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CHROMATOGRAPHIC ANALYSIS OF 4-DIMETHYLAMINOAZOBENZENE-4'-SULPHONYL AND 4-NAPHTHALENE-1-AZO-(4'-DIMETHYLAMINOBENZENE)SULPHONYL DERIVATIVES OF ALIPHATIC ALCOHOLS

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SUMMARY

Seven 4-dimethylaminoazobenzene-4'-sulphonyl (dabsyl) and 4-naphthalene-1-azo-(4'-dimethylaminobenzene)sulphonyl (dabnsyl) derivatives of aliphatic alcohols have been analysed chromatographically in adsorption and reversed-phase systems. It has been found, rather unexpectedly, that the derivatives can be separated in both types of system. The results obtained for dabsyl and dabnsyl derivatives of aliphatic alcohols satisfy the well-known equations describing the influence of the mobile phase concentration on the capacity factor k'. The influence on the dabsylation and dabnsylation yield of the molar ratio of the derivatizing reagent to the substrate, and of the pH value of the reaction medium, has been examined.

INTRODUCTION

Derivatization is a widely applied procedure during various chromatographic operations. Its use enables detection, enhances sensitivity and improves the selectivity of an analytical separation, mainly thanks to a decrease in the polarity of the substances examined[1–3].

Alcohols and phenols are strongly polar compounds because of the presence of the hydroxyl group. Hence alcohols are good solvents for various organic and inorganic compounds. They are also applied as components of mobile phases in thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The use of alcohols as solvents is sometimes limited owing to the reactivity of the hydroxyl group.

The hydroxyl group is a functional element of many naturally occurring compounds; however, these compounds predominantly have a complex structure and also contain other groups, *e.g.* aldehyde, carboxyl and amine.

Detection of the hydroxyl group is still not a completely solved analytical problem. None of applied reactions for this group is sufficiently characteristic to allow unambiquous confirmation on the basis of a single test of the presence of the hydroxyl group. Several reactions are necessary[4].

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The liquid chromatographic analysis of aliphatic alcohols is usually performed by the gel chromatographic method on Sephadex[5,6] with the use of a refractometric detector[6]. Another possibility is derivatization of alcohols with phenyl isocyanate[7] or naphthyl isocyanate[8], and subsequent analysis of the derivatives by adsorption[8] or reversed-phase chromatography[7]. Parkin and Lau[9] have separated aliphatic alcohols by the reversed-phase method, and used for their detection various substances absorbing UV radiation at 270 nm.

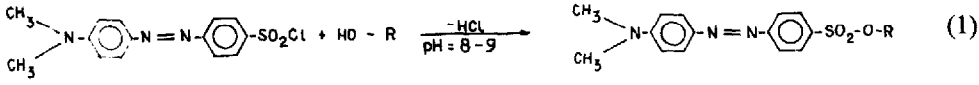
It is known that dabsyl chloride (DABS-Cl, 4-dimethylaminoazobenzene-4'-sulphonyl), which has recently been applied in analytical practice[10], reacts with amino, hydroxyl and sulphydryl groups; hence it can be used as derivatizing reagent for alcohols. Examples of the application of DABS-Cl in chromatographic analysis have been presented in numerous publications. Lammens and Verzele[11] and Chang *et al.*[12] have described a rapid and easy method for the chromatographic analysis of amino acids. Lin and Wang[13] have determined amino acids in urine. Other authors have applied dabsyl chloride to the determination of primary and secondary amines[14] and sphingosine[15].

Recently we have suggested a new derivatizing reagent, dabnsyl chloride (DABNS-4-Cl, 4-naphthalene-1-azo-(4'-dimethylaminobenzene)sulphonyl chloride)[16,17] for amino acid analysis. The compound is a modification of dabsyl chloride.

Both DABS-Cl and DABNS-4-Cl react not only with amino groups but also with hydroxyl groups of phenols and alcohols. That is why the compounds seem to be good reagents for alcohol analysis. Studies of the reaction of aliphatic alcohols with DABS-Cl and DABNS-4-Cl can also give information on the reactivity of alcoholic and phenolic hydroxyl groups of amino acids (*e.g.* serine and tyrosine) with these reagents. The data are useful during derivatization of polyfunctional amino acids and help to determine whether mono- or polysubstituted derivatives are formed.

EXPERIMENTAL

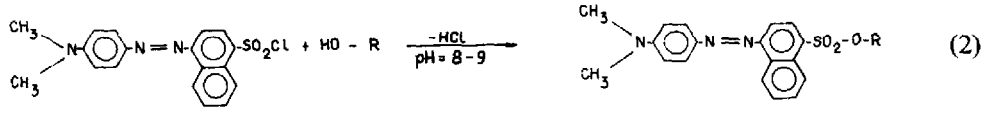
Dabsyl chloride reacts with aliphatic alcohols according to the reaction shown in eqn. 1:



(1)

where R is an alkyl chain.

The reaction between dabnsyl chloride and aliphatic alcohols[16,17] is similar:



(2)

The derivatives obtained in both reactions are coloured solids of good solubility in organic solvents. The conditions for alcohol (Polish Reagents, POCh Gliwice) dabsylation are similar to those for amino acid derivatization[10,18].

Derivatives of alcohols used as standards were prepared in the following way.

Pure alcohol (1 cm^3) was mixed with 10 cm^3 of 0.1 *M* sodium hydrogen carbonate and 20 cm^3 of acetone solution of DABS-Cl or DABNS-4-Cl (10 $\mu mol/cm^3$),

and the reaction mixture was allowed to react at 343 K in a water-bath for 10 min under reflux. The mixture was cooled, and the derivative was extracted with diethyl ether. The DABS-ONa or DABNS-4-ONa, which originated from DABS-Cl and DABNS-4-Cl, respectively, was re-extracted from the organic phase with citrate buffer (pH 3). The diethyl ether was evaporated off and the solid derivative was obtained. Quantity determination of DABS- and DABNS-4-alcohols showed that the molar ratio of derivatizing reagent to alcohol lay in the range 2:1 to 16:1.

The conditions for serine (produced by E. Merck, Darmstadt, F.R.G.) dabnsylation are the same as for its dabsylation, and are described elsewhere[10,18].

The experiments were carried out using a liquid chromatograph Type 302 (Institute of Physical Chemistry of the Polish Academy of Sciences, Warsaw), which consisted of a syringe pump, a UV detector (fixed wavelength 254 nm) and a four-port injection valve. Two 250 × 4 mm I.D. stainless-steel columns (ZOCh, Lublin, Poland) packed with 10 μm LiChrosorb Si 60 and LiChrosorb RP-18 (E. Merck) were used for measurements of capacity factors.

The dead volume of the column packed with LiChrosorb Si 60 was calculated after injection of a heptane solution of benzene. The determination was performed at various concentrations of ethyl acetate in the mobile phase. The dead volume of the column packed with LiChrosorb RP-18 was calculated after injection of an aqueous solution of potassium chloride when the mobile phase consisted of methanol–water (60:40, v/v).

RESULTS AND DISCUSSION

Structural effects in molecules of organic compounds can exert an influence on the separation selectivity of adsorption chromatography[19]. On the other hand it is known that organic compounds that differ by only one $-CH_2-$ group in an alkyl chain are separated with difficulty in typical adsorption systems[19,20]. That is why DABS- and DABNS-4 derivatives of aliphatic alcohols differing by only one $-CH_2-$ group could be expected to have poor selectivity in adsorption chromatographic systems too. However, the experiments performed show that separation is possible. Fig. 1 shows that the DABS derivatives of alohols can be separated without difficulty. Jusiak and Soczewiński[21] suggested that dabsyl derivatives of aliphatic amines are adsorbed by the $-SO_2-$ group (structure 1); so it can be assumed that differentiation of capacity factors k' of the alcohol derivatives is caused by the steric hindrance due to the alkyl chain of each alcohol. Soczewiński and Golkiewicz[22,23], Snyder[24], Ościk *et al.*[25] and Jandera and Churaček[26] have all shown that the following equation is appropriate for adsorption chromatography:

$$\log k' = \log k'_S - n \log X_S \tag{3}$$

where k' is the capacity factor, n is the slope of the plot corresponding to eqn. 3 ($n = A_S/n_b$ is the ratio of the molecular areas on the adsorbent surface occupied by the solute A_S and the solvent n_b), and X_S is the mole fraction of the stronger solvent S in the binary mobile phase.

Figs. 2 and 3 show the correlation between the logarithm of the capacity factor k' and the logarithm of the concentration of the more polar solvent S in the binary

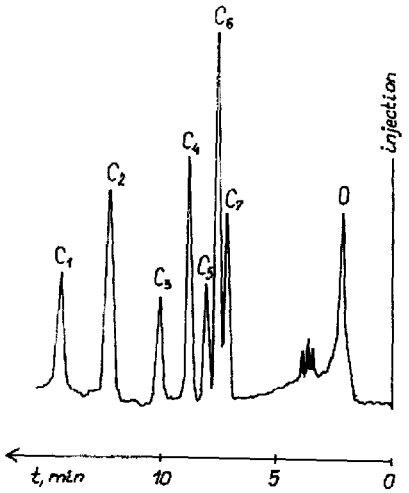


Fig. 1. HPLC chromatogram of seven DABS-alcohols. Stationary phase, LiChrosorb Si 60 (10 μm); mobile phase, *n*-heptane–ethyl acetate (85:15, v/v); stainless steel column, 250 × 4 mm I.D.; flow-rate, 1.2 cm^3/min; detection at 254 nm. Peaks: C_1 = DABS-methanol; C_2 = DABS-ethanol; C_3 = DABS-propanol; C_4 = DABS-butanol; C_5 = DABS-pentanol; C_6 = DABS-hexanol; C_7 = DABS-heptanol; 0 = DABS-Cl.

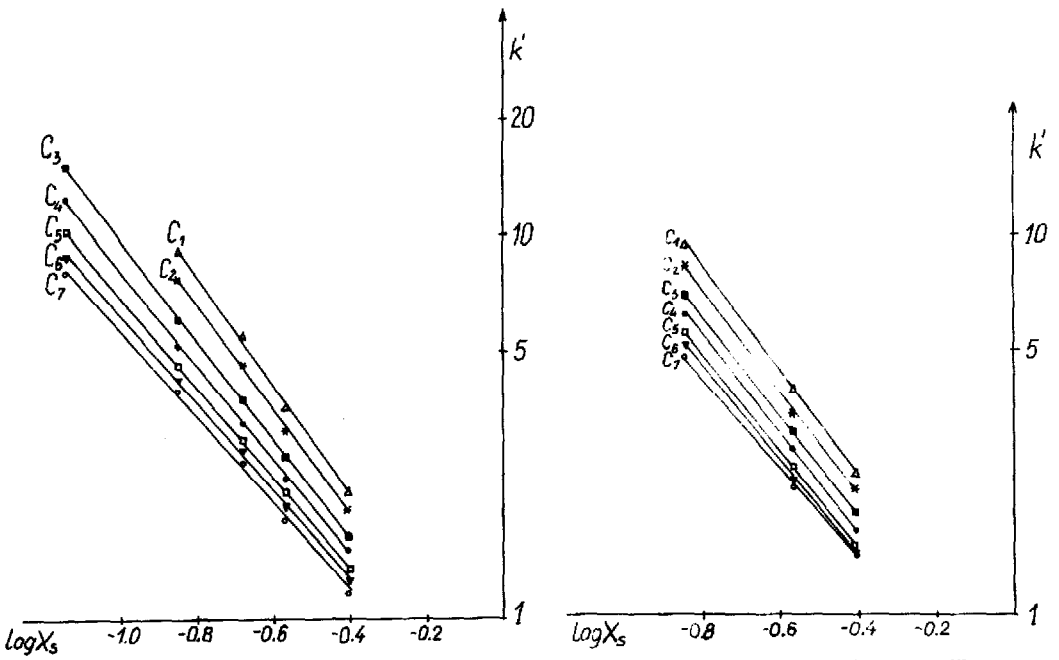


Fig. 2. The log k' *vs.* log X_S relationship for some DABS-alcohols. Polar solvent, ethyl acetate; diluent, *n*-heptane. Notation of compounds as in Fig. 1.

Fig. 3. The log k' *vs.* log X_S relationship for some DABNS-4-alcohols. Conditions as in Fig. 1. Notation of compounds: C_1 = DABNS-4-methanol; C_2 = DABNS-4-ethanol; C_3 = DABNS-4-propanol; C_4 = DABNS-4-butanol; C_5 = DABNS-4-pentanol; C_6 = DABNS-4-hexanol; C_7 = DABNS-4-heptanol.

TABLE I

ABSOLUTE SLOPES FOR THE PLOTS OF log k' OF DABS- AND DABNS-4 DERIVATIVES OF ALCOHOLS, AGAINST LOGARITHM OF MOLE FRACTION OF ETHYL ACETATE (NORMAL PHASE) AND VOLUME FRACTION OF METHANOL (REVERSED PHASE)

Solute	*Code*	*n-Heptane–ethyl acetate on SiO_2*		*Water–methanol on RP-18*
		DABS	*DABNS-4*	*DABNS-4*
Methanol	C_1	1.45	1.36	4.05
Ethanol	C_2	1.37	1.33	4.68
Propanol	C_3	1.30	1.31	5.31
Butanol	C_4	1.24	1.28	5.95
Pentanol	C_5	1.19	1.25	6.55
Hexanol	C_6	1.15	1.22	7.20
Heptanol	C_7	1.12	1.19	7.83

mobile phase. The linear correlation log $k' = f(\log X_S)$ obtained for both DABS- and DABNS-4 derivatives proves that the chromatographic behaviour of the compounds is accurately described by eqn. 3.

The values of the plot slopes n compiled in Table I also seem to confirm that adsorption of the derivatives is caused by the $-SO_2-O-$ group. If it is assumed, according to the Snyder theory[19,24], that the slope n in eqn. 3 equals the ratio of the areas of the adsorbed solute and solvent, the n value in the case of DABS- and

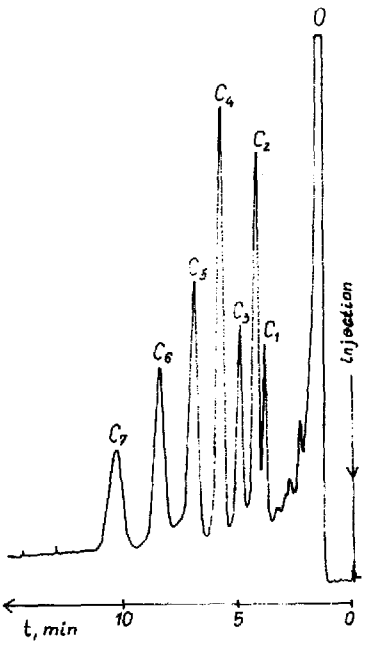


Fig. 4. HPLC chromatogram of seven DABNS-4-alcohols. Stationary phase, silica gel ODS (10 μm); mobile phase, water–methanol (90:10, v/v). Notation of compounds as in Fig. 3; 0 = DABNS-4-OH.

DABNS-4 derivatives of aliphatic alcohols and ethyl acetate should be very high. The n values in the range 1.12–1.45 indicate that only one group, probably the $-SO_2-O-$ group, occupies the space on the adsorbent surface but the rest of the molecule is in the bulk phase.

The slopes of the plots describing the correlation $\log k' = f(\log X_S)$ differ slightly and decrease as the alkyl chain lengthens (Table I).

The differences in the slopes for DABNS-4 derivatives of neighbouring alcohol homologues are constant and equal to 0.03 units, but for DABS derivatives they decrease regularly as the alkyl chain lengthens (notice that the differences in slopes are 0.08, 0.07, 0.06... between the pairs DABS-methanol and -ethanol, -ethanol and -propanol, -propanol and -butanol, etc., respectively).

When preparing DABS- and DABNS-4 derivatives of alcohols we expected that they would be easily separated by reversed-phase chromatography. These expectations are confirmed by the chromatogram (Fig. 4).

The molecular mechanism of adsorption on adsorbents with a chemically bonded stationary phase is a matter of controversy. It has been shown experimentally that generally $\log k'$ is approximately a linear function of the volume fraction of a modifier[21,27,28] in the binary mobile phase:

$$\log k' = \log k'_w - n\varphi \tag{4}$$

where the subscript w denotes pure water and φ is the volume fraction of the modifier.

Sometimes, in the case of a wider range of concentrations, curved lines described by a quadratic equation[29]:

$$\ln k' = A\varphi^2 + B\varphi + C \tag{5}$$

are obtained (A, B and C are empirical coefficients of the quadratic equation).

It is of interest that our results confirm the validity of eqns. 4 and 5. The correlation for DABS derivatives of alcohols in the system LiChrosorb RP-18–methanol–water is curvilinear (Fig. 5) and accurately described by eqn. 5[29], but in the case of DABNS-4 derivatives, chromatographed in the same system, the correlation obtained is rectilinear according to eqn. 4.

An analysis of the slopes described by the equation $\log k' = f(\varphi)$ and shown in Table I (reversed phase), is also of interest. The values of the slopes increase regularly from 4.05 to 7.83 for DABNS-4-methanol to DABNS-4-heptanol, respectively (Fig. 6). The increase in the slope value corresponding to one $-CH_2-$ group is constant and equals 0.63 units. It is noteworthy that in theories of gradient elution the value of the slope is of great importance during elaboration of optimum conditions of the chromatographic analysis[30,31].

Quantitative determination of DABS- and DABNS-4-alcohols was performed chromatographically using an external standard method. A rectilinear relationship between peak heights and concentrations of the alcohol derivatives in the range 0.01–0.2 mg/cm^3 was obtained.

Quantitative determination of alcohols in the form of their derivatives with dabsyl and dabnsyl chloride was performed mainly in order to establish to what extent alcoholic hydroxyl groups of amino acids can react with these reagents.

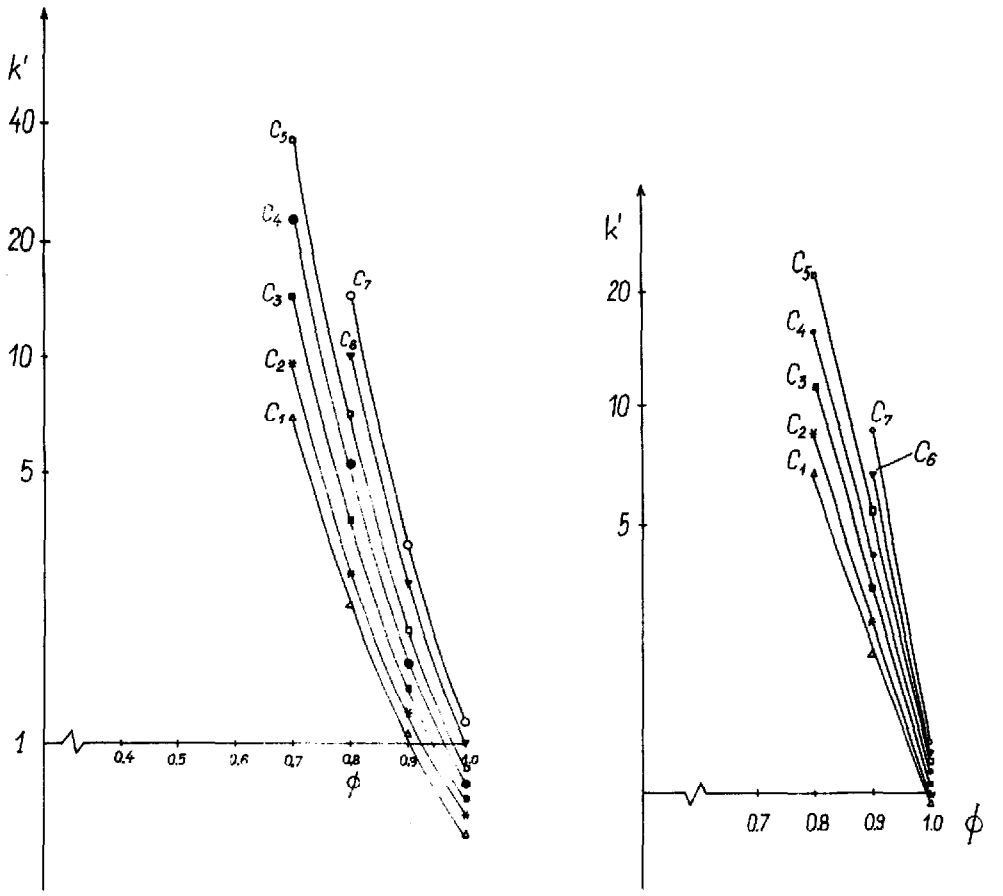


Fig. 5. The plots of log k' *vs.* volume fraction of methanol for some DABS-alcohols. Mobile phase, water–methanol. Notation of compounds as in Fig. 1.

Fig. 6. The plots of log k' *vs.* volume fraction (φ) of methanol for some DABNS-4-alcohols. Mobile phase, water–methanol. Notation of compounds as in Fig. 3.

Initially the influence of the pH of the reaction medium on the dabsylation yield was examined. It turned out that maximum yield was attained at pH 9, the same as in the case of amino acid derivatization.

Next the influence of the reagent excess on the dabsylation and dabnsylation yield was investigated. The reactions were performed with various molar ratios of derivatizing reagent to alcohol. The derivatization reactions of alcohol proceed with a very low yield (Fig. 7), so dabsyl and dabnsyl chloride cannot be applied to the quantitative determination of alcohols. The problem of the formation of disubstituted derivatives of amino acids may have a significant influence on the results of chromatographic analysis of amino acids containing both $-NH_2$ and $-OH$ groups: it is known that both groups react with dabsyl chloride.

Lin and Chang[10] and Chang *et al.*[32] have suggested that disubstituted derivatives of amino acids can be formed with a great excess of dabsyl chloride; the molar ratio of dabsyl chloride to amino acid should be 5:1 or even 40:1.

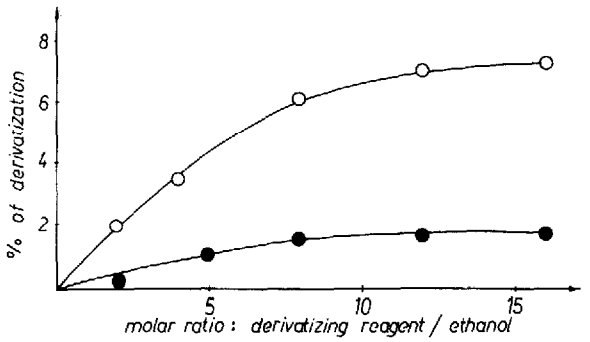


Fig. 7. Dependence of the yield of ethanol derivatization on the molar ratio of reagent to alcohol. Key: 0 = DABS-ethanol; ● = DABNS-4-ethanol.

It is interesting that in the case of derivatization with DABNS-4-Cl, the dabnsylation yield even with a great excess of the reagent does not exceed 2%. The low yield of dabnsylation of alcoholic hydroxyl groups and the high yield (95–100%) of dabnsylation of amino groups[33], seem to prove that DABNS-4-Cl is a more selective derivatizing reagent for amino acids than DABS-Cl.

This conclusion is confirmed by results obtained during serine derivatization with excess amounts of DABS-Cl and DABNS-4-Cl (molar ratio of derivatizing reagent to serine = 15:1). Two peaks of the derivatives were obtained on chromatographic analysis of DABS-serine. The capacity factor for the less retained peak was almost identical with the k' value of DABS-alanine: this suggests that the serine was contaminated with alanine. After addition of DABS-Ala to DABS-Ser (Fig. 8a) three peaks of derivatives were observed: DABS-Ala, monosubstituted DABS-Ser and probably disubstituted DABS-Ser.

The low yield of the reaction between DABNS-4-Cl and ethanol suggests that the peak of disubstituted DABNS-4-Ser should be considerably lower than that of disubstituted DABS-Ser. Fig. 8b shows a chromatogram of DABNS-4-Ser spiked with DABNS-4-Ala. The peak of disubstituted serine is indeed very small, which confirms the earlier supposition of low reactivity of DABNS-4-Cl with alocholic hydroxyl groups.

It is noteworthy that if quantitatively determined amino acids forms mono- and disubstituted derivatives (two peaks on the chromatogram), the quantitative results obtained on the basis of the monosubstituted derivative will be several per cent lower than they should be.

The results obtained permit us to draw some conclusions concerning the derivatization of bifunctional amino acids such as serine. In the case of serine dabsylation and dabnsylation, it is mainly the amino group that participates in the reaction to form the monosubstituted derivative. With large excesses of derivatizing reagents, the hydroxyl group can also react and form small amounts of disubstituted serine. Such amounts of disubstituted derivatives are sometimes chromatographically revealed as unidentified peaks.

It has been shown[33] that DABS-Cl reacts with phenolic hydroxyl groups of phenolic acids and amino acids in good yield.

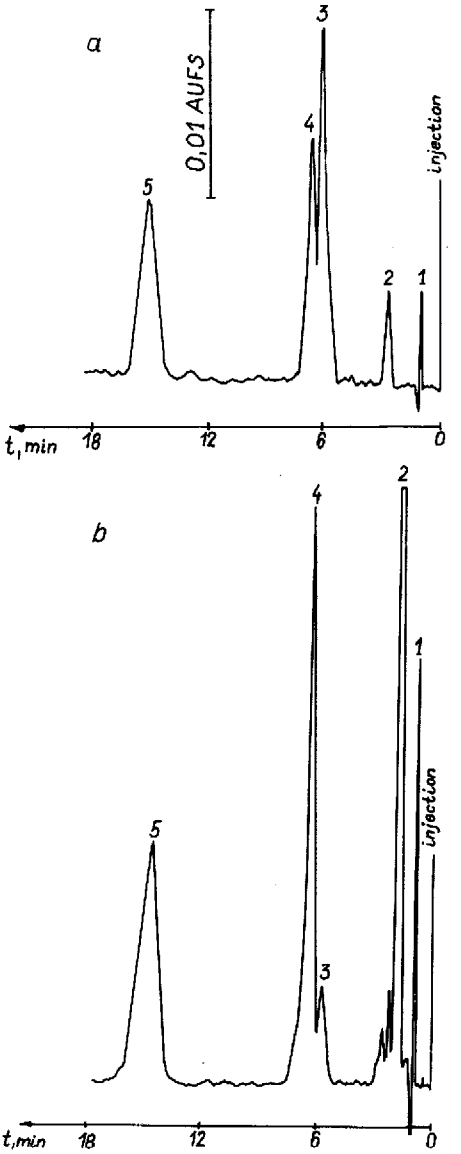


Fig. 8. HPLC chromatogram of mono- and disubstituted derivatives of serine spiked with alanine derivative. Conditions as in Fig. 1, except the mobile phase, *n*-heptane–acetone–acetic acid (75:20:5, v/v). (a) DABS-Ser spiked with DABS-Ala; peaks: 1 = solvent; 2 = DABS-Cl; 3 = disubstituted DABS-Ser; 4 = DABS-Ala; 5 = monosubstituted DABS-Ser. (b) DABNS-4-Ser spiked with DABNS-4-Ala; peaks: 1 = solvent; 2 = DABNS-4-Cl; 3 = disubstituted DABNS-4-Ser; 4 = DABNS-4-Ala; 5 = monosubstituted DABNS-4-Ser.

CONCLUSIONS

Both DABS-Cl and DABNS-4-Cl are good reagents for amino[10,16–18,32] as well as phenolic hydroxyl groups[33], but considerably worse for alcoholic hydroxyl

groups. The derivatization yield of aliphatic alcohols does not exceed 10% and 2% with DABS-Cl and DABNS-4-Cl, respectively. This means that in the case of amino acids with both amino and hydroxyl groups (*e.g.* serine), mainly monosubstituted derivatives are formed as a result of reaction between the amino group and dabsyl chloride.

However, in the presence of a large excess of derivatizing reagent, disubstituted derivatives can be formed (Fig. 8a and b); this effect is more noticeable for DABS-Cl than for DABNS-4-Cl.

Dabsyl and dabnsyl derivatives of aliphatic alcohols can be separated by liquid–solid or reversed-phase chromatography.

In spite of poor yields, DABS-Cl or DABNS-4-Cl can be used for the detection of compounds with primary alcoholic hydroxyl groups.

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