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METHODS FOR IMPROVING THE RELIABILITY AND INFORMATION CONTENT OF CHROMATOGRAPHIC ANALYSES OF BIOLOGICALLY ACTIVE COMPOUNDS

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SUMMARY

A series of methods was developed for improving the reliability of chromatographic analyses and to augment their information content and reproducibility. In high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) the possibility of utilizing azeotropic and isobaric solvent systems was substantiated. To control variations of the composition of the eluent systems during storage and analysis in TLC and HPLC, gas chromatography (GC) was found to be applicable. To improve the reliability of GC analyses, a membraneless assembly for introduction of samples, which allows false peaks in a chromatogram to be removed, is recommended. In order to augment the reliability and information content of chromatographic analyses of several bioactive compounds, complex chromatographic methods based on GC, TLC and HPLC were found to be desirable.

INTRODUCTION

The analysis of complex bioactive compounds is difficult but can be achieved, for example, by separating the constituents of a sample and by subsequent analyses of the components by conventional physico-chemical methods. This can be realized by using various individual chromatographic techniques [gas (GC), high-performance liquid (HPLC) and thin-layer (TLC)] or by combining these chromatographic techniques with each other or with other analytical methods. However, when developing complex methods with acceptable reliabilities and information content, it is desirable for each component of the combined techniques to possess similar reliabilities. This applies particularly to complex chromatographic methods based on GC, TLC and HPLC.

One of the main disadvantages of TLC and HPLC is that the eluent systems can change their compositions during storage and analysis. In GC "false peaks" can occur, which distorts the results. These drawbacks can be removed by using eluents with components with similar volatilities for TLC and HPLC and devices for the membraneless introduction of samples for GC.

This work was aimed at the development of complex chromatographic

methods for analyses of several bioactive compounds by improving the reliability of the component techniques.

EXPERIMENTAL

Reagents of pure, pure for analysis and chemically pure grades and distilled water were used.

GC measurements were carried out with a Varian-3700 instrument with a flame-ionization detector and an LKhM-8MD Model 5 instrument with a thermal conductivity detector. For liquid column chromatography we used a Milichrom-1 microcolumn chromatograph with a UV detector (254 nm), a Jasco (Japan) liquid chromatograph with a UV detector (254 nm) and a Waters Assoc. (U.S.A.) liquid chromatograph with a UV detector and with refractometric detectors. For TLC, Silufol UV-254 plates (15 cm × 15 cm) were used. The chromatograms were recorded with a Beckman (U.S.A.) densitometer.

The conditions of chromatography were as follows.

Varian-3700 chromatograph

A stainless-steel column (100 × 0.2 cm I.D.) containing 5% XE-60 on Chromaton N-AW of particle size 0.16–0.20 mm was used. The injector and detector were kept at 300°C and the column temperature was programmed from 100 to 250°C at 4°C/min. The carrier gas was nitrogen. The flow-rates of nitrogen and hydrogen were 30 ml/min and that of air was 300 ml/min. Solutions of N-TFA derivatives of butyl esters of amino acids were introduced. With synthetic steroids the column temperature was kept at 260°C.

LKhM-8MD Model 5 chromatograph

A stainless-steel column (200 × 0.3 cm I.D.) containing Polysorb-1 of particle size 0.25–0.5 mm was used. The injector and detector temperatures were 200°C and the column temperature was 160–180°C. This chromatograph was used to study the compositional stability of eluent systems.

Milichrom-1 chromatograph

A stainless-steel column (12.8 × 0.2 cm I.D.) containing Silasorb-600 of particle size 5 μm was used. The eluent was *n*-heptane–*n*-propanol–formic acid (80.2:17.1:2.7) at a flow-rate of 100 μl/min. Solutions of phenolcarbonic acids were introduced into the chromatograph.

Jasco chromatograph

A stainless-steel column (30 × 0.4 cm I.D.) containing μBondapack C_{18} of particle size 10 μm was used. The eluent was ethanol–water (80:20) at a flow-rate of 0.8 ml/min. Solutions of synthetic steroid compounds were analysed in this device.

Waters Assoc. chromatograph

The conditions were the same as for the Jasco chromatograph, except that the eluent flow-rate was 0.5 ml/min. This device was used to analyse solutions of 2-methyldihydrotestosterone and its esters.

Thin-layer chromatography

The Silufol UV-254 plates were used for the analysis of solutions of amino acids, phenolcarbonic acids, polyethylene glycols of different molecular weight and synthetic steroid compounds with various eluent systems such as dichloroethane–toluene–acetic acid (53:34:13) (for phenolcarbonic acids), dioxane–water (50:50) (for polyethylene glycols), benzene–methyl ethyl ketone (80:20) (for steroids) and *n*-propanol–water–formic acid (80:10:10) (for amino acids).

RESULTS AND DISCUSSION

It is clear that in order to obtain reliable and informative complex chromatographic methods, the individual specific techniques must be also reliable and reproducible. In thin-layer and column chromatography, this aim can be achieved by increasing the stability of the eluent systems, *i.e.*, by using systems that do not change their compositions during storage and analysis. One of the means of doing this is to formulate systems of components with close volatilities. However, the volatilities depend on a number of factors, *e.g.*, saturated vapour pressures and concentrations of the components, temperature and the reactivity of the components. Stable eluent systems can be obtained, in particular, with use of azeotropic and isobaric systems in which the boiling temperatures of the components do not differ by more than 1.6°C[1,2]. However, this approach does not readily result in acceptable eluent systems. Therefore, in order to establish the stability of a system, a technique must be applied that allows variations in solvent composition to be measured. For example, GC can be used to monitor variations in composition connected with evaporation and to trace possible chemical changes in a system.

Table I gives the extreme values of boiling temperatures in eluent systems and the variations in concentration of the components in several systems. System 1 exhibits the lowest value of $\Delta t^0 = 1.3$°C, but the most stable with time is system 2,

TABLE I

MAXIMUM DIFFERENCES OF BOILING TEMPERATURES AND THE VARIATIONS IN CONCENTRATION OF THE COMPONENTS IN ELUENT SYSTEMS

No.	*Components*	*Δ (boiling temperature) (°C)*	*Content (%)*	*Variation (%/h)*	*Duration (h)*
1	Dioxane	1.3	50	−0.3	>72
	Water		50	+0.3	
2	Dichloroethane	34.6	53	+0.05	>72
	Toluene		34	+0.05	
	Acetic acid		13	−0.1	
3	*n*-Propanol	2.8	80	+0.5	⩽3
	Water		10	−0.1	
	Formic acid		10	−0.4	
4	*n*-Heptane	3.7	80.2	−0.25	⩽5
	n-Propanol		17.1	+0.1	
	Formic acid		2.7	+0.15	

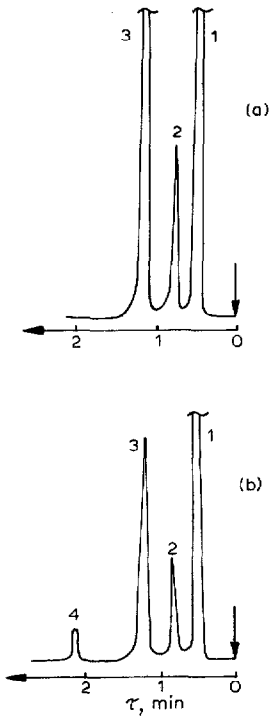

Fig. 1. Chromatograms of the eluent system consisting of *n*-propanol–water–formic acid (80:10:10): (a) initial system; (b) after 4 h. Components: 1 = water; 2 = formic acid; 3 = *n*-propanol; 4 = new component.

with $\Delta t^0 = 34.6°C$. Moreover, with systems 3 and 4 not only the changes in the compositions but also the appearance of new components was detected using GC (see Fig. 1).

Hence GC allows all the changes in eluent systems to be followed qualitatively and quantitatively. Using these data one can either choose systems with the best long-term stability (*e.g.*, 1 or 2) or specify the maximum duration of storage of less stable systems (*e.g.*, 3 and 4).

The results of analyses of specific samples by of thin-layer and column liquid chromatography become very reproducible provided that the stability of the eluent systems is monitored. The reproducibility of R_F values in the best eluent systems was as follows: phenolcarbonic acids and polyethylene glycols, $\leqslant 3\%$; and steroid preparations, $\leqslant 5\%$.

The results of GC analyses can also be made more reliable by using membraneless assemblies for the introduction of samples, which eliminates undesirable memory effects. Fig. 2 shows the chromatograms of N-TFA derivatives of butyl esters of amino acids, which were obtained with the use of membrane and membraneless assemblies. It can be seen that the membraneless assembly removes the false peaks so that the results become more reliable.

The proposed methods for improving the reliability of analyses were applied in the development of complex chromatographic methods for the analysis of amino

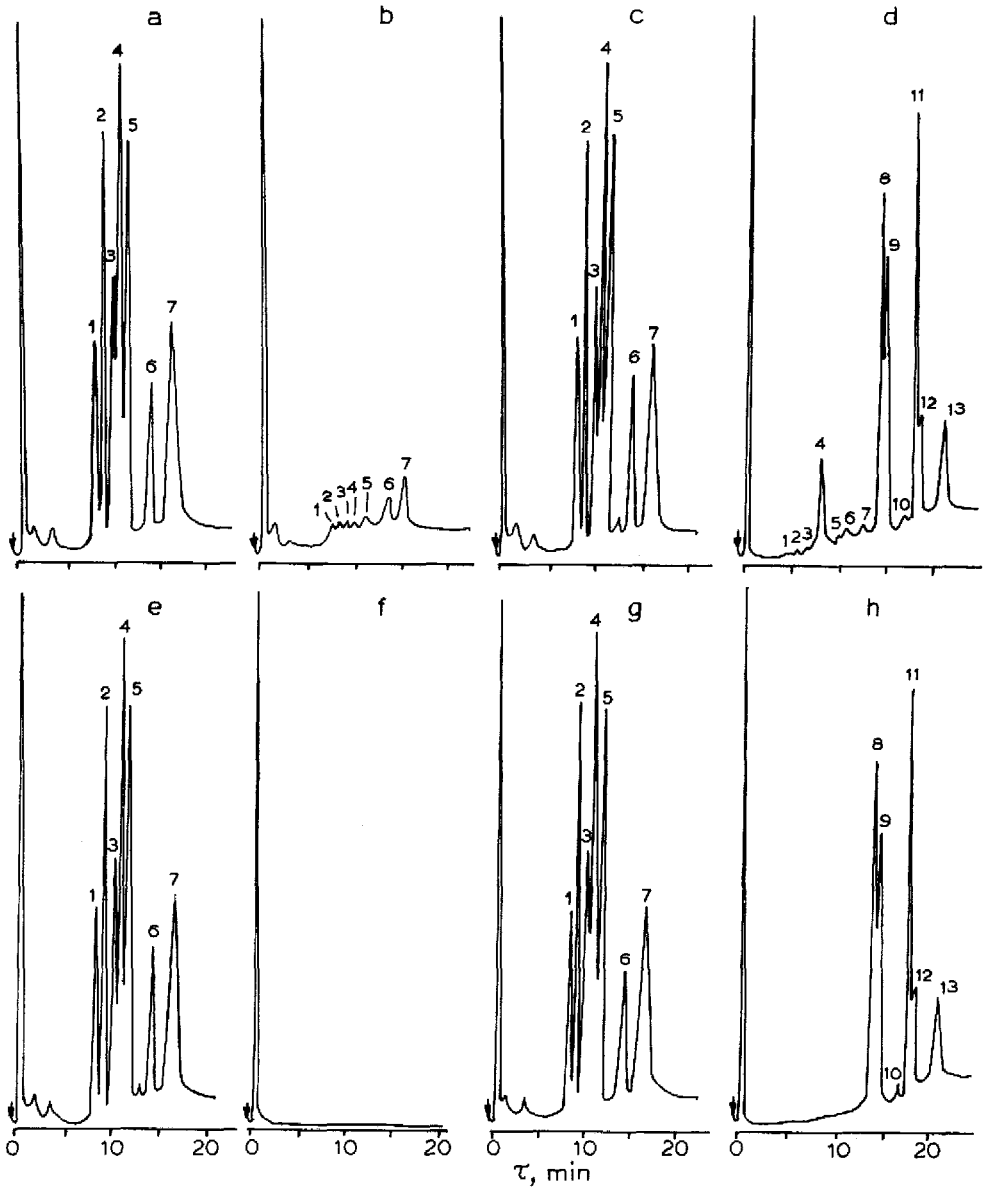

Fig. 2. GC analysis of N-TFA derivatives of butyl esters of protein amino acids with membrane (a–d) and membraneless (e–h) introduction of samples. Derivatives of: 1 = alanine; 2 = valine; 3 = isoleucine; 4 = glycine; 5 = leucine; 6 = serine; 7 = proline; 8 = hydroxyproline; 9 = cysteine; 10 = aspargine acid; 11 = methionine; 12 = phenylalanine; 13 = glutamine acid.

acid compositions of Oxytocinum and Vazopressinum preparations, for the analysis of phenolcarbonic acids in grape juice, in wines, and in propolis-based preparates such as Propoceum unguent and Proposolum aerosol and for the analysis of the purities of synthetic steroid compounds.

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