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Evaluation of reversed-phase columns for the analysis of very basic compounds by high-performance liquid chromatography

Application to the determination of the tobacco alkaloids

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ABSTRACT

A rapid separation of the major tobacco alkaloids with high efficiency and acceptable peak tailing is demonstrated on a RP-HPLC column containing a high-purity silica substrate, without use of silanol masking agents in the eluent. A test procedure based on that of Engelhardt and co-workers, with pyridine as an additional probe compound, was used to aid the selection of a suitable stationary phase from a number of the best commercially available columns for the separation of strongly basic compounds.

INTRODUCTION

The production and characterisation of packing materials suitable for the analysis of basic compounds by HPLC using RP columns has been an area of much recent work due to important applications in pharmaceutical analysis and in the separation of proteins [1–3]. The poor chromatography of these compounds has been largely attributed to undesirable interactions with residual silanol groups on the silica substrate. Engelhardt and co-workers [4,5], after careful review of a number of procedures, have proposed a test for the evaluation of the properties of RP columns, which includes assessment of their suitability for the analysis of basic compounds.

The quantitation of the tobacco alkaloids can be of importance in the development of breeding programmes designed to influence the levels of these compounds in the final product. Furthermore, much interest has been shown in biotechnological studies in determining these alkaloids in cell suspension and other plant cultures [6]. Although analysis of tobacco alkaloids can be performed by GC using deactivated columns [7], the ease of direct injection of aqueous extracts of culture materials by HPLC is a distinct advantage, especially when considering the potential volatility of nicotine and its possible loss in sample preparation procedures. These alkaloids include strong bases; the reported pK_a value of the pyrrolidine nitrogen atom in nicotine is 8.02 [8]. A recent study has indicated that peak tailing in RP-HPLC generally increases with increasing pK_a of the analyte [9]. For the analysis of the tobacco alkaloids, column performance characteristics have not been formally reported

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but a popular HPLC procedure shows rather low column efficiency and tailing peaks, even though the silanol masking agent triethylamine, which is widely recommended to improve peak symmetry, was incorporated in the mobile phase (pH 7.25) [10]. In addition, the μBondapak C_{18} column used is considered good for the analysis of basic compounds, amongst the long-established commercially available phases [9,11]. A more recent study [12] in which a triethylamine containing buffer at pH 3.5 was used seemed to show little improvement in column performance. Dolan and Snyder [13] have noted that the addition of triethylamine fails to yield symmetrical peaks for some compounds. Several authors, including Kimata *et al.* [14] have shown that metal impurities in alkylsilated silica packing materials may contribute detrimental interactions for some types of compound; this might explain why use of silanol masking agents has not given better results in the analysis of these alkaloids. Thus, in the present study we have investigated the performance of columns without use of amine additives. Such a study should also give an idea of the inherent overall activity of the column towards basic compounds; as noted by Vervoort *et al.* [9] the use of silanol blockers may conceal differences between columns. Some disadvantages are also inherent when amine additives are used: for instance, they may be difficult to remove from the column when changing mobile phases [13]. Some additives may be aggressive towards the stationary phase [9] or contribute to the complexity and detector background of the mobile phase (for instance when LC–mass spectrometry is used). Finally, although not important for the compounds of the present study, some analytes containing carbonyl groups may chemically react with the amine additive in the mobile phase [15]. Nevertheless, with triethylamine and other basic masking agents, good column efficiency and peak shape have been obtained with basic drugs and similar compounds using a variety of columns [9,16].

A test procedure based on that of Engelhardt and co-workers was investigated for its ability to select a suitable RP column for this application. The columns were chosen to be representative of some different approaches that have been taken in column manufacture in order to give good performance for basic compounds. Some of the columns tested here have been recommended as the best in previous comparative studies. The columns tested included a packing based on high-purity silica with low metal content, an "electrostatically shielded" reversed phase, a silica column with a high (polymeric) ODS loading, a porous graphitic carbon column and a polymer-coated pH-stable alumina column.

EXPERIMENTAL

The HPLC system consisted of a SP8800 pump, a Spectra 100 variable-wavelength UV detector with time constant 0.1 s and a 9-μl flow cell (all from Spectra-Physics, San Jose, CA, USA) and a valve injector equipped with a 5-μl loop (Rheodyne, Cotati, CA, USA). Efforts were made to keep instrumental dead volume to a minimum; in these comparative studies of column performance we did not wish to use mobile phases of differing organic solvent composition. This could affect both the wetting of the stationary phase (see below) and the degree of ionisation of ionisable analytes. Column efficiency values (N) were determined from peak widths at half height. Asymmetry factors (A_s) were calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak, using a Model 2000 data station (Trivector, Bedford, UK) in conjunction with a BASIC program. Peak identities were verified in biological samples using a photodiode array detector system (Integral 4000, Perkin-Elmer, Beaconsfield, UK). The new columns used included LiChrospher RP-8 Select B 5 μm, 25 × 0.4 cm I.D. (Merck, Darmstadt, Germany), Hypercarb 7 μm, 10 × 0.32 cm I.D. (Shandon, Runcorn, UK), Inertsil ODS 5 μm, 25 × 0.46 cm I.D. (GL Sciences, Tokyo, Japan), Nucleosil C_{18} AB 5 μm, 25 × 0.4 cm (Macherey–Nagel, Düren, Germany), pH-stable alumina–C_{18} 3 μm, 10 × 0.46 cm (Phase Sep, Deeside, UK) and Suplex pK_b-100 5 μm, 15 × 0.46 cm (Supelco, Bellefonte, PA, USA). All columns were operated at 20°C. Buffers were prepared by dissolving 6.803 g of KH_2PO_4 in 1 l of pure

water, and adjusting the pH (before addition of organic modifier) with either concentrated H_3PO_4 or 0.05 *M* KOH. The test mixture for use with unbuffered mobile phases consisted of uracil (*ca.* 1 mg l^{-1}), pyridine (*ca.* 5 mg l^{-1}), aniline (*ca.* 10 mg l^{-1}), phenol (*ca.* 30 mg l^{-1}), *o*-toluidine (*ca.* 5 mg l^{-1}), *m*-toluidine (*ca.* 5 mg l^{-1}), *p*-toluidine (*ca.* 5 mg l^{-1}) and benzene (*ca.* 150 mg l^{-1}). Standard solutions of the tobacco alkaloids contained 5–50 mg l^{-1} of each alkaloid. All column performance results are the average of duplicate determinations.

RESULTS AND DISCUSSION

The test mixture proposed by Engelhardt and co-workers contains toluene and ethylbenzene as probes of hydrophobic interaction, phenol and ethyl benzoate as neutral polar probes, aniline, N,N-dimethylaniline and the isomeric *o*-, *m*- and *p*-toluidines as basic probes, and thiourea as an inert probe. Methanol–water (55:45, v/v) without addition of buffer or salt solutions is used as the mobile phase. Under these conditions the RP is totally wetted (less than 60% water) giving the greatest influence of silanol groups on solute retention. According to Engelhardt *et al.* [5], at higher water concentrations, the interactions of the alkyl groups become stronger, and they can collapse to a film, shielding and covering the surface silanols. A good column for analysis of bases is signified by the elution of aniline before phenol with the ratio of A_s for aniline/phenol being 1.3 or less, and by the co-elution of the isomeric toluidines (or ratio of k' values being below 1.3). We have found this test procedure extremely useful for the characterisation of different columns. For the present study which concentrates on the evaluation of the properties of columns with regard to basic solutes, we have made some modifications to the test. We have preferred to use uracil as a void volume marker after the work of Bidlingmeyer *et al.* [17]. We omitted ethyl benzoate which can be used to distinguish between C_8 and C_{18} phases. Furthermore we have substituted benzene as a hydrophobic probe because when using the specified mobile phase, the retention of compounds such as toluene and ethyl benzene was excessive on some of the more heavily loaded phases utilised in this work. Finally, we have substituted pyridine, which has been used as a probe by some authors and commercial companies [18] for N,N-dimethylaniline (N,N-DMA). Engelhardt and co-workers state that N,N-DMA is not a particularly sensitive tracer for silanophilic interactions. In our own work we have found that aniline, despite having a lower pK_a value than N,N-DMA, usually gives more asymmetric peaks than N,N-DMA. Pyridine is a stronger base than either aniline or N,N-DMA according to the pK_a values (5.25, 4.63 and 5.15, respectively [8]); however, the ability of less sterically hindered amines to penetrate the phase and interact with active sites is also important [9,18].

We did not wish to evaluate a large number of well established ODS phases; much information is available from previous studies and from our own work [4,9,11,18]. We chose LiChrospher RP-8 Select B as a "benchmark" phase since it was stated as one of the best by Engelhardt and co-workers; we have also used this material successfully in the separation of the basic cinchona alkaloids [15]. The other columns chosen for further study here were more recently available materials all designed specifically for the separation of basic (and in some cases also acidic compounds). Nucleosil-type silicas were first identified by Köhler *et al.* [3] as giving low adsorption of basic solutes. The new Nucleosil C_{18} AB column, is according to the manufacturers, a cross-linked C_{18} phase with 25% carbon content, giving increased shielding of the silica matrix. The Suplex pK_b-100 column incorporates anion-exchange sites in the stationary phase which repel positively charged amines and thus give electrostatic shielding of the surface. This column gave superior performance for bases in two comparative surveys of deactivated columns [9,18]. Inertsil ODS is, according to the manufacturers, a material with 14% carbon content based on a spherical silica of 99.9% purity. Metal impurities are known to participate in the retention of some solutes (see above). So-called pH-stable silica columns have enhanced stability at high pH which allows work with buffers in which basic analytes may not be protonated,

eliminating undesirable ion-exchange interactions. They are generally prepared by encapsulation of the substrate with a polymeric layer such as polybutadiene [1]. Alumina columns of this type are also available. Finally, porous graphitic carbon phases are not designed especially for basic solutes, but being based on a different substrate, might not be expected to show the same problems as silica phases.

The silica-based columns other than Nucleosil C_{18} AB yielded 60–80 000 plates m^{-1} for benzene under the normal test conditions. The 3-μm alumina column showed slightly higher efficiencies for this test in terms of plates m^{-1}. However, such figures for short columns exaggerate their potential because the back pressure required for the operation of longer columns may not allow this performance to be realised in practice. Nucleosil C_{18} AB gave a somewhat lower plate number ($N = 50\,000$ plates m^{-1}) which we attribute to the high loading of polymeric ODS [19]. All of these columns gave A_s for benzene of between 1.00 and 1.35. Table I shows the other results obtained using the modified test of Engelhardt and co-workers for four of the above columns. All would be considered "good" for the analysis of basic compounds based on the criteria of Engelhardt and co-workers: all show coelution of the isomeric toluidines (ratio of $k' < 1.3$), all elute aniline before phenol (*i.e.* $k'_{phenol}/k'_{aniline} > 1$) and all show a ratio of the asymmetry of aniline/phenol peaks <1.3. However, it does not seem possible to distinguish "excellent" from merely "good" columns using this method because the test results are similar in each case. The ratio of the capacity factors of phenol and aniline (Table I) indicates by how much aniline is eluted before phenol. However, it does not seem possible to use this figure in a quantitative sense. The column efficiency and asymmetry for pyridine (or the asymmetry ratio pyridine/phenol) does appear to reveal some differences between the columns. Furthermore, on the Inertsil and Suplex columns, pyridine eluted before aniline. On Nucleosil pyridine coeluted with aniline, and on Lichrospher it coeluted with phenol, making separate measurement of pyridine data necessary. The chromatogram of these test compounds obtained using Inertsil ODS is shown in Fig. 1.

None of the columns gave satisfactory elution of benzylamine when using methanol–water mixtures with *no* additional components. Benzylamine is technically an aliphatic amine, with pK_a 9.3 [8]. Table I also indicates that nicotine gave similar results with distorted peaks and very low efficiency in these unbuffered

TABLE I

ANALYSIS OF TEST COMPOUNDS ON DEACTIVATED SILICA ODS COLUMNS

an = Aniline; phen = phenol; pyr = pyridine; bzylam = benzylamine. Mobile phase methanol–water (55:45, v/v), flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C. Asymmetry factors are not reported for peaks with $N < 1000$ plates m^{-1}.

Column	k' ratio toluidines	an/phen A_s ratio	k' phen/an	pyr		pyr/phen A_s ratio	bzylam		nicotine	
				$N(m^{-1})$	A_s		$N(m^{-1})$	A_s	$N(m^{-1})$	A_s
Nucleosil C_{18} AB	1.0	1.16	1.4	5 320	3.80	2.66	<1000		<1000	
Suplex pKb-100	1.0	1.01	2.0	33 000	1.57	1.18	2700	0.58	2890	0.40
Inertsil ODS	1.0	1.05	1.5	44 400	1.68	1.36	<1000		1300	2.55
LiChrospher RP-8 Select B	1.1	1.10	1.3	26 500	2.30	1.56	retained		5760	4.55

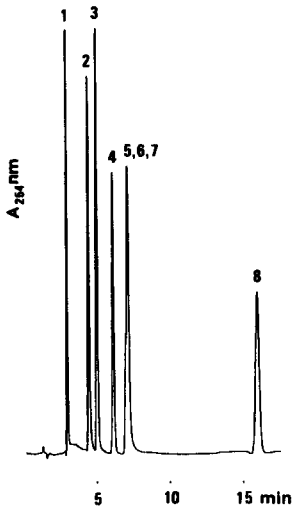

Fig. 1. Separation of test compounds using Inertsil ODS. Mobile phase methanol–water (55:45, v/v), flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C. Peaks: 1 = uracil; 2 = pyridine; 3 = aniline; 4 = phenol; 5 = *o*-toluidine; 6 = *m*-toluidine; 7 = *p*-toluidine; 8 = benzene.

mobile phases. Aliphatic amines are much more difficult to analyse than aromatic amines. Thus it seems they cannot as yet be included in test mixtures for silica based columns designed for use with simple organic solvent–water mobile phases that do not contain buffer components. Buffer solutions not only stabilise the pH environment for chromatography of ionisable compounds but also can provide cations which can counter the ion exchange interaction of basic compounds with dissociated silanol groups in the packing. Table II shows the column performance for pyridine, benzylamine, nicotine and quinine using methanol–0.05 *M* phosphate buffer pH 6.25 (55:45, v/v). This pH was not necessarily optimum for analysis of the compounds; indeed it may highlight ion exchange with dissociated silanols. Silanol group dissociation is suppressed at lower pH; thus, nicotine and quinine, another basic alkaloid, gave improved results on the Lichrospher column using a pH 3 buffer [15]. In general, basic compounds tend to give sharper, more symmetrical peaks at pH 3 [20]; however, a recent study advocates the use of a higher pH (7.4) for compounds with moderate pK_a which, unlike nicotine, would not be protonated at this pH [9]. Column performance for pyridine seemed little changed by use of the pH 6.25 buffer rather than water in admixture with methanol. However, substantial improvements for the chromatography of benzylamine and nicotine were noted, presumably due to the competitive effect of buffer K^+ ions. Examination of Tables I and II shows that the performance for pyridine in unbuffered mobile phases does appear to give a reasonable indication of the likelihood of success for analysis of basic alkaloids in buffered mobile phases. This suggests that pyridine is a useful simple test

TABLE II

PERFORMANCE DATA FOR NICOTINE AND OTHER BASIC COMPOUNDS ON DEACTIVATED SILICA COLUMNS

Mobile phase methanol–0.05 *M* phosphate pH 6.25 (55:45, v/v), flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C.

Column	Pyridine		Benzylamine		Nicotine		Quinine	
	$N(m^{-1})$	A_s	$N(m^{-1})$	A_s	$N(m^{-1})$	A_s	$N(m^{-1})$	A_s
Nucleosil C_{18} AB	4 920	3.81	1 990	6.47	5 100	4.87	7 660	3.64
Suplex pKb-100	36 200	1.64	9 130	1.60	22 300	2.47	21 700	1.72
Inertsil ODS	40 700	1.62	31 800	1.73	42 300	1.77	29 500	1.91
Lichrospher RP-8 Select B	24 300	2.10	10 600	2.04	8 400	4.62	6 720	4.60

compound which can be incorporated in the system of Engelhardt and co-workers. Benzylamine appears to be too severe a test compound, giving poor results even with the buffered mobile phase on most columns. Furthermore, this compound showed little retention on some columns, even when low concentrations of organic modifier were used.

The pH-stable alumina column gave poor results for the chromatography of basic compounds using the methanol–water mobile phase, and did not pass the asymmetry ratio test for aniline and phenol. This indicates a high activity of the underlying material. Nevertheless, these columns can be used at high pH without apparent ill effect. Excellent results were obtained for the chromatography of pyridine (40 000 plates m^{-1}, A_s 1.53) using acetonitrile–ammonium nitrate buffer (pH 10). Surprisingly, however, nicotine still gave some peak tailing even when a pH 12 sodium hydroxide buffer was used (best results about 30 000 plates m^{-1} with $A_s = 2.4$). This indicates the presence of undesirable interactions even when the compound is uncharged. A new pH-stable alumina RP material for basic compounds (Aluspher RP-select B) has recently become commercially available [21]. Manufacturers' literature would suggest that this material is more inert, but gives somewhat lower efficiency than the material tested here.

The porous graphitic carbon column tested showed low efficiency for phenol and benzene ($N < 18\,000$ plates m^{-1}) with considerable peak asymmetry ($A_s > 2$) when using organic solvent/water mixtures. Nevertheless, similar results were shown for basic compounds and nicotine gave $N = 17\,000$ plates m^{-1} with A_s 2.3 using an acetonitrile–water mixture without addition of salts, a result which is better than any of the other columns. However, we were not able to improve on these results by modification of the mobile phase, and the column was not considered further. Further development of such columns may yield improved performance for all types of compound.

Of the columns investigated in our study, the high-purity silica ODS column seems to give the best performance for the analysis of the tobacco alkaloids. However, in agreement with other studies [9,18], the Suplex pK_b-100 column also seems to give very good general performance for basic compounds. In addition to the detrimental effects of silanol groups, metals can provide surface acidity, ion-exchange sites or promote interactions with analytes that have chelating properties; it is possible that the alkaloids, having two basic nitrogen atoms, could possess some chelating properties, in addition to Lewis base properties. However, the stereochemistry appears unfavourable. We were unable to improve significantly the performance of the LiChrospher and Nucleosil columns for the analysis of nicotine by further addition of EDTA to the eluent used to obtain the results for Table II [13]. The Inertsil column also had the advantage of greater retention of nicotine than many of the other columns allowing greater flexibility of choice of the mobile phase composition and pH. Finally, it is in other respects a conventional ODS phase which can be used in the normal way. Fig. 2a shows the separation of the four major tobacco alkaloids nicotine, nornicotine, anabasine and anatabine together with the minor

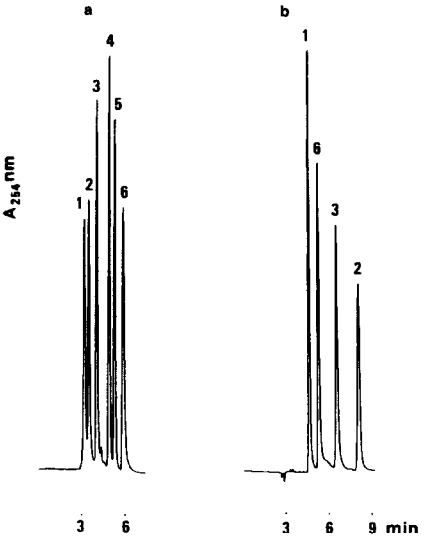

Fig. 2. Separation of tobacco alkaloids using Inertsil ODS. Detection UV at 254 nm. Flow-rate 1 ml min^{-1}. Column temperature 20°C. Mobile phase (a) methanol–0.05 *M* potassium phosphate pH 6.25 (60:40, v/v); (b) methanol–0.05 *M* potassium phosphate pH 3.00 (10:90, v/v) Peaks: 1 = nornicotine; 2 = anabasine; 3 = anatabine; 4 = myosmine; 5 = 2,3-dipyridyl; 6 = nicotine.

compounds myosmine and 2,3-dipyridyl on the Inertsil column using a similar mobile phase to that in Table II. In all cases, we injected small amounts of the alkaloids (see Experimental section). The injection of large amounts can contribute to peak tailing, presumably due to saturation of column active sites [9]. Doubling the concentration of the buffer solution to 0.1 *M* did not give significant improvements in the peak shape of these compounds, although interesting selectivity effects could be obtained. The retention of nicotine relative to the other alkaloids was noticeably affected by buffer strength; ion exchange still seems to give some contribution to the retention of these compounds at pH 6.25 even with this column. Reduction of the mobile phase pH increases the degree of protonation of the alkaloids and thus reduces hydrophobic retention of the compounds; furthermore ion-exchange retention of the alkaloids should be reduced due to suppression of silanol dissociation. On some of the columns, a reduction in pH produced very low capacity factors for the tobacco alkaloids even when using low concentrations of organic modifier in the mobile phase. However, the relatively high phase loading of the Inertsil ODS column gave reasonable retention of the alkaloids when using a pH 3.0 phosphate buffer containing 10% methanol. For routine analysis of the four major tobacco alkaloids we would recommend use of the pH 3.0 buffer system (Fig. 2b); column performance data are shown in Table III. Some improvement in peak shape, particularly for nornicotine, was noted at the lower pH. Furthermore, the first peak is eluted further from the void volume of the column, reducing the possibility of co-elution of the analytes with matrix compounds when biological samples are analysed. Finally, column deterioration may occur more rapidly at the higher pH [9]. On the other hand, improved results for the analysis of the minor alkaloids 2,3-dipyridyl and myosmine were obtained using the pH 6.25 eluent. 2,3-Dipyridyl gave excessive retention with the methanol–pH 3 buffer (10:90) mobile phase necessitating a gradient elution separation with increasing methanol concentration and the peak shape for myosmine was poorer using this eluent. Nevertheless, analysis of the major alkaloids is possible using either eluent and since the order of elution of the analytes (and potentially matrix constituents) is different in each, the possibility of peak overlap can be ascertained by comparison of results. This procedure can be used in addition to peak purity checks by diode array spectrophotometry. However, for samples containing low levels of alkaloids, considerable care must be taken in sample preparation procedures used to purify the

TABLE III

PERFORMANCE DATA FOR TOBACCO ALKALOIDS ON INERTSIL ODS

Mobile phase methanol–0.05 *M* phosphate buffer pH 3.0, flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C.

	Nornicotine	Nicotine	Anatabine	Anabasine
$N(m^{-1})$	47 600	43 200	46 000	48 400
A_s	1.51	1.72	1.60	1.46

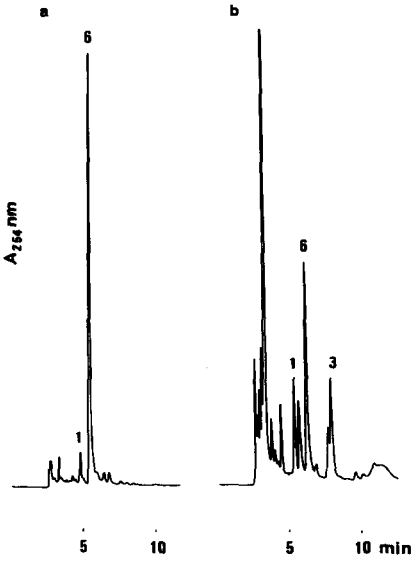

Fig. 3. Analysis of alkaloids in (a) commercial cigarette tobacco [mobile phase methanol–0.05 *M* potassium phosphate pH 3.00 (10:90, v/v)]. (b) hairy root culture of Wisconsin 38 [mobile phase methanol–0.05 *M* potassium phosphate pH 3.00 (8:92, v/v)]. Flow-rate 1 ml min^{-1}. Column temperature 20°C. Detection UV at 254 nm. Peaks as in Fig. 2.

extracts prior to HPLC; we are presently reviewing alternative procedures.

Fig. 3 shows the application of the method to the determination of the tobacco alkaloids in a phosphate buffer extract [10] of (a) a sample of commercial cigarette tobacco and (b) a hairy root culture of the tobacco plant Wisconsin 38. The chromatograms indicate an improvement on previously published methods for the HPLC analysis of these compounds.

CONCLUSIONS

Some of the best available stationary phases for RP-HPLC of basic substances were tested, with the objective of establishing an improved method for the analysis of the tobacco alkaloids. Superior results were obtained using a high purity silica-ODS phase. Pyridine is a useful additional test compound for evaluation of these phases. Rapid analysis of the tobacco alkaloids with symmetrical peaks was achieved without use of silanol masking agents. The HPLC method is especially suitable for applications where injection of aqueous extracts is desired. Moreover, the high performance of the method may make the method competitive with GC in some other applications.

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