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High-sensitivity fluorescence derivatization for the determination of hydroxy compounds in aqueous solution by high-performance liquid chromatography☆

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Abstract

The utility of the fluorescent labelling reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) for the determination of alcohols in aqueous media was evaluated. The labelling conditions were optimized for C_1–C_8 alcohols. Under the very mild reaction conditions of 25°C for 1 min in a basic buffer, all the alcohols tested were derivatized with the reagent to yield highly stable fluorescent carbamate derivatives. The maximum excitation (290 nm) and emission (365 nm) wavelengths were the same for all the alcohols tested. The stability of the derivatives and their yield were affected by the chosen pH in the range 6–9.5 and by the steric hindrance of the alcohol function. All the derivatives obtained with AQC were completely separated by reversed-phase high-performance liquid chromatography on a 20-cm C_{18} column with a gradient of aqueous sodium acetate buffer (pH 6) and acetonitrile. The detection limit ($S/N = 3$) with fluorescence detection is at the picomole level. Some preliminary applications in the fields of foods and beverages are described.

Keywords: Derivatization, LC; Beverages; 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate; Alcohols

1. Introduction

As fluorescence derivatization has been found to be one of the most sensitive methods for the determination of analytes at low concentrations, considerable efforts have been directed to the development of new fluorescence labelling reagents, as documented by the review literature [1,2].

Numerous fluorescence reagents have been reported for the labelling of amino [3–6], carbonyl [7–9] and carboxyl [10,11] functional groups. Although hydroxy groups of lipophilic molecules such as sterols [12,13], prostaglandins [14] and enantiomeric [15] and bifunctional alcohols [16] can be derivatized in water-free media, the derivatization is less satisfactory for the fluorescent labelling of the hydroxy groups of low-molecular-mass hydrophilic alcohols. However, these alcohols belong to one of the most important classes of organic compounds, as they are found in foods, beverages, pharmaceuticals and biological and clinical fluids. Among these, ethanol is a fundamental constituent in many

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beverages, but a wrong or unreliable fermentation process could lead to the formation of high concentrations of other alcohols known to be more toxic than ethanol itself. Volatile low-molecular-mass alcohol monitoring in fermentation processes is currently accomplished by headspace gas chromatography [17] or high-performance liquid chromatography (HPLC) with refractometric [18] or enzymatic detection. However, the detection of trace amounts of alcohols is neither easy nor sensitive enough and derivatization such as UV labelling [19] of the selected molecules is required to achieve sufficient sensitivity.

In order to improve the detection limit of hydrophilic and volatile alcohols, the utility of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [20], known as an amino acid fluorescent derivatizing agent, was evaluated. This work was aimed at the derivatization of achiral hydrophilic alcohols with AQC reagent and the separation and fluorescence detection of the derivatives by RP-HPLC.

2. Experimental

2.1. HPLC apparatus

The HPLC system was composed of two Model 510 pumps, and a Model 712 WISP automatic sample processor from Waters (Milford, MA, USA). The analytical column consisted of two stainless-steel cartridge columns (100 × 4.6 mm I.D.) and an R2 precolumn (10 × 2 mm I.D.) packed with Microspher 3-μm C_{18} from Chrompack (Middelburg, Netherlands). The column was maintained at 33°C using a SPH 99 column oven supplied by Chrompack. A Schoeffel–Kratos (Westwood, NJ, USA) FS-970 fluorescence detector equipped with a 5-μl cell was used for detection of the derivatives. The excitation wavelength was selected with a monochromator at 290 nm and a 345-nm bandpass filter (25-nm bandwidth) was used to filter the emission radiation. A Waters Model 810 baseline chromatographic station was used to control the pumps for gradient generation and the autosampler, to acquire and compute the signal obtained from the fluorescence detector. A Trilab 2500 data station (Trivector, Sandy, UK) was also used to acquire, reprocess and save the chromatograms. Optimized chromatographic conditions were obtained with the aid of the HIPACG computer optimization program purchased from Phase Separations (Deeside, UK).

2.2. Materials and reagents

6-Aminoquinolyl-N-hydroxysuccimidyl carbamate (AQC) was obtained from Waters (Brussels, Belgium) as a kit for amino acid labelling and used without further purification. AQC powder is stable for at least 6 months, but when dissolved even in super-dry far-UV-grade acetonitrile, the solution was found to be effective for less than 1 week at room temperature. Also, the vial containing the solution had to be flushed with a dry, inert gas after each use to prevent air humidity hydrolysing the AQC.

The eluents, water, HPLC-grade methanol and far-UV grade acetonitrile were purchased from Lab-Scan (Dublin, Ireland). All the mobile phases were filtered and degassed on a vacuum filtration system fitted with a 0.2-μm Durapore filter from Millipore (Molsheim, France). The alcohol standards were purchased from Acros (Janssens Chimica, Beerse, Belgium). Acetic acid and sodium acetate were purchased from Fluka (Basle, Switzerland). All the chemicals were of analytical-reagent grade and used as received.

2.3. Derivatization procedure

A 30-μl volume of reagent (3 mg/ml AQC in dry far-UV-grade acetonitrile), prepared as directed by Waters, was added to 40 μl of an alcohol sample mixed with 130 μl of 0.5 *M* borate buffer (pH 7.5). The reaction mixture was mixed by agitation for 2 min at 25°C. The vial was placed in the sample rack of the automated sample processor and an aliquot (5 μl) of the solution was subjected to liquid chromatography.

Table 1
Optimized gradient conditions obtained with the aid of the computer optimization program (see text) and used for the chromatographic separation of the derivatized alcohols

Running time (min)	Flow (ml/min)	A (%)	B (%)	Curve[a]
Initial	1.0	80	20	–
40	1.0	27	73	6
41	1.0	0	100	6
43	1.0	0	100	6
45	1.0	80	20	11
50	1.0	80	20	11
60	0.01[b]	80	20	11

[a] Curve 6 = linear gradient; curve 11 = instantaneous change.
[b] Minimal flow hold between batches of analysis.

Reagent blanks (without alcohols) were treated in the same manner.

2.4. HPLC separation

Elution was effected by using a gradient of two eluents. Eluent A was a mixture of 5% (w/v) sodium acetate aqueous buffer, adjusted to pH 6 with acetic acid, and acetonitrile (95:5, v/v) and eluent B consisted of 95% acetonitrile and 5% acetate buffer. A 40-min linear gradient from 20% to 73% B in A–B was used, followed by column cleaning and column conditioning as described in Table 1.

3. Results and discussion

3.1. Optimization of the alcohol derivatization

The reaction of AQC with alcohols is analogous to that reported by Cohen and Michaud [20] for amino acid derivatization and is shown in Fig. 1. Excess of reagent is rapidly hydrolysed to yield 6-aminoquinoline (AMQ), *n*-hydroxysuccinimide (NHS) and carbon dioxide; thus all reactions are stopped after 1 min. Yields of alcohols in a standard mixture (30 m*M*) were studied using sodium borate buffers with pH ranging from 6 to 9. Derivatization yields are plotted in Fig. 2, and show that there is a substantial effect of the buffer pH, with the maximum response at pH 7.5. As is also shown in Fig. 2, two different groups of derivatives could be observed. The first group, giving the best recoveries, was the *n*-alcohols; the second group contained only secondary alcohols. The difference in the steric hindrance of the vicinal methyl groups is so predominant that tertiary alcohols did not react at all. No derivatives of *tert.*-butyl alcohol could be obtained.

The buffer concentration had little effect on the yields as long as the reaction pH was maintained at 7.5, but some salt precipitation was observed at higher concentration levels. Sodium carbonate, sodium acetate and sodium phosphate were also effective buffers for derivatization but

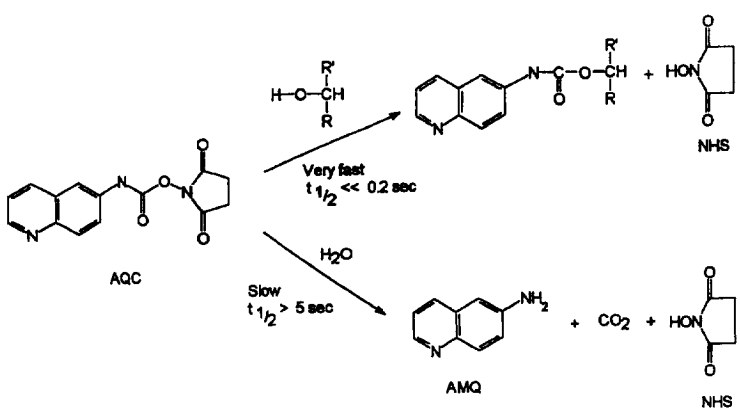

Fig. 1. Scheme of reaction of AQC with alcohols and water.

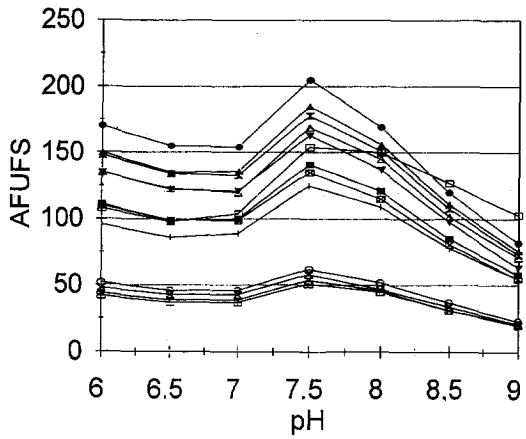

Fig. 2. Alcohol derivatization yields obtained as a function of pH. Chromatographic conditions as described in the text. Symbols as in Table 2.

some contaminants from the buffers, as observed in the derivatization blanks, were worse than with sodium borate. In conclusion, the best results were obtained with 0.5 *M* sodium borate buffer, and this was used for the subsequent experiments.

All the reactions were performed in 0.5 *M* sodium borate buffer (pH 7.5); individual alcohol concentrations were 30 μM, giving a total alcohol content of 390 μM. It was observed that using a fivefold molar excess of reagent afforded the maximum yield, as was described for the same reaction of amino acid derivatization. Heating the solution above 25°C had no effect on the yields of the alcohol derivatives, so the derivatization reactions were routinely carried out at room temperature.

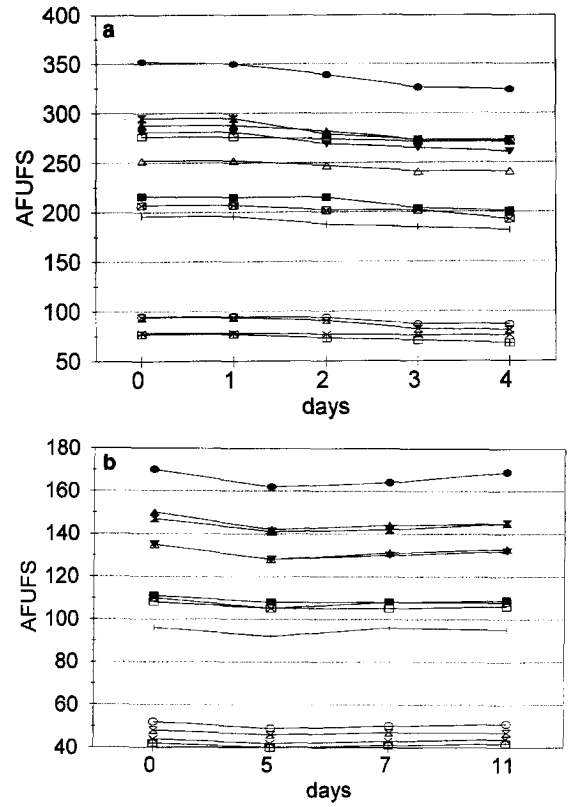

Fig. 3. Stability of the fluorescent derivatives as a function of time and temperature. (a) At 25°C; (b) at 4°C. Symbols as in Table 2.

3.2. Fluorescence properties, response of the derivatized alcohols and observed stability

As described, AMQ, the major reagent-related by-product, has a maximum of fluorescence

Table 2
Fluorescence response relative to heptanol

Symbol	Alcohol	Relative fluorescence	Symbol	Alcohol	Relative fluorescence
□	Methanol	0.82	▽	3-Pentanol	0.30
+	Ethanol	0.56	○	2-Pentanol	0.29
□	2-Propanol	0.22	▼	1-Pentanol	0.78
■	1-Propanol	0.64	▲	2-Methyl-1-butanol	0.84
×	2-Butanol	0.28	⧗	1-Hexanol	0.86
⊠	2-Methyl-1-propanol	0.58	●	1-Heptanol	1
△	1-Butanol	0.75			

[a] For reaction and chromatographic conditions used, see Experimental.

around 425 nm while the alcohol derivatives were found to have a maximum at 365 nm. This shift in emission observed for the alcohol derivatives was similar to that described for the amino acid derivatives. For this reason, it was possible to use a 345-nm bandpass filter to minimize the off-scale fluorescence peak of the AMQ present in the injection mixture. The relative fluorescence responses obtained using excitation at 290 nm are given in Table 2.

At pH 7.5, the peak areas of the derivatized alcohols were essentially stable for at least 1 day at room temperature, allowing manual preparation and automatic injection of 30 samples per day. As shown in Fig. 3a after 2 and 4 days the main losses were about 5% and 10%, respectively. At 4°C and pH 6 the observed stability was even better, as shown in Fig. 3b. The differences were found to be within the confidence limits of the chromatographic system used and were as low as 2% even after 11 days.

In these experiments, the best stability of all the derivatized alcohols was obtained at pH 6 and this pH was chosen to store derivatized samples and to buffer the aqueous mobile phase.

3.3. Chromatography of the derivatized alcohols

Complete resolution of the derivatized alcohol standard mixture could be obtained at pH6, where the derivatives had the greatest stability. Optimized conditions, as described under Experimental, were obtained with the aid of a computer optimization program and yielded reproducible retention times with almost baseline resolution of all the alcohols in about 40 min.

Fig. 4 shows the very good chromatographic separation obtained and it is also evident that the

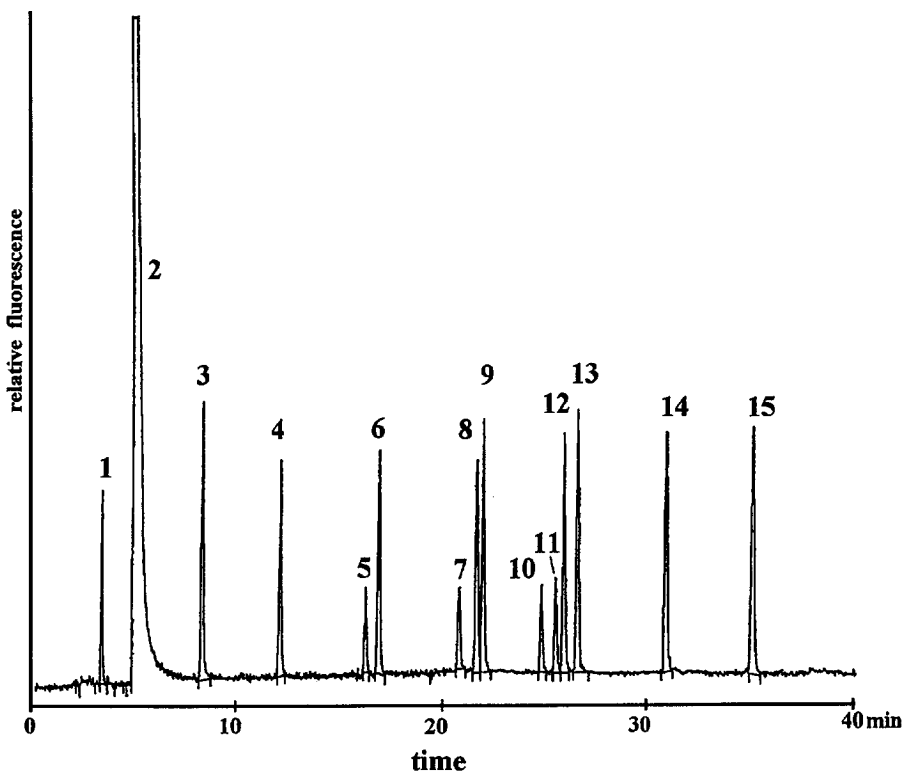

Fig. 4. Chromatographic separation of the standard alcohol derivatives. The amount of each derivatized alcohol was 5 nmol. Peaks: 1 = ammonia; 2 = reagent peak (AMQ); 3 = methanol; 4 = ethanol; 5 = 2-propanol; 6 = 1-propanol; 7 = 2-butanol; 8 = 2-methyl-1-propanol; 9 = 1-butanol; 10 = 3-pentanol; 11 = 2-pentanol; 12 = 1-pentanol; 13 = 2-methyl-1-butanol; 14 = 1-hexanol; 15 = 1-heptanol.

AMQ peak, detected at a retention time (t_R) of 4.72 min, did not disturb the chromatogram at all. In our experience, 1-butanol (t_R 21.38 min) and 2-methyl-1-propanol (t_R 21.68 min) needed too long a run time to be fully baseline separated.

Six identical standard samples were derivatized and analysed according to the procedure described under Experimental. The final sample concentration was 5 μM, the total derivatization volume was 200 μl and a 100 nM solution was injected for analysis.

The data in Table 3 demonstrates excellent reproducibility for both retention times and areas for all the derivatized alcohols excepted for the most hindered 3-pentanol, which had a peak area R.S.D. of 6.00%.

The day-to-day repeatability was also checked and showed peak area R.S.D.s and retention time R.S.D.s in the same range as those shown in Table 3.

On the basis of the experiment shown in Fig. 4, detection limits (signal-to-noise ratio of 3) of about 100 pmol were calculated for the *n*-alcohols and 250 pmol for the secondary alcohols. Analysis of serial dilutions of the standard mixtures ranging from 5 to 150 μM demonstrated very good linear responses with excellent correlation coefficients. The detection limits and correlation coefficients are shown in Table 4.

Table 3
Reproducibility of peak response and retention time (100 pmol injected, $n = 6$)

Alcohol	Peak area R.S.D. (%)	Retention time R.S.D. (%)
Methanol	1.61	0.01
Ethanol	0.82	0.01
2-Propanol	1.04	0.02
1-Propanol	1.05	0.02
2-Butanol	1.77	0.02
2-Methyl-1-propanol	0.91	0.03
1-Butanol	3.68	0.01
3-Pentanol	6.00	0.01
2-Pentanol	0.8	0.02
1-Pentanol	3.63	0.01
2-Methyl-1-butanol	0.81	0.02
1-Hexanol	0.7	0.03
1-Heptanol	4.01	0.03

Table 4
Linearity and detection limits

Alcohol	r^2 [a]	Detection limit (pmol)[b]
Methanol	0.988	90
Ethanol	0.989	100
2-Propanol	0.994	250
1-Propanol	0.991	100
2-Butanol	0.983	250
2-Methyl-1-propanol	0.987	110
1-Butanol	0.990	100
3-Pentanol	0.981	250
2-Pentanol	0.985	250
1-Pentanol	0.986	100
2-Methyl-1-butanol	0.987	100
1-Hexanol	0.981	100
1-Heptanol	0.972	100

[a] Correlation coefficients; samples of standard alcohol mixtures derivatized were in the range 5–150 nmol.
[b] Detection limits were calculated from a 5-nmol injection and based on a signal-to-noise ratio of 3.

3.4. Preliminary applications

As an application of the proposed method, the determination of the methanol concentration in commercial alcoholic beverages and beers was tried. The chromatograms, shown in Fig. 5, were obtained from (a) the reagent blank, (b) a derivatized sample of a light beer, (c) a sample of spirits (Cognac) and (d) Genever. The presence of methanol was detected in one sample (b) at a level as low as 0.02% (v/v). These results were confirmed using a static headspace gas chromatographic method [17].

3.5. Effect of diverse compounds

AQC reagent has been used for the determination of amino acids [20] and free mono- and polyamines [21], it will react with these compounds during the described reaction for the derivatization of low-molecular-mass alcohols. As shown in Fig. 5b, all the amino acids were found to elute before the reagent peak (t_R 4.72

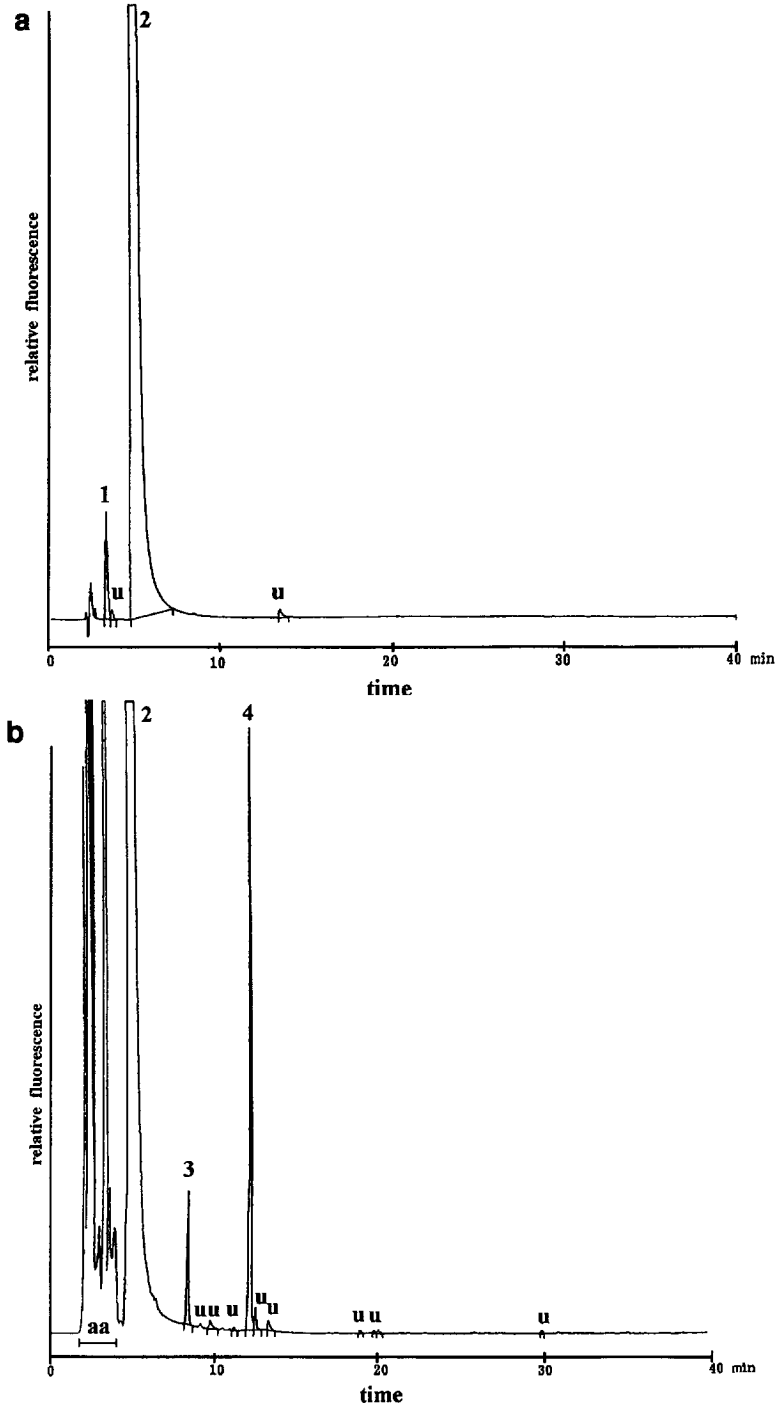

Fig. 5 (*continued on p. 340*)

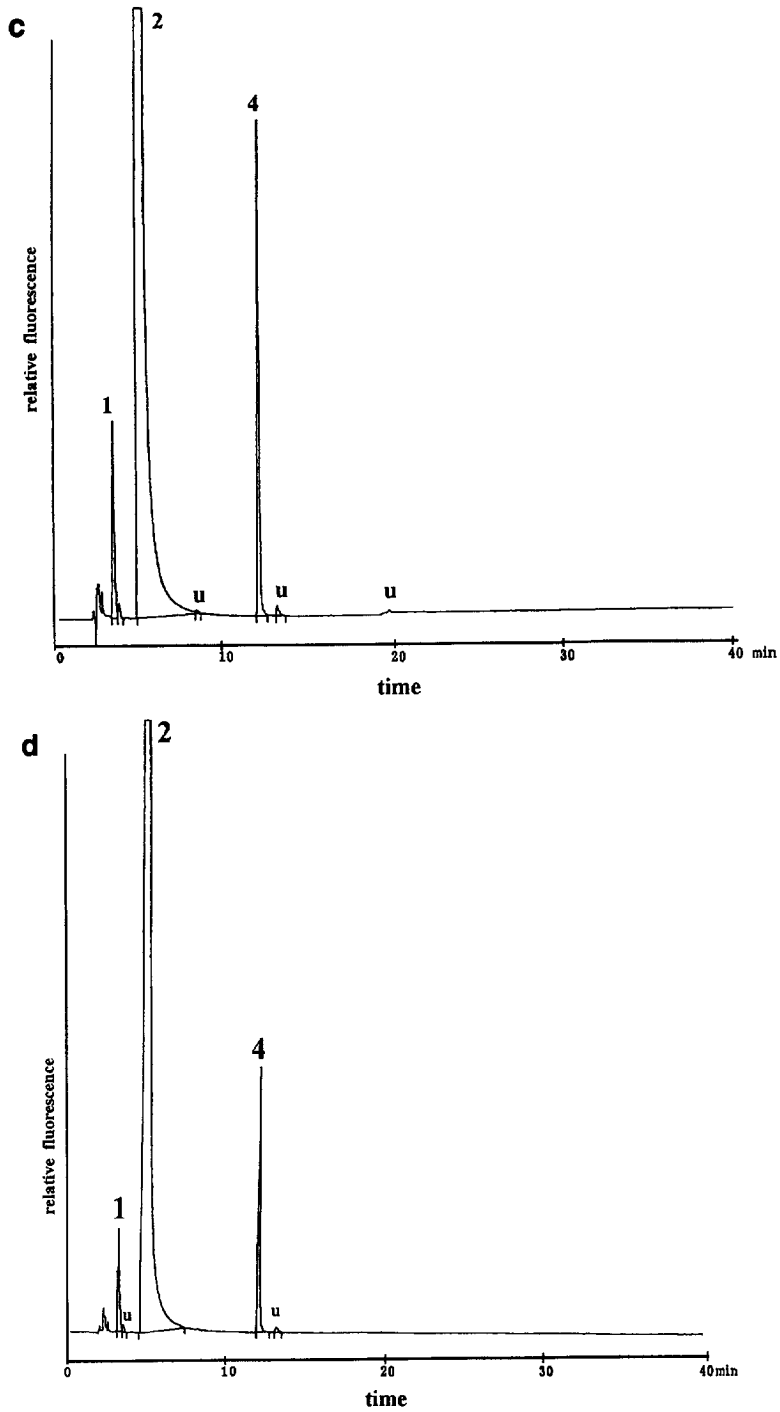

Fig. 5. Chromatographic separation of beverages. (a) Blank; (b) light beer; (c) spirit (Cognac); (d) Genever. Peaks: 1 = ammonia; 2 = reagent peak; 3 = methanol; 4 = ethanol; aa = amino acids; u = unknown.

min). Only some biological polyamines were eluted in the retention time window of methanol and ethanol, but did not disturb the analysis [21].

In the experiment shown in Fig. 5b, vacuum filtration was the only pretreatment of the beer sample analysed. Sugars could also be found to react with AQC reagent but they are not soluble under the reaction conditions used and no derivatives were found [21].

4. Conclusions

The proposed HPLC procedure for the trace determination of the hydrophilic aliphatic alcohols offers advantage in terms of ease of reactions, sensitivity and stability of the reagent and its derivatives. The detection limits are similar to the levels achieved with gas chromatography and much lower than in previous liquid chromatographic determinations. The proposed method can be used for the determination of the alcohol concentrations in food and beverages, in aqueous pharmaceutical preparations and in dissolution tests.

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