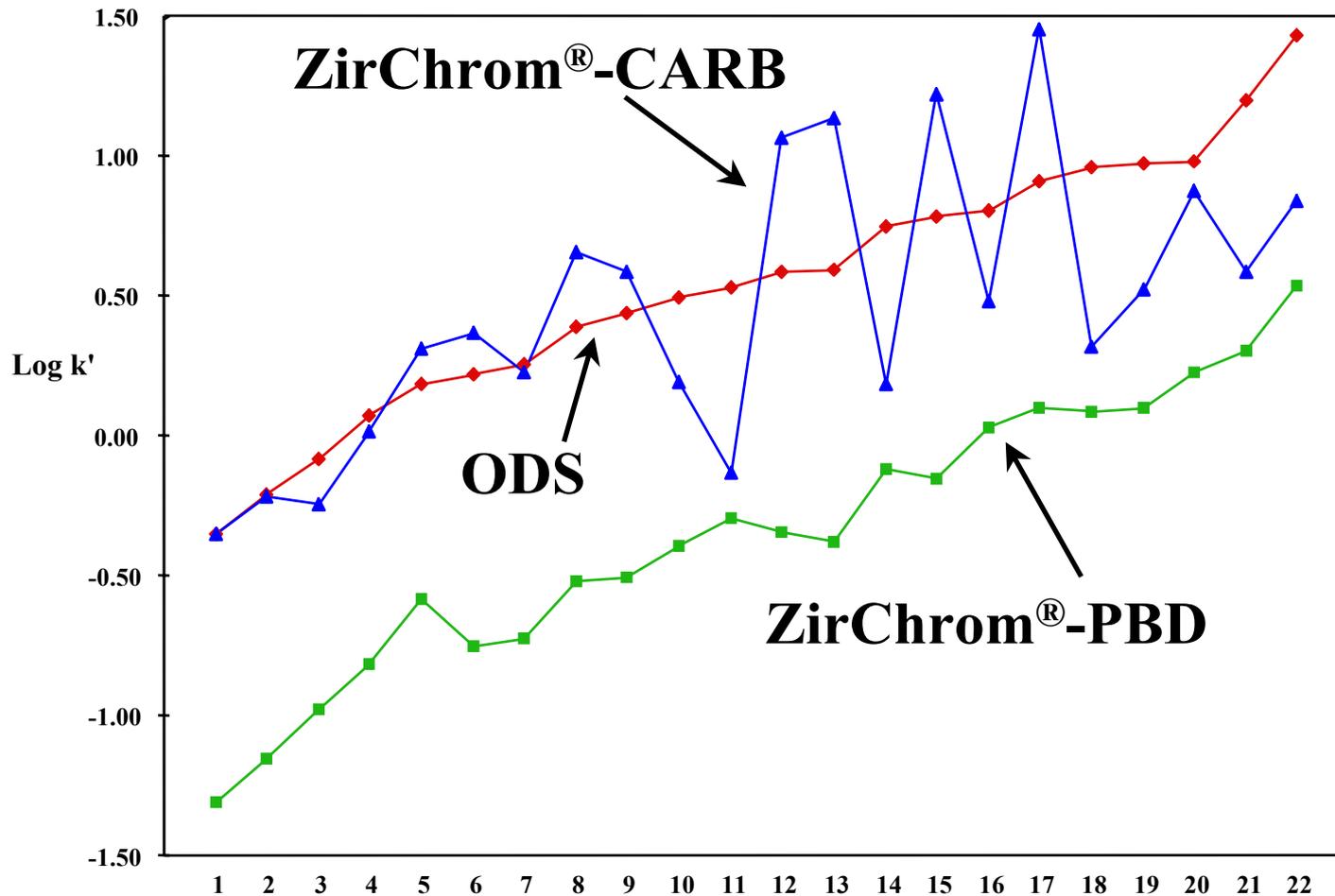
Graphitic carbon and carbon-clad zirconia reversed-phase materials historically have suffered from peak tailing, low column efficiency, and irreversible adsorption of some analytes. Despite recent improvements in carbon-clad zirconia materials, poor performance is still observed for very strongly retained analytes. In this work we systematically studied the use of small amounts (0.1-10% (v/v)) of aliphatic alcohols, nitriles, and amines of increasing chain length as mobile phase additives. The retention, peak shape, plate count, and selectivity for a number of anabolic and estrogenic steroids were studied as a function of the type and amount of additive in eluents consisting primarily of water and acetonitrile. We show that long chain alcohols and nitriles significantly decrease the retention, and also improve the peak shape and plate count of these highly retained steroidal analytes on carbon-clad zirconia phases. In some cases extraordinary selectivities were achieved in some otherwise very challenging separations. Furthermore, the use of long chain alcohols and nitriles is advantageous compared to previously recommended tetrahydrofuran and methyl-tert-butyl ether additives when working with UV detection at low wavelengths because a substantially smaller amount is needed and thus the resulting mixtures are more transparent. Finally, the practical utility of these novel mobile phase additives is demonstrated in the fast (< 5 minutes) separation of some difficult mixtures of anabolic steroids. The inherent chemical stability of the carbon-clad zirconia stationary phase allows the use of elevated column temperatures and flow rates with only modest column backpressures to greatly decrease analysis time.

Outline

- Why carbon phases for HPLC?
- Test solutes and solvent additives used in this work
- Solvent effects on retention of steroids
- Solvent effects on peak shape and efficiency of steroids
- Solvent effects on chromatographic selectivity
- UV spectra of eluent mixtures containing solvent additives
- Ultra-fast separations of closely related steroids at elevated temperature

Project Goal: To explore alternatives to the use of THF and MTBE for improving the performance of carbon-clad zirconia phases

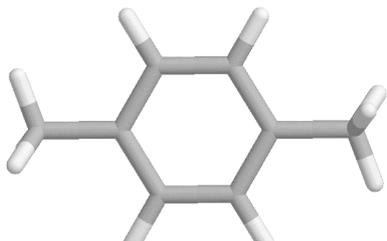
Why Carbon Phases for HPLC? Radically Different Selectivity!



- | | | | | |
|-----------------------|--------------------|----------------------------|-----------------------|--------------------|
| 1. N-benzyl formamide | 6. Acetophenone | 11. Benzene | 16. Bromobenzene | 21. Propylbenzene |
| 2. Benzylalcohol | 7. Benzonitrile | 12. p-chlorotoluene | 17. Naphthalene | 22. n-butylbenzene |
| 3. Phenol | 8. Nitrobenzene | 13. p-nitrobenzyl chloride | 18. Ethylbenzene | |
| 4. 3-phenyl propanol | 9. methyl benzoate | 14. Toluene | 19. p-xylene | |
| 5. p-chlorophenol | 10. Anisole | 15. Benzophenone | 20. p-dichlorobenzene | |

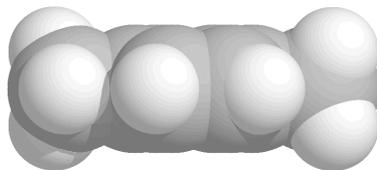
Selectivity and Shape: Isomeric Analytes

p-xylene

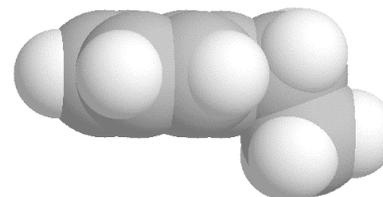
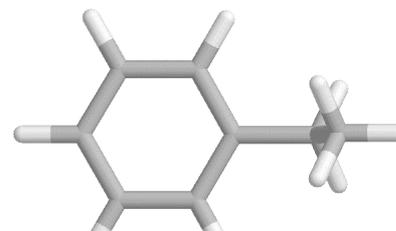


$$\alpha_{\text{ODS}} = 1.03$$

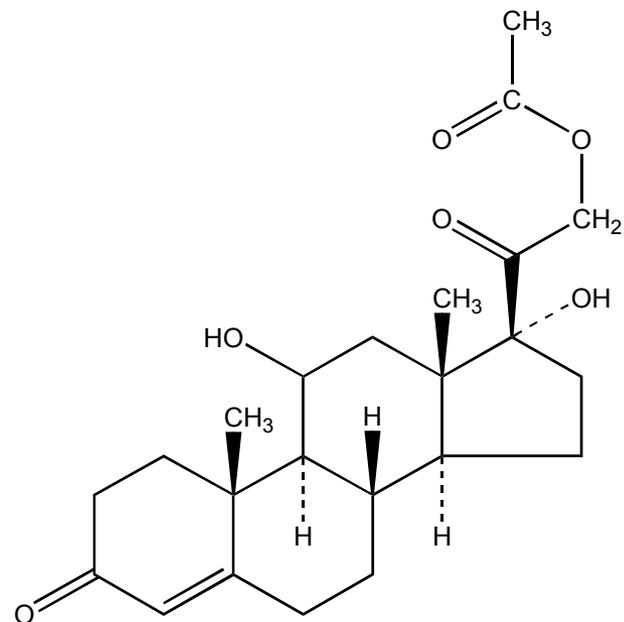
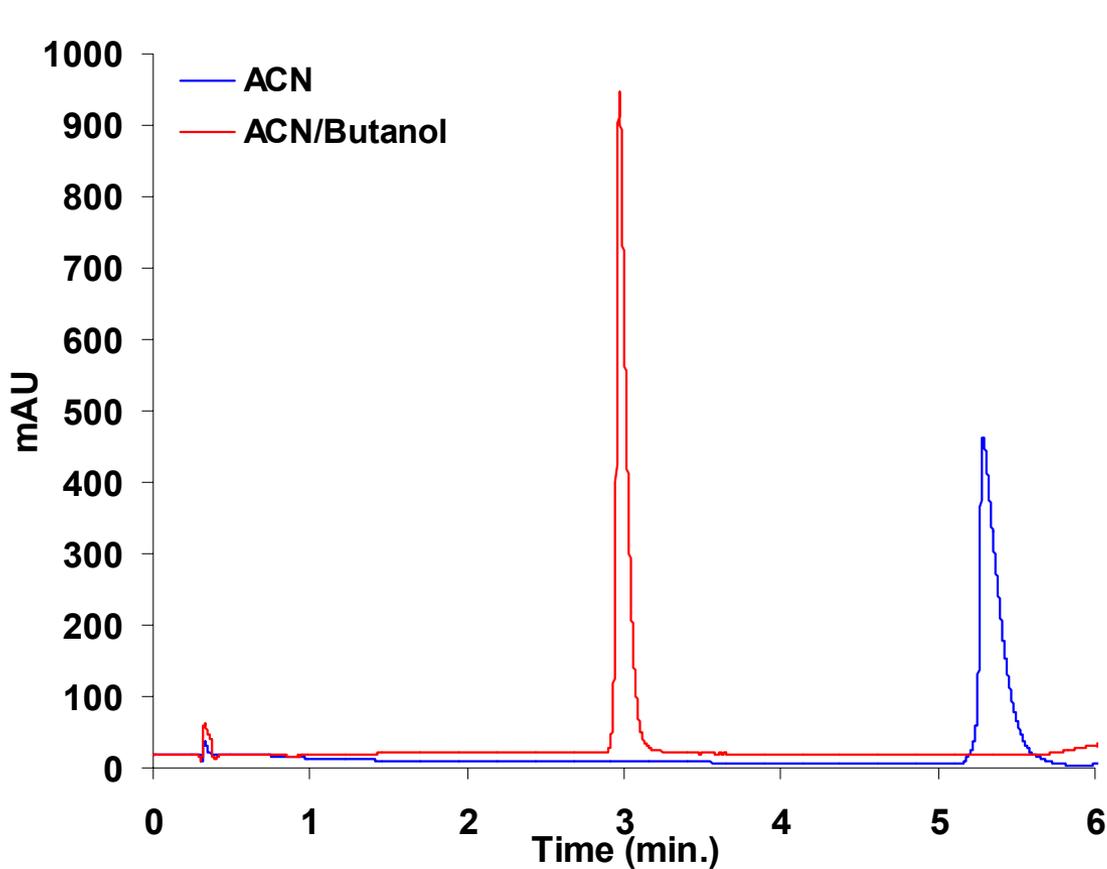
$$\alpha_{\text{C-Zr}} = 1.58$$



ethylbenzene



Long Retention and Poor Peak Shape in Traditional Reversed-Phase Solvents



Hydrocortisone acetate

LC Conditions: Mobile phase, gradient elution from 10-90% of organic modifier from 0-5 minutes, with 20mM ammonium carbonate, pH 9.0 buffer; Flow rate, 2.0 ml/min.; Temperature, 80 °C; Injection volume, 1 µl; Detection at 240 nm; Column, 50 mm x 4.6 mm i.d. ZirChrom®-CARB

How Can We Overcome the Long Retention and Poor Peak Shape?

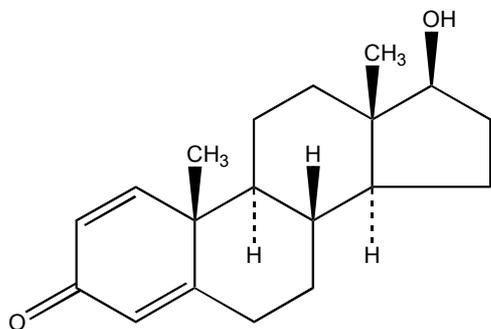
Historically organic modifiers such as tetrahydrofuran (THF) and methyl-tert-butyl ether (MTBE) have been used to decrease retention of strongly retained analytes, and improve their peak shape, however these solvents have several undesirable characteristics including:

- High UV-cutoff
- Incompatible with PEEK tubing
- Peroxide formation
- Toxicity

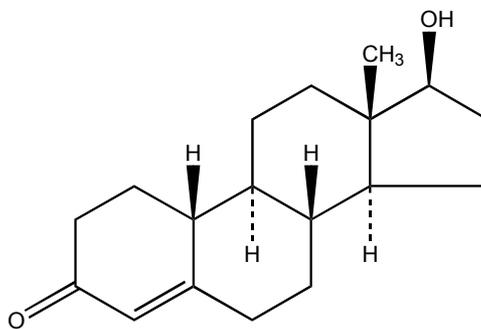
Project Goal: To explore alternatives to the use of THF and MTBE for improving the performance of carbon-clad zirconia phases

Test Solutes Used in this Work

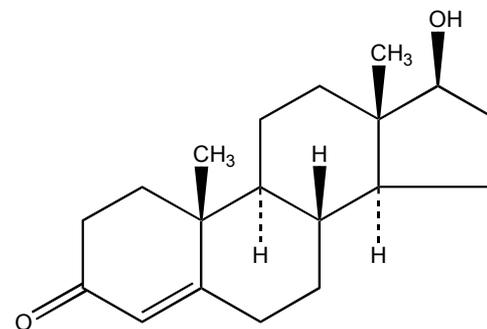
Anabolic Steroids



Boldenone

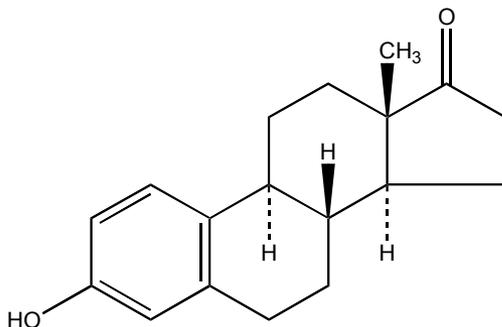


Nandrolone

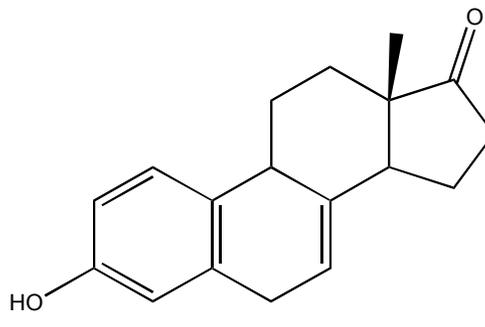


Testosterone

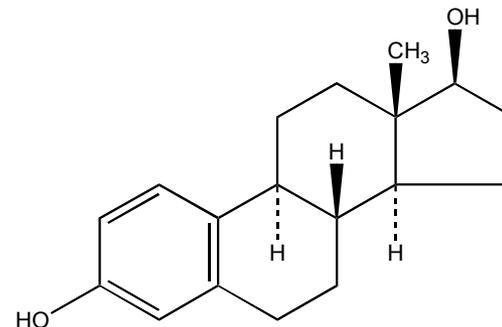
Estrogenic Steroids



Estrone



Equilin



Estradiol

Solvent Additives Used in this Work

Alcohols

Methanol (HPLC grade, Pharmco)

Ethanol (HPLC grade, Pharmco)

Isopropanol (HPLC grade, Pharmco)

1-Butanol (HPLC grade, Fisher Sci.)

1-Octanol (HPLC grade, Aldrich)

Cyclohexanol (99%, Aldrich)

Nitriles

Butanenitrile (98%, Pfaltz & Bauer)

Hexanenitrile (98%, Pfaltz & Bauer)

Octanenitrile (98%, Pfaltz & Bauer)

Octanedinitrile (98%, TCI)

Others

Tetrahydrofuran (HPLC grade, Pharmco)

Methyl-tert-butyl ether (HPLC grade, Aldrich)

Octylamine (99%, Aldrich)

The General Experimental Design

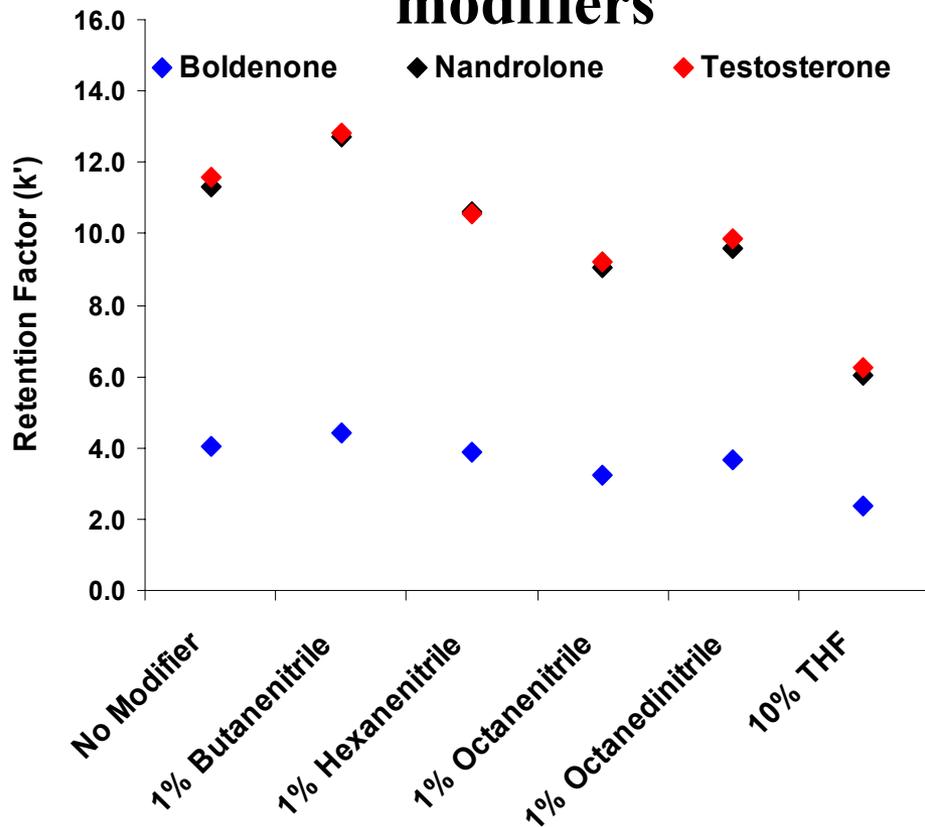
The composition of the mobile phase was the main parameter studied in this work. The mobile phase used in all of the work shown here consisted of a base of 70/30 ACN/water (v/v). As an example, if 1% octanol was tested as an additive, 1% of ACN was removed from the base mobile phase such that addition of the octanol resulted in a mobile phase whose total organic solvent composition was constant at 70% by volume.

All of the other chromatographic parameters were maintained constant as follows:

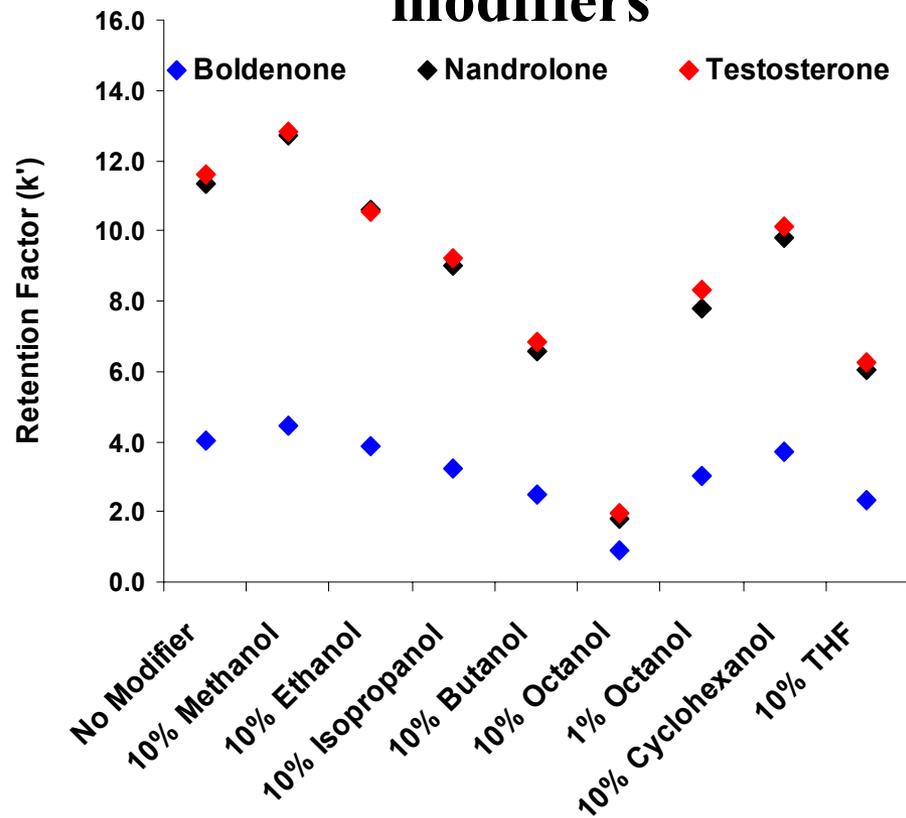
- Column – 50 mm x 4.6 mm i.d. ZirChrom-CARB
- Flow rate – 2.0 ml/min.
- Injection volume, 5 μ l
- Sample concentration – 10 μ g/ml
- Temperature, 80 °C
- Diode array detection at 240 and 280 nm

Effects of Solvent Additives on *Retention* of Steroids

Comparison of nitrile modifiers



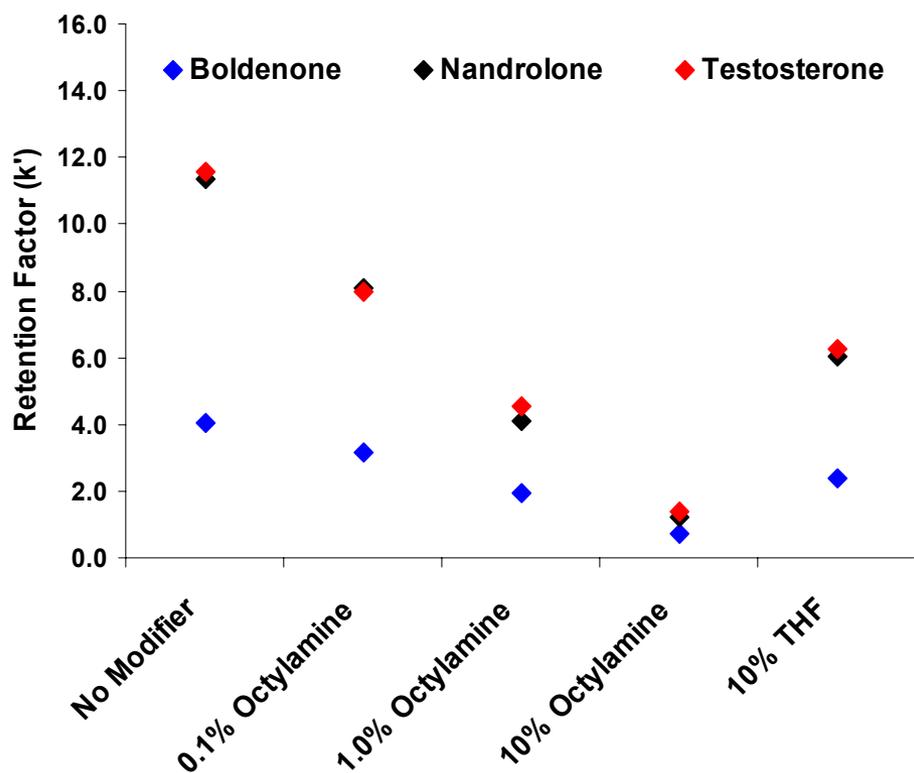
Comparison of alcohol modifiers



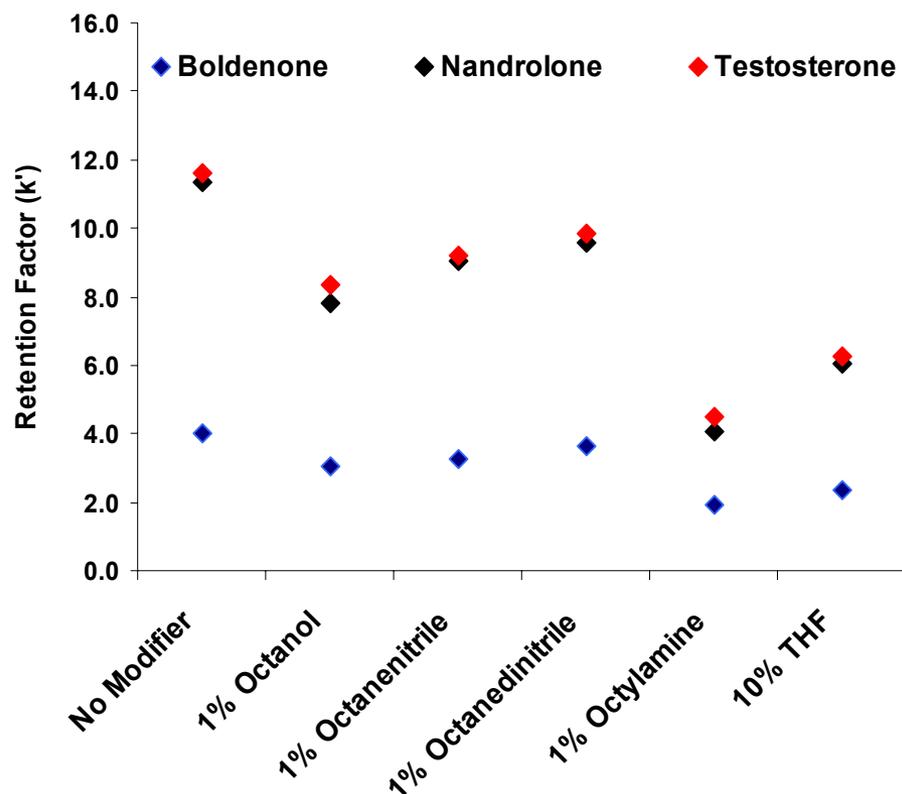
➤ The highest carbon number additive has the largest effect on retention

Effects of Solvent Additives on *Retention* of Steroids

Comparison of different concentrations of modifier

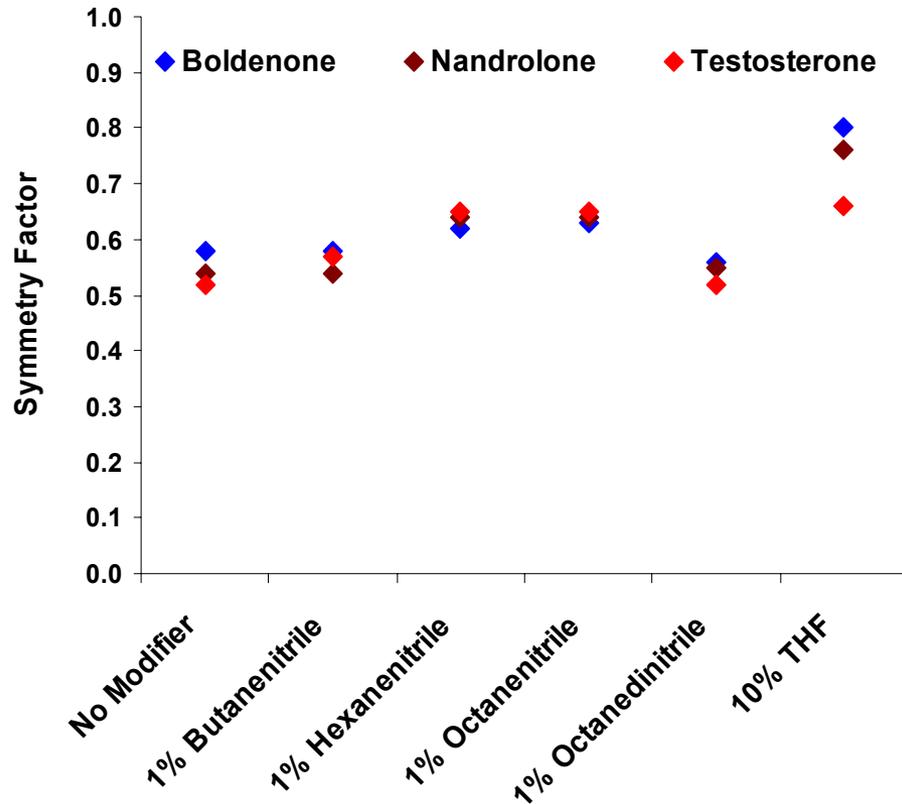


Comparison of 8-carbon modifiers

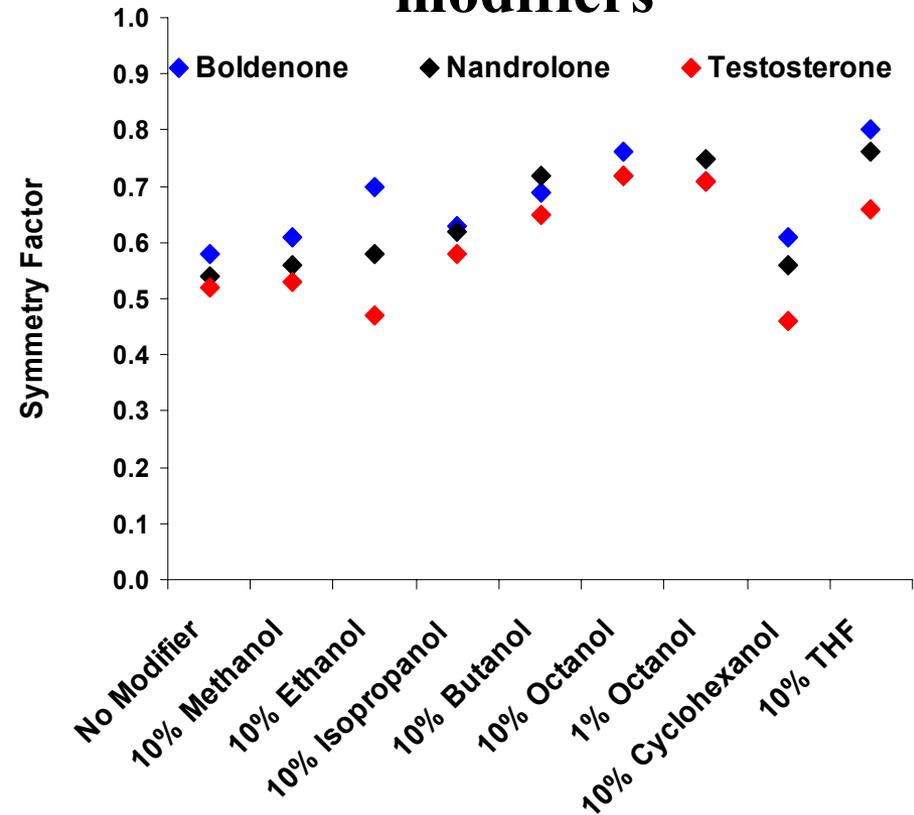


Effects of Solvent Additives on *Peak Shape* of Steroids

Comparison of nitrile modifiers

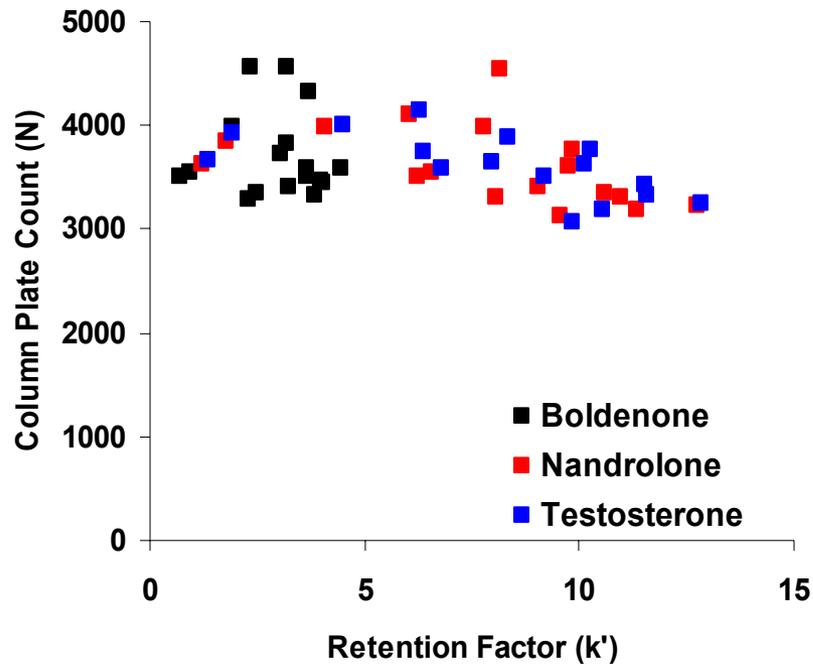


Comparison of alcohol modifiers

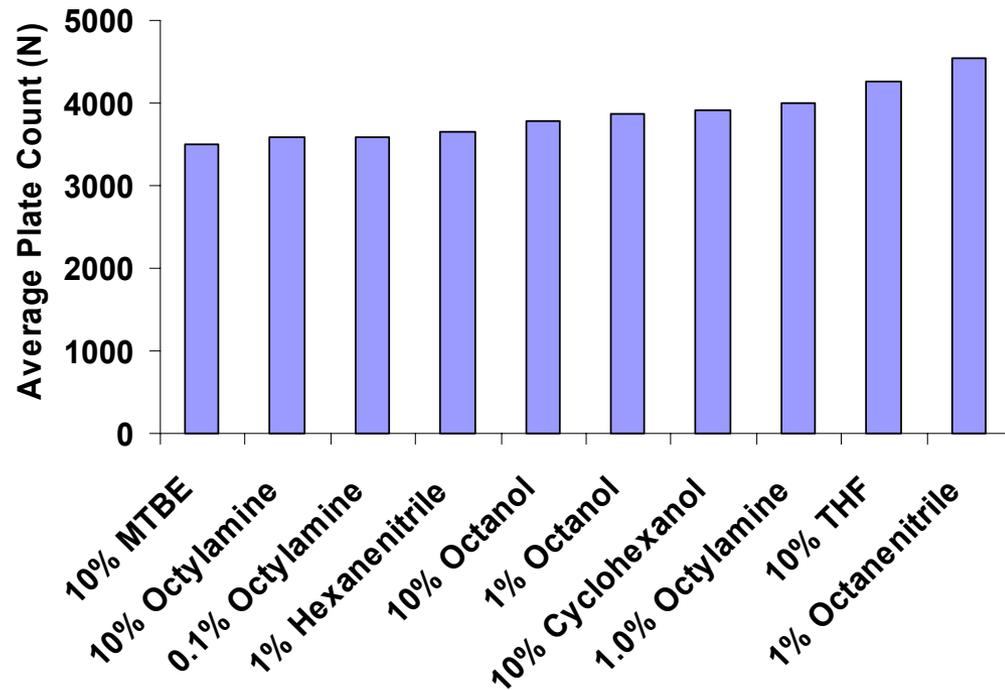


➤ There is considerable peak tailing, even in the best solvents

Effects of Solvent Additives on *Plate Count* of Steroids

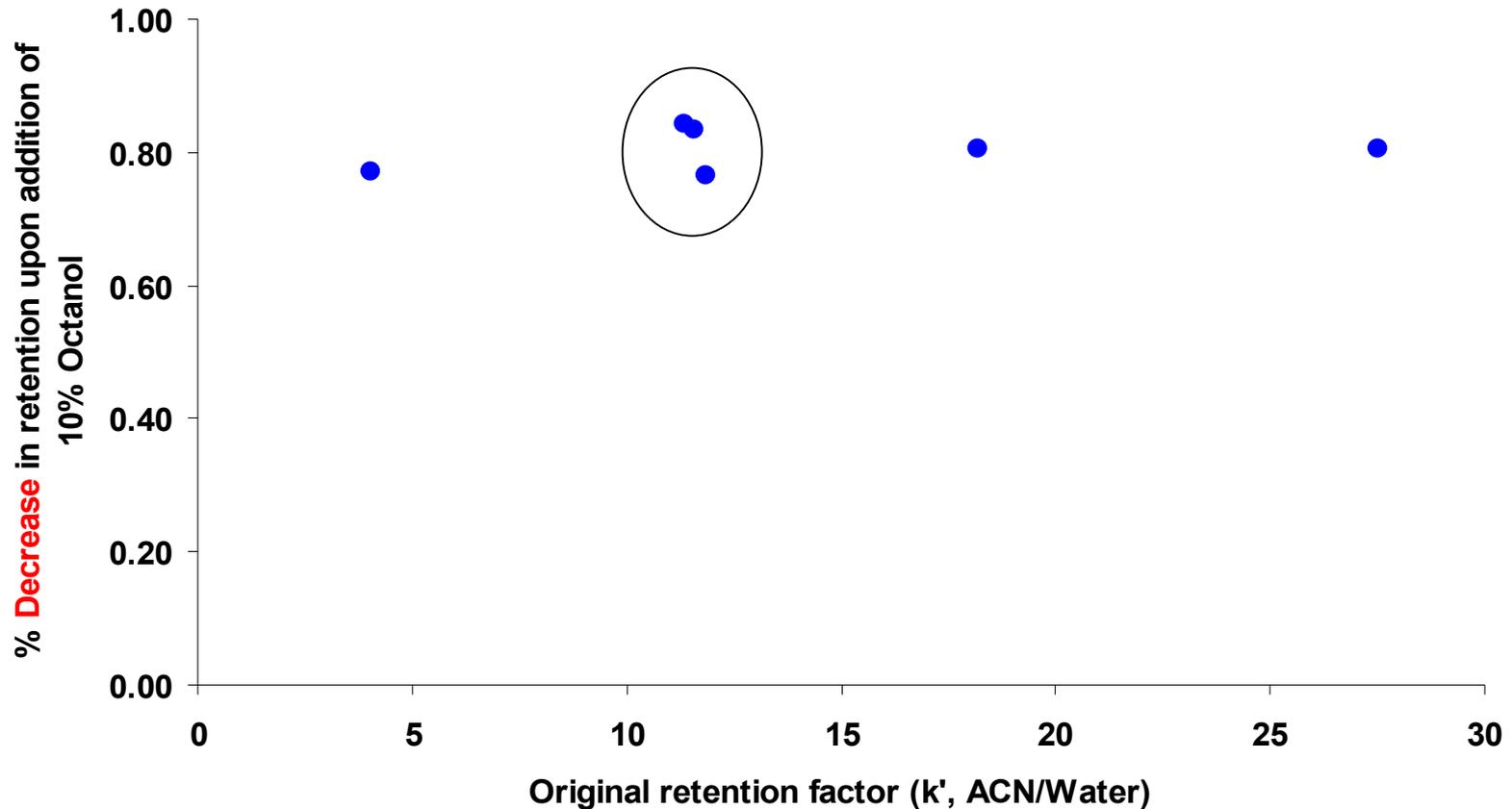


Average plate count for three anabolic steroids in top ten solvents



➤ The effect of the type of additive on the plate count is random

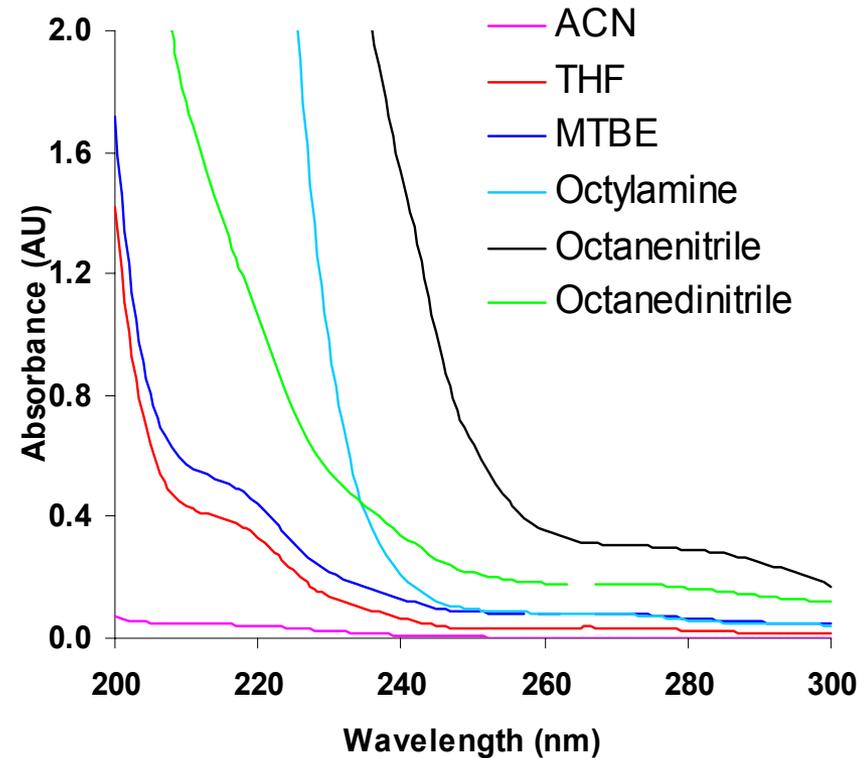
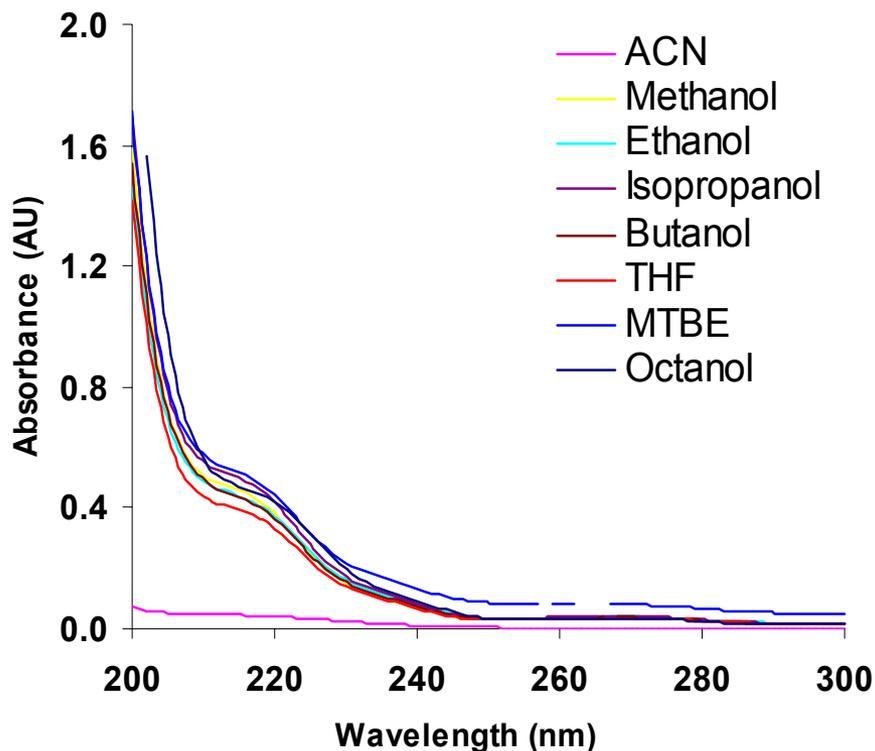
Effect of Solvent Additives on *Selectivity* for Six Steroids



- There are only *small changes in chromatographic selectivity* over a wide range in retention factor

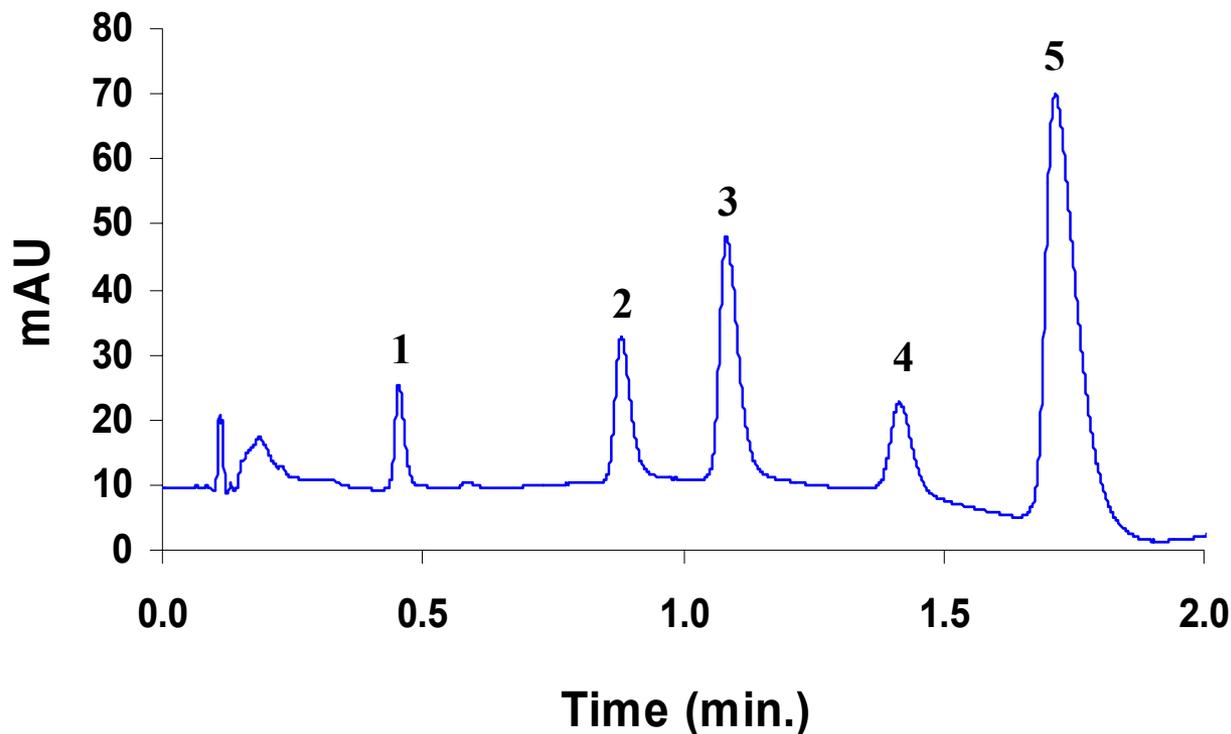
UV Spectra of ACN/Water Eluents Containing Additives

Solvent mixtures containing 65/5/30 (v/v) of ACN/Indicated Additive/Water were prepared and scanned from 200-300 nm.



- The relative similarity of the spectra for all mixtures containing HPLC grade solvents indicates that the very strongest solvent additive should be used, so that the relative amount of the additive can be reduced.

A Fast Separation of Reproductive Steroids



Gradient Elution Profile

Time (min.)	% A	% B
0.0	90	10
1.5	10	90
2.0	10	90

A: 70/30 ACN/Water

B: 85/5/10 ACN/Octanol/Water

Flow rate: 5.0 ml/min.

Temperature: 80 °C

Injection volume: 5 μ l

Detection at 220 nm

Backpressure: 205 bar

Sample concentration: 2 μ g/ml

Solutes: 1-Boldenone, 2-Testosterone, 3-Equilin, 4-Estrone, 5-Estradiol

Conclusions

- The addition of small amounts (0.1-10%) of higher alcohols, nitriles, and amines to typical RPLC eluents dramatically reduces the retention of highly retained steroids on carbon-clad zirconia
- Octylamine was by far the most potent in terms of reducing the retention of the molecules tested
- Only minor effects on efficiency and peak shape were observed for the steroid compounds under the conditions of this work
- Only minor effects on chromatographic selectivity were observed upon addition of small amounts of the solvent additives
- A major limitation to the practical use of these unconventional solvent additives is their general unavailability in sufficient purity for use in HPLC
- Judicious choice of the correct organic modifier and additive, along with the use of elevated column temperature, allows very fast separations of closely related compounds on a carbon-clad zirconia RPLC column

Acknowledgements

