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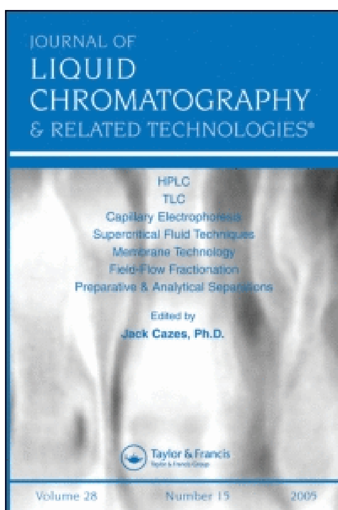

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INTEREST OF A SIMPLE MOBILE PHASE CONTAINING DIETHYLAMINE-ACETIC ACID FOR HPLC ASSAY OF NICOTINE AND COTININE

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ABSTRACT

We have developed a simple mobile phase containing a diethylamine/acetic acid mixture free of salts and ion-pairing compounds for the assay of nicotine and cotinine in biological media by means of high-performance liquid chromatography. The best composition was found to be methanol/acetonitrile/acetic acid/water (230/20/2/748 v/v) adjusted to pH 5.00 or 6.40 with diethylamine. The results were excellent when compared with those obtained from

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mobile phases containing the following buffers: monopotassium phosphate/phosphoric acid, dipotassium phosphate/citric acid and sodium acetate/acetic acid. We found that 1) its buffering power is similar to that of the others and 2) it reduces pump-head wear and column washing time. Various columns may be used with this mobile phase, including the C18, 5µm Ultrasphere TM

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INTRODUCTION

Respirable particles and toxic gases released by tobacco combustion and exhaled by active smokers may have important public health implications (1-2). It is now well established that the degree of exposure to tobacco smoke among smokers and non-smokers can be estimated by the measurement of nicotine and its major metabolite, cotinine, in body fluids, particularly urine (3). Following extraction, several analytical techniques can be used for determining these analytes, including radioimmunoassay (4), enzyme-immunoassay (5), gas-liquid chromatography, gas chromatography-mass spectrometry (6) and reversed-phase isocratic high-performance liquid chromatography (HPLC), with (7) or without (8) an ion-pairing procedure. In the last decade, HPLC has been the preferred technique for rapid analysis for nicotine and cotinine in biological specimens. In epidemiological studies, interference by caffeine, found in 95% of samples in a recent

study (9), must be taken into account. Machacek (7), Hariharan (10) Godin (11) and ourselves (12) have overcome this interference by using C18 stationary phases and mobile phases containing water, methanol and/or acetonitrile. The fundamental difference between these mobile phases is the type of buffer and the working pH. The choice of pH is capital in avoiding the coelution of cotinine and caffeine. Furthermore, the addition of large quantities of salts to these mobile phases can overload the stationary phase and reduce its resolving power. Finally, it diminishes column and pump-head life and necessitates lengthy washing. These phenomena are aggravated when ion-pairing sodium heptane sulfonate is used.

In this work, we developed and evaluated a simple mobile phase (water, methanol, acetonitrile and acetic acid) which gives a good resolution of nicotine and cotinine and avoids interference by caffeine. Using varying diethylamine (DEA) concentrations, we determined optimum pH. We then optimized the concentration of each component, evaluated the buffering power of the DEA/acetic acid mixture and compared it to that of the mobile phases described by Machacek, Hariharan and Godin.

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MATERIALS AND METHODS

* Reagents :

Solvents (HPLC-grade reagents), acetic, citric and phosphoric acids, mono and dipotassium phosphate, sodium

acetate and diethylamine were from Prolabo (75011 Paris, France). Nicotine, cotinine and caffeine were from Sigma (St Louis, MO 63178).

•Standard Solutions :

For the optimization of the mobile phase and the precision study, nicotine, cotinine and caffeine were dissolved in methanol to give working solutions with concentrations of 25, 50 and 100 µg/mL. The detection limits were determined using 0.05 to 1.00 µg/mL nicotine and cotinine in methanol.

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*Instrumentation :

We used an isocratic HPLC system (Waters, Milford, MA 01757) comprising a model-501 solvent pump, a U6K injector, a variable-wavelength model-481 spectrophotometer (λ = 260 nm) and a model-745 data recorder and electronic integrator.

•Chromatographic Conditions :

We used a 22 X 0.48 (i.d) cm column of C18, 5µm Ultrasphere™ UL 225 (Société Française Chromato Colonne, 93360 Neuilly-Plaisance, France).

The mobile phase was optimized using mixtures of water, methanol, acetonitrile and acetic acid at variable concentrations, and pH was adjusted with diethylamine. The composition of the optimal mobile phase is reported (D) in Table I, which also shows the characteristics of the buffers described by Machacek (A), Hariharan (B) and Godin (C).

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TABLE I Composition of Studied Mobile Phases.
A, B, C, and D represent the mobile phases of Machacek, Hariharan, Godin and ourselves, respectively.

	MOBILE PHASES			
	A	B	C	D
WATER (mL)	750	750	750	748
METHANOL (mL)	230	230	230	230
ACETONITRILE (mL)	20	20	20	20
ACETIC ACID (mL)	–	–	–	2
DIETHYLAMINE	to pH 5.00	to pH 5.00	to pH 5.00	to pH 5.00
BUFFER COMPOSITION (FINAL CONCENTRATION)	Monopotassium phosphate/ Phosphoric acid (0.04 M)	Dipotassium phosphate/ Citric acid (0.02 M)	Sodium acetate/ Acetic acid (0.04 M)	–

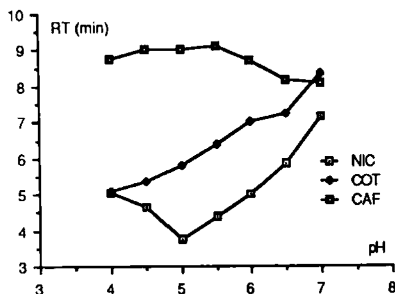

Figure 1: pH effects on retention time (RT) of nicotine (NIC), cotinine (COT) and caffeine (CAF). Mobile phase = water/methanol/acetonitrile/acetic acid (748/230/20/2 v/v) and DEA to vary pH.

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RESULTS AND DISCUSSION

•Mobile Phase Development :

-Optimal pH :

In order to avoid tailing peaks, some authors adjust mobile-phase pH by using triethylamine or N-butylamine. We preferred diethylamine, which can be used at lower concentrations because of its higher alkalinity. Figure 1 shows the effects of pH on nicotine, cotinine and caffeine resolution when adjusted using variable amounts of DEA in a 748/230/20/2 (v/v) mobile phase of water/methanol/acetonitrile/acetic acid. The retention time (RT) of caffeine was almost constant (about 9 minutes), that of cotinine varied from 5.10 to 8.15 min and that of nicotine from 3.75 to 7.15 min between pH values from 4.00 to 8.00. In agreement with Machacek's results, the best pH value was 5.00

($RT_{nicotine}$ = 3.75 min) but, as stated by Hariharan and Godin, a pH of 6.40 ($RT_{nicotine}$ = 5.80 min) can also be used, since both give excellent separation. Urinary specimens gave interfering peaks at RT of between 0 and 3.50 minutes. "Clean" chromatograms are therefore obtained when the RT of the first eluted substance (nicotine) is above 3.50 minutes. pH values above 6.40 should be avoided in order to prevent the rupture of the siloxane-stationary phase bridges.
In the remainder of the study we used pH 5.00, which offers greater mobile phase stability than pH 6.40.

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-Composition :

The ideal proportion of each mobile phase component was determined by studying the variations in the resolution and RT of nicotine, cotinine and caffeine with different methanol, acetonitrile and acetic acid concentrations. As shown in Figure 2, the methanol concentration can vary from 190 to 250 mL/L, that of acetonitrile must be less than 40 mL/L and that of acetic acid must be above 0.8 mL/L. The optimum mixture was 230 mL methanol / 20 mL acetonitrile / 2 mL acetic acid / 748 mL water and DEA to pH 5.00 (or 6.40).

-Mobile Phase Specifications :

Using the mobile phase described above, we obtained a chromatogram with sharp peaks (Fig 3) and low ∂ and values. The resolution was excellent. Nicotine was eluted

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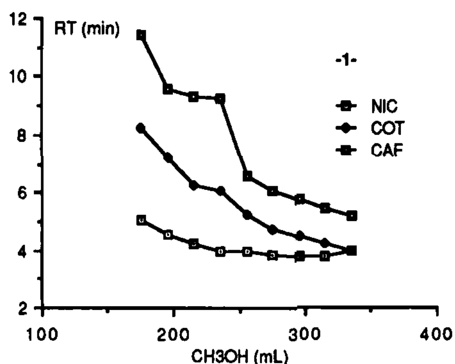

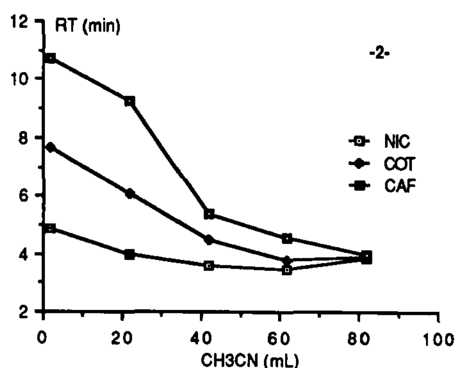

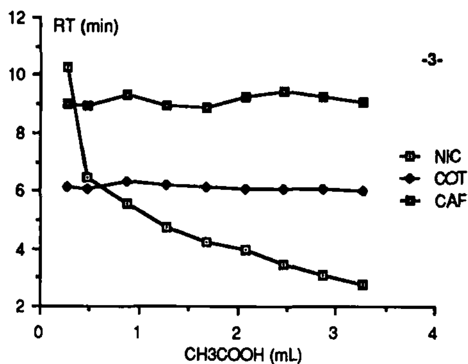

Figure 2: Influence of methanol (-1-), acetonitrile (-2-) and acetic acid (-3-) concentration on the retention of nicotine, cotinine and caffeine. Mobile phases composition: -1- acetonitrile 20 mL, methanol 170 to 340 mL, water to complete to 1000 mL; -2- methanol 230 mL, acetic acid 2 mL, acetonitrile 0 to 80 mL, water to 1000 mL; -3- methanol 230 mL, acetonitrile 20 mL, acetic acid 0.2 to 3.2 mL, water to 1000 mL. pH ajusted to 5.0 with DEA.

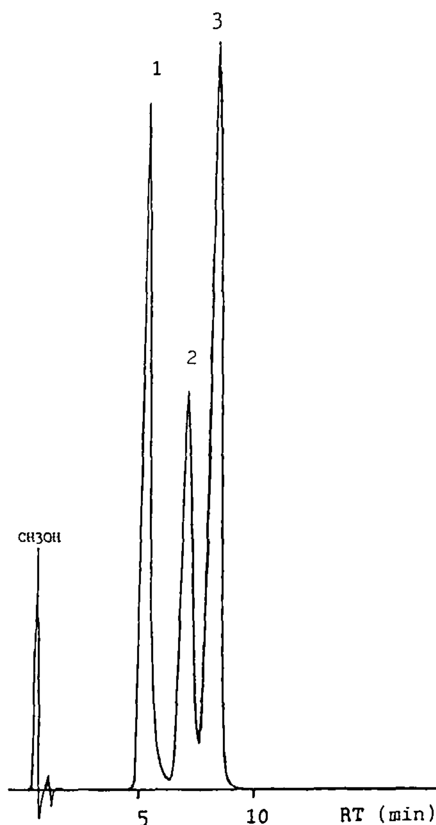

FIGURE 3. HPLC chromatogram of nicotine (1 : 100 µg/mL), cotinine (2 : 100 µg/mL) and caffeine (3 : 150 µg/mL) standard solutions.

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sharply, followed by the cotinine peak, distinctly separated from interfering caffeine which is eluted last. The capacity factor k' = RT - RT_0 / RT_0 (RT being the retention time of the analyte and RT_0 the retention time of methanol) is better than 2 and the separation factor $\alpha = k'_{caffeine} / k'_{cotinine}$ better than 3.

This method is sufficiently sensitive to detect nicotine and cotinine concentrations of 4.0 and 2.4 ng, respectively, in a 20µL injection volume.

Data on the precision of the assay are summarized in Table II. Reproducibility was evaluated by determining within- and between-run precision for three concentration standards.

The mobile phase was used successfully with other columns, including Nucleosil™ C18, 5µm, 25cm, 4.6mm i.d. (Société Française Chromato Colonne) ; Novapak™ C18, 4µm, 15cm (Waters) ; Partisil™ ODS2, 10µm, 22cm, 4.6mm i.d. (Whatman, Santa-Clara CA 95050) ; Spherisorb™ RP18, 5µm, 22cm, 4.6mm i.d. (Brownlee, Santa-Clara CA) and Hypersil™ C18 ODS, 5µm, 25cm, 4.6mm i.d. (Shandon, Southern Instruments, Servickley, PA). The use of C18 columns with too high a retention power (e.g. Zorbax™) should be avoided..

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• Comparative Study With Other Mobile Phases :

-Buffering Power :

Figure 4 shows the titration curves for our DEA/acetic acid mixture and the other buffers listed in Table I. System A (monopotassium phosphate/phosphoric acid) is a classical buffer developed by Sorensen (13). System B (dipotassium phosphate/citric acid) is a universal buffer with a low buffering power but over a wide pH range. It was derived from Mac Ilvaine's buffer (14). System C (sodium acetate/acetic acid) is a biological buffer with a lower neutralizing capacity

TABLE II Within- and Between-run Precision of Nicotine, Cotinine and Caffeine Assays.

	NICOTINE		COTININE		CAFFEINE	
	Mean (SD) µg/mL	CV%	Mean (SD) µg/mL	CV%	Mean (SD) µg/mL	CV%
WITHIN (n=30)						
LOW	25.3 (0.28)	1.11	24.8 (0.23)	0.93	23.7 (0.33)	1.39
MEDIUM	49.8 (0.81)	1.63	50.1 (0.59)	1.18	51.2 (0.48)	0.94
HIGH	102.6 (2.28)	2.22	97.3 (1.24)	1.27	101.6 (1.15)	1.13
BETWEEN (n=15)						
LOW	26.2 (1.59)	6.07	26.5 (1.40)	5.28	25.8 (1.50)	5.81
MEDIUM	47.4 (1.90)	4.01	49.7 (1.59)	3.20	52.3 (1.65)	3.15
HIGH	103.5 (4.39)	4.24	96.2 (4.10)	4.26	102.7 (3.42)	3.33

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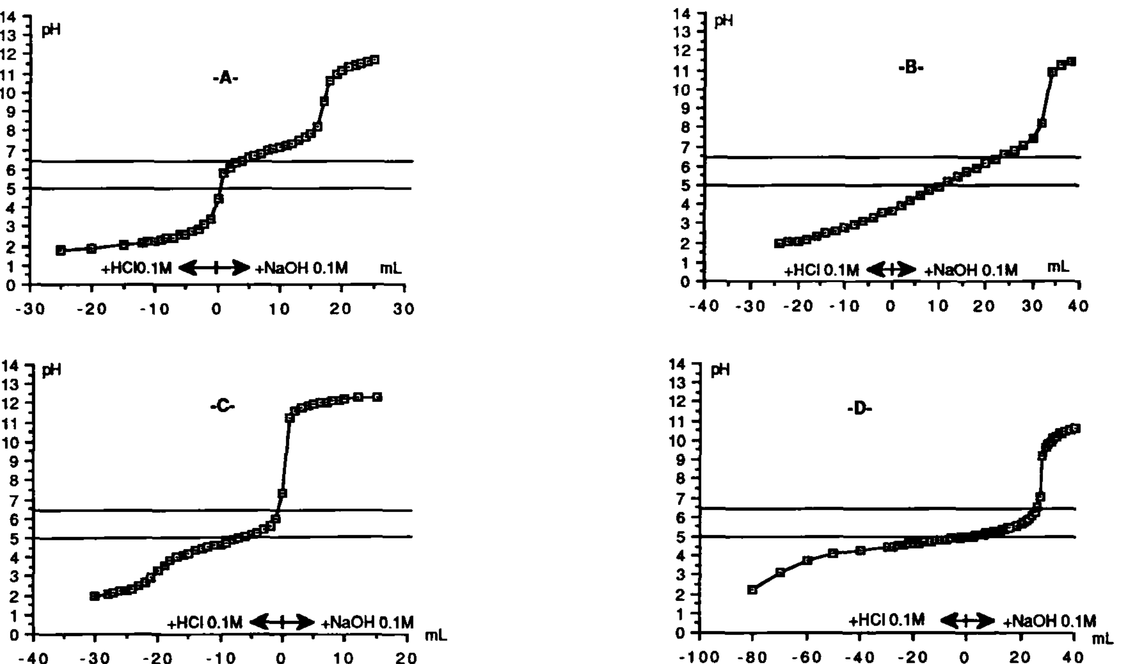

Figure 4: Determination of the Buffering Power of the Four Systems Studied (-A- monopotassium phosphate/phosphoric acid 0.04 M; -B- dipotassium phosphate/citric acid 0.02 M; -C- sodium acetate/acetic acid 0.04 M; -D- diethylamine/acetic acid 0.1 M) by addition of 0.1 M NaOH or HCl .The horizontal lines indicate the two pH values studied.

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TABLE III Mobile Phase Specifications .

	BUFFER COMPOSITION	BUFFERING RANGE	MOBILE PHASE pH
PRESENTED MOBILE PHASE	Diethylamine/ Acetic acid	4.50 to 6.00	5.00 or 6.40
MACHACEK'S MOBILE PHASE	Monopotassium phosphate/ Phosphoric acid	2.50 to 3.50 and 6.50 to 8.00	4.80
HARIHARAN'S MOBILE PHASE	Dipotassium phosphate/ Citric acid	2.50 to 7.50	6.10
GODIN'S MOBILE PHASE	Sodium acetate/ Acetic acid	4.00 to 5.50	6.40

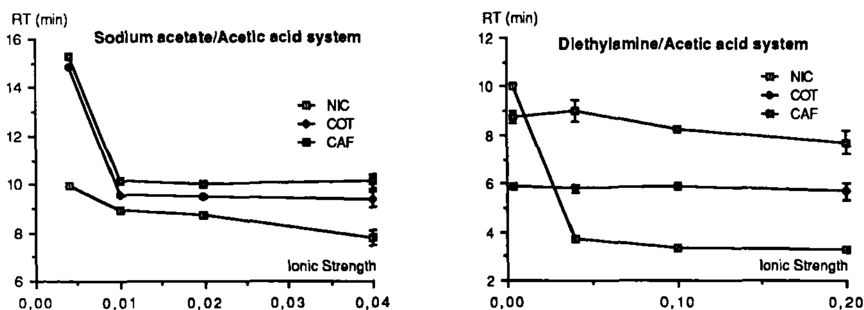

Figure 5: Ionic Strength Effects on Retention Time (RT) of Analytes Studied.

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(15). It is particularly stable and does not contain phosphates (responsible for undesirable reactions in biological fluids). System D (diethylamine/acetic acid) is a buffer with characteristics highly comparable to those described above. It stabilizes the mobile-phase pH between 4.50 and 5.50 according to the following reaction : $CH3COOH + (C_2H_5)_2\ NH \longleftrightarrow CH_3COO^- + (C_2H_5)_2\ NHH^+$. In the presence of acid : $CH_3COO^- + H_2O \longrightarrow CH_3COOH + OH^-$; in the presence of base : $(C_2H_5)_2\ NHH^+ + H_2O \longrightarrow (C_2H_5)_2\ NH + H_3O^+$.

System A buffered little at pH 6.40 and not at all at pH 5.00. System B buffered little at the two pH values. System C and D buffered at pH 5.00 but not at 6.40. Although the relevant authors did not always choose a working pH within the buffering zone (Table III), the reported results were good: at pH 5.00 and 6.40, mobile phases containing the three buffers studied gave similar results to those obtained with our DEA/acetic acid system. We infer that no buffering power is

required in mobile phases, and that the major role of the substances used is in the ionization of the analytes (e.g. nicotinium ion formation) and interactions with the stationary phase.

The reason we chose pH 5.00 was therefore not because it fell within the buffering zone of the DEA / acetic acid system, but simply because it gave the best stability.

-Influence of Ionic Strength :

In agreement with Godin's results (11), we found that ionic strength was an important parameter on the resolution power. At our working pH, sodium acetate and diethylamine are completely ionized. Therefore, we calculated their ionic strength (I) using the formula :

$I = 1/2 \Sigma Ci Zi^2$ (Ci = Mol/L ionic concentration; Zi = ion charge). Resolution varied with the sodium acetate/acetic acid or DEA/acetic acid systems when ionic strength varying from 0.004 to 0.04 with the best results at the higher value (Fig 5). But in the case of the sodium acetate/acetic acid system, it was forbidden to overstep the limit of 0.04 above what salt crystallization in pump-heads was observed. In contrast, increasing the ionic strength of the DEA/acetic acid system up to 0.2 had no effect on the retention time of the analytes studied.

Finally, the mobile phase described presents usual practical

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advantages for rapid, reliable and accurate measurement of nicotine and cotinine in urine, plasma or saliva samples.

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