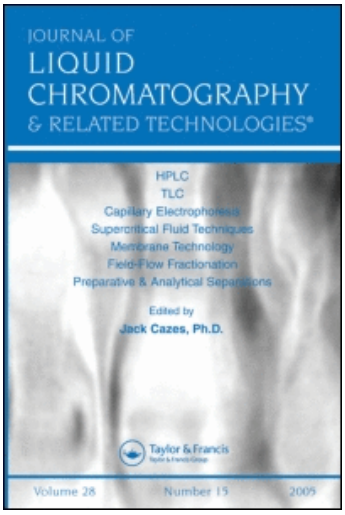

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SAMPLE SOLVENT AS ANALYTE IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract:

Solvents vary in their behavior in high performance liquid chromatography (HPLC). Water and methanol, among others, are widely used in the mobile phase as well as solvents for the solute. Few reports indicate that the solvent used for the solute can behave as an analyte. Normally, it is generally accepted that the solute solvent, a non-constituent of the mobile phase will be the first eluent. However, a solvent which is a component of the sample can show up as an unexpected peak with its own identity. This solvent may show a similar retention time as some of the unknown components of the sample. This indicates that in some cases the quantitative results may be the sum of the absorptivity of the solute and solvent used for the sample. It is assumed that some solvents show no absorption in the ultraviolet region at which the analysis is being conducted. Depending on the mobile phase composition some solvents can be detected at the wavelength or wavelengths used for analysis. Water, ethylacetate, and methanol showed absorption at 210 nm when present in the sample being analyzed with a mobile phase of acetonitrile-methanol using a C_{18} column. These solvents overlapped or showed retention times the same as estriol and testosterone.

Introduction:

The fact that resolution and detection of solvents (nonconstituents of the mobile phase) used as solvent for an analyte has not been fully investigated. Few reports mention the appearance of the sample solvent. Solvents can show absorbance in the region of absorption by the analyte (1). The resolution of this solvent varies and depends on the chromatographic conditions. A solvent can appear as an independent peak or may overlap with another unkown component. The wavelength selected for the assay, if it is the same as that at which the solvent shows absorbance, can result in a broad peak which can overshadow some resolved analytes. Usually sample volume varies from submicroliter to 100 µl (µg to 100 µg/ injection) in each of the injections made. It means solute-solvent ratio varies between 1 to 1000 fold and this may create " holes" and artifactual vacancy (negative) and ghost (positive) peaks (2). This may be true in the case of solvents which are a component of the mobile phase and as well as solvent for the solute. Generally, these solvents do not show appreciable absorbance in UV regions at which most analyses are conducted. But they can show absorption and may cause some difficulty in quantitation and detection. There have been reports on the role of "system peaks" in HPLC (3-5). However, these relate to conditions existing in the mobile phase and not to the solvent used for the solute. Whatman (6) uses a mixture of toluene and ethylbenzene to evaluate C_{18} columns using methanol - water (80:20) as mobile phase.

This report shows that solvent used as a solvent for the solute, can interfere with the analyte assay.

Experimental:
Material and Methods:

All solvents were EM Omnisolv, purchased from E. M. Science. Steroids were from Sigma Chemical Company, USA. Water was, demineralized, filtered and degassed before use. Sample solutions were made in methanol and kept at 4 °C. All solvents were degassed by filtration.

A modular liquid chromatograph (LC) equipped with LDC pump and a variable wavelength UV Spectroflow 773 detector, was interfaced with a Hewlett Packard HP 3385A integrator. A standard Waters LC equipped with model 510 pumps, auto injector (WISP model 512) and a diode array model 990 detector with APC111

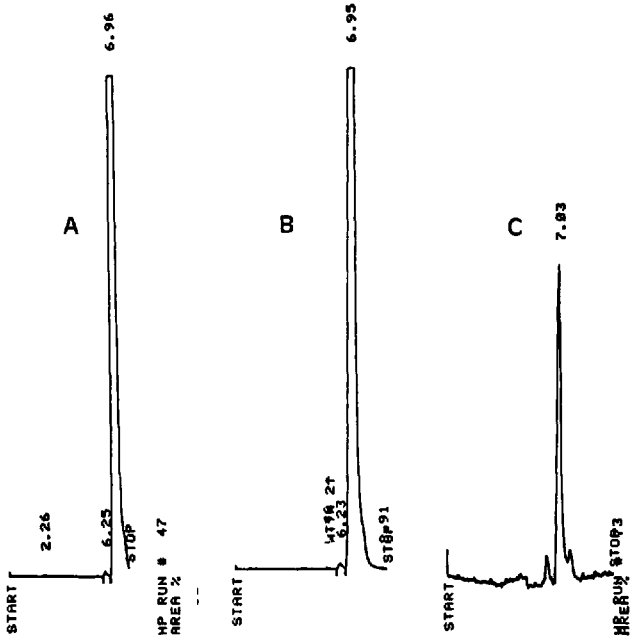

Figure 1: Chromatograms showing separation of various analytes. Mobile phase flow rate was1.5 ml/min. Detection (210 nm); A = t_R acetonitrile = 6.96; B = t_R 16-epiE3 = 6.95; C = t_R ethanol = 7.03

Computer was also used. The column was a Whatman Partisil 10 ODS (250 x 4.6 mm). The mobile phase was acetonitrile-methanol (65:35). Detection was at 210 nm. Other wavelengths were used to scan the spectra of analytes as required.

Results and Discussion:

The chromatograms in Fig 1 show a) acetonitrile; b) 16-epiestriol and c) ethanol detected at 210 nm. All of these solutes showed the same t_R but not absorbance at higher wavelengths. Selective detectors are sensitive only to some property of "solute" (e.g. UV absorption) (7). The absorption maxima of methanol and acetonitrile were found at 205 and 190 nm respectively, which agreed with published reports (8). An injection of both of these

mobile phase components shows absorption (peak) near 205 nm (210 nm) but not at higher wavelength (data shown in Table 1). Although there is change in mobile phase refractive index when analytes are resolved and the detector shows a peak, it demonstrates the bulk property of the mobile phase. It is not necessary that this change in refractive index of mobile phase should also show some absorbance at the chosen wavelength. This phenomena could be further elaborated when resolved analytes are monitored at different wavelengths.

The data in Table 1 show the retention times (t_R) and relative peak height of components assessed at various wavelengths. Solutes were dissolved in a solvent with little or no absorbance in a conventional UV region. The use of UV spectrometry for monitoring the resolution of analytes by HPLC is preferred for those components which show strong absorbance above 220 nm. The absorption depends upon the nature of the bonds within a molecule. Most σ or σ^* absorption takes place below 200 nm. Absorption from π-π^* and n-π^* bonding is also in the lower UV region (1). Benzene (200; 255; 280), methanol (210), acetonitrile (213) chloroform (247) , dichloro methane (233) are common solvents which show absorption in the lower UV region (1).

Selection of solvents to extract compounds of interest using liquid:liquid extraction (LLE) and solid phase extraction (SPE) and to prepare the mobile phase is important in two respects. First the solvent should not show absorbance either as solvent used to dissolve the sample or as a component of the mobile phase in an isocratic or gradient system. Secondly, the sample solvent should have t_R different than the analyte. Assay in the lower UV region may increase instrumental noise and solvent background due to the presence of ultraviolet absorbing compounds from impurities which may be in trace amounts either in the mobile phase or in the solvent. Instrumental noise and background may be overcome using area rejection and attenuation modes. However, dissolving the analyte in the mobile phase may minimize the problem but this may not be suitable in all cases.

16-Epiestriol (16-epiE_3), methanol or acetonitrile when resolved in the methanol-acetonitrile mobile phase have similar t_{Rs} when detection at the wavelength 210 nm was used (Figure 1). Testosterone extracted in toluene and injected directly showed the

Table 1
HPLC of Solvents

Mobile phase : acetonitrile : methanol (65 : 35 v/v)

Analyte	Wavelength nm	t_R	Dilution with mobile phase	Peak height in cm.
	Flow rate 1.0 ml/min			
chloroform	210	10.4	1 : 50	12
carbon disulfide	210	10.5	1 : 40	12
benzene	210	10.4	1 : 50	12
methylene chloride	210	10.6	1 : 5	12
ethanol	210	9.5	1 : 5	12
ethanol	280	ND	1 : 5	
toluene	210	11.4	1 : 5	12
water	210	11.2	1 : 5	4
water	280	ND	1 : 5	
testosterone (in toluene)	245	11.5		5.4
testosterone (in ethanol)	210	11.4		12
testosterone (in toluene)	210	11.4		12
	Flow rate 1.5 ml/min			
methanol	280	6.22	1 : 5	1.4
methanol	210	6.22	1 : 5	4.0
acetonitrile	210	6.95	1 : 5	12
acetonitrile	280	ND	1 : 5	
16-epiE_3 (in mobile phase)	210	6.95		12
16-epiE_3 (in methanol)	280	6.95		7.0
16-epiE_3 (in methanol)	210	6.95		12

t_R = retention time ; Analyte solution was made in mobile phase; ND = not detected; 0.5 µl sample injection was made in all cases.

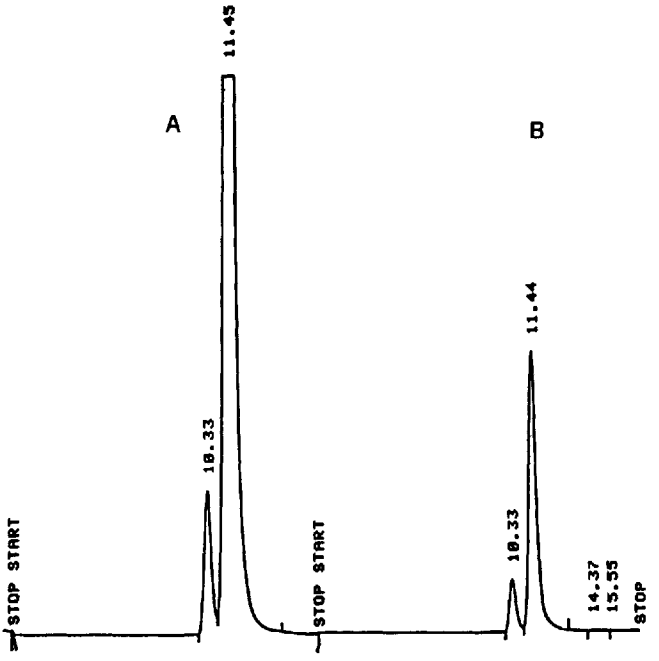

Figure 2: Resolution of testosterone extracted with toluene and aliquot injected (A) = t_R (Testosterone + toluene) = 11.45; (B) = t_R testosterone = 11.44 (reconstituting in ethanol after evaporation of toluene); Detection 210 nm, flow rate 1 mL/min.

same t_R as the toluene. When injected using the mobile phase as solvent the testosterone had the same retention time as toluene injected separately in the mobile phase as the solvent. When reconstituted in the mobile phase the tR was identical for both (Figure 2). Similar results were obtained for chloroform (A) and carbon disulfide (B) (Figure 3). Estradiol -17β (E_2-17β) overlapped H_2O in retention time.

However, 210 nm can sometimes be better. The spectra of H_2O, and E_2-17β between 190 - 240 nm are shown in Figure 4. Both analytes were detected at 210 nm. The spectra demonstrate the lmax for both of the components between 205-208 nm. Chromatograms

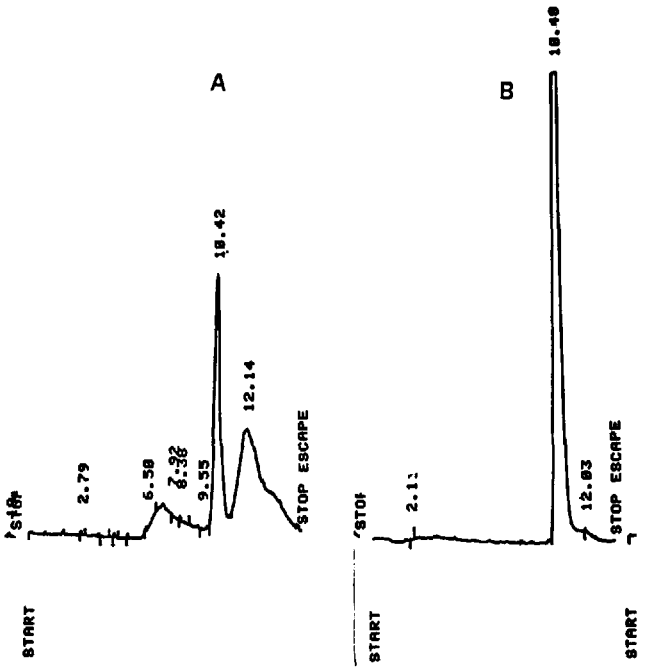

Figure 3: Retention time for chloroform = 10.42; carbon disulfide = 10.48, parameters are same as for Figure 2.

shown in Figure 5, represent methanol, and water and give the relative peak height at various wavelengths between 210 - 230 nm. (Figure 5). They all show maximum absorbance at lower wavelengths. These σ - σ bonded compounds showed λ_{max} in lower UV regions.

In case of thin layer chromatography (TLC), the solute solvent is evaporated prior to development and there is no solvent problem. In HPLC the situation is different since solvent which is used to dissolve and prepare the sample solution can show absorption. An injection of sample consists of solvent and analyte which can be eluted together. If the solvent has the same t_R of any analyte, then change of solvent is required.

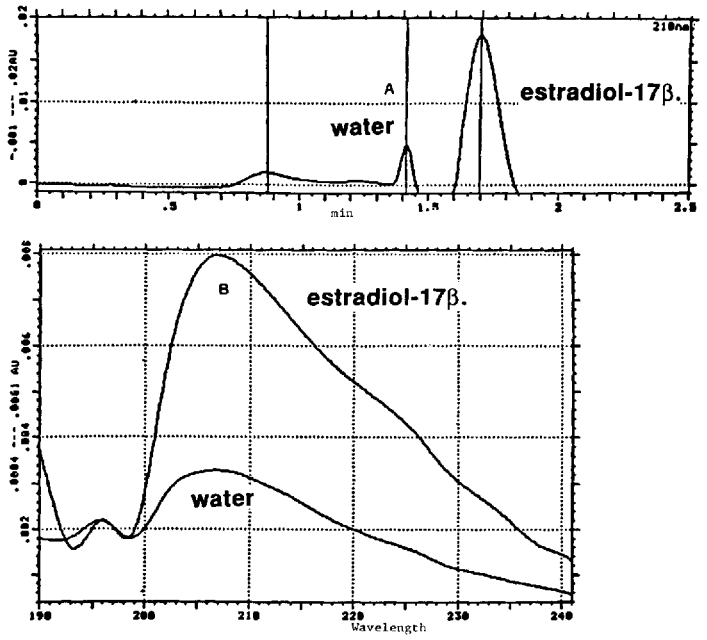

Figure 4: (A): Resolution of water and estadiol-17β.(Detection at 210) (B): Spectra showing the absorption maxima of water and estadiol-17β.

Analytes can be dissolved in the mobile phase to circumvent solvent problems. In certain cases this is not possible e.g. testosterone extracted in toluene. When injected they showed a single peak or overlapped. The solvent and analyte had the same t_R and absorbed at 210 nm but this could be circumvented by dissolving the extracted specimen in methanol or mobile phase after evaporating the toluene. For more precise quantitative HPLC more attention must be paid to the solvent behaving as analyte.

During previous work in this laboratory it was noticed that the "solvent" peak (freak) was more readily separated from the desired analyte when the column was cooled to subambient temperature. Some analytes do not resolve at ambient temperatures but separated at subambient temperatures. This was previously shown when

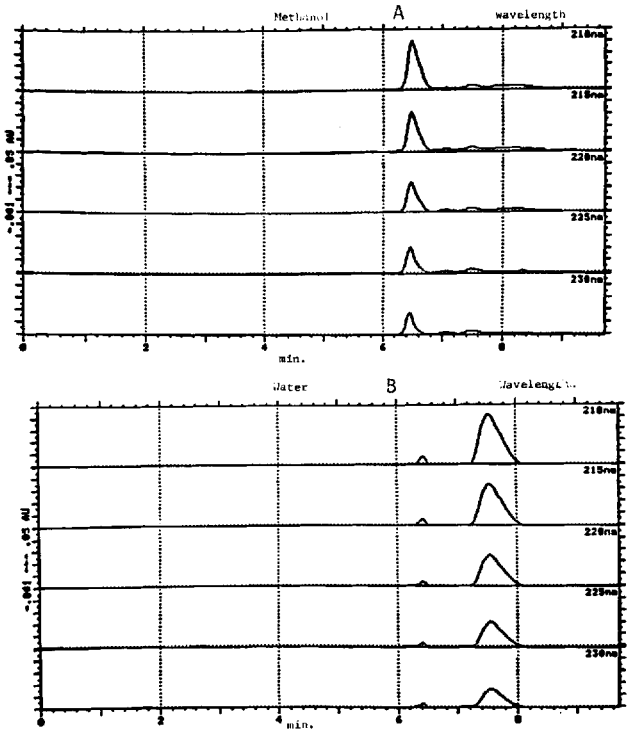

Figure 5: A: Peak height variation at different wavelenghts for water(A) and methanol(B).

chromatograms were developed at 25, 0, -30 and -50 °C to resolve diasteromers androst-5-ene 3β, 17 α-diol and androst-5-ene- 3β, 17β-diol (9). Thus, cooling can also be used to overcome vagaries due to sample solvent (nonconstituent of the mobile phase).

Some reports indicate system peaks in ion-chromatogrphy(IC). These showed up due to solvent dilution factor which may be an outcome of disturbances of chromatography equilibria (4) or pH of buffers in isocratic and gradient systems (5). In the present report these factors are not involved. There were no negative, vacancy or ghost peaks involved. That water shows absorbance in the lower wavelengths of the ultraviolet has been well

documented. Mc Nesby and Okabe (10) showed that two continua exist 1250 - 1860 A (125.0 - 186.0 nm) with a maximum about 1665 A (166.5 nm). In the presence of media it would be reasonable to expect that the maxima for the water would shift. This can explain why water shows a maximum at 205 nm as shown in Figure 4.

Zhang and Imre(11) recently reported studies of mathematical models of the photodissociation dynamics of water. The results of experiments on both the ground and excited states are discussed. Emission spectra (resonance Raman) of water vapor excited at 160 nm were described. Furthermore, Smith and Baker (12) showed that the absorption coefficient of water at 200 nm was greater than at the higer wavelengths. Further study is needed to evaluate the absorption characteristics of water or other solvents in a medium presented by a mobile phase.

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