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Short Communication

Use of chromatographic peak-heights ratios for quantitative analysis: application to the separation of enantiomers

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ABSTRACT

In analytical chromatography, both peak heights and peak areas are proportional to the mass of compound injected. However, it is not possible to calculate the ratio of enantiomers from the respective peak heights without calibration. In isocratic or isothermal chromatography, the second peak of a pair is wider than the first and therefore its relative height is smaller; this effect is described here in mathematical form. With solvent gradients or temperature programming, peak-height ratios are influenced to a non-predictable extent and therefore calculations (without calibration) are not appropriate.

INTRODUCTION

It is well known that either peak areas or peak heights can be used for quantitative analysis by chromatography. Usually peak areas are used for this purpose, but peak heights can give more accurate results in the case of low signal-to-noise ratios, if the peaks of interest are very different in size or if the constancy of the mobile phase flow-rate cannot be guaranteed in studies where a concentration-dependent detector is used [1].

A special situation arises if enantiomeric ratios need to be determined by direct "chiral" chromatography (the formation of diastereomers before or after chromatographic separation is not discussed here). Then the detector properties of the two compounds are identical and peak areas can be used directly for quantification, *i.e.*, calibration is not needed (at least in principle, because the manner in which electronic integrators operate can be the reason for deviations form a "true" value [2]). In the author's experience this approach is in widespread use in laboratories which need to determine ratios of enantiomers. However, there are some laboratories which obtain "better" results if the peak-height ratio is used. This paper shows that such a procedure without calibration is not allowed in gas or liquid chromatography with isothermal, isocratic or programmed elution and with concentration-sensitive (*e.g.*, UV) or mass-sensitive (*e.g.*, flame ionization) detectors.

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PEAK-HEIGHT RATIO IN ISOCRATIC OR ISOTHERMAL CHROMATOGRAPHY

Calculations

In the following calculations it is assumed that the plate numbers for the two peaks 1 and 2 of a pair of enantiomers are identical, *i.e.*, $N_1 = N_2$. It is calculated from the retention time t_R and baseline peak width w:

$$N = 16\left(\frac{t_R}{w}\right)^2 \tag{1}$$

By solving for w and using the expression $t_R = k't_0 + t_0$, *i.e.*, replacing the retention time by the capacity factor k' and breakthrough time t_0, we obtain

$$w = \frac{4\,(k't_0 + t_0)}{N^{\frac{1}{2}}} \tag{2}$$

This equation is valid for both peaks, and therefore the ratio of the two peak widths is given by

$$\frac{w_1}{w_1} = \frac{k'_2 + 1}{k'_1 + 1} \tag{3}$$

To obtain an expression for peak-height ratios we can use the relationship between peak height h, peak area A and standard deviation σ, with $\sigma = w/4$ as is valid for peaks of Gaussian shape [3]:

$$h = \frac{A}{\sigma\sqrt{2\pi}} = 0.3989 \cdot \frac{A}{\sigma} = 1.596 \cdot \frac{A}{w} \tag{4}$$

Therefore, eqn. 3 becomes

$$\frac{h_1}{h_2} = \frac{A_1(k'_2 + 1)}{A_2(k'_1 + 1)} \tag{5}$$

or, if $A_1 = A_2$, as applies with a racemate,

$$\frac{h_1}{h_2} = \frac{k'_2 + 1}{k'_1 + 1} \tag{6}$$

Eqn. 5 shows why the ratio of peak heights is not proportional to the ratio of the peak areas. Therefore, an uncalibrated peak-height ratio must not be used for quantitative analysis.

Eqns. 5 and 6 are also valid for tailed peaks which can be described by the exponentially modified Gaussian function if their asymmetry, described as b/a (width of the trailing "half" of the peak to the width of the leading "half") at a given fraction of peak height, is equal; the area of this type of peak is described by equations of the type [4]

$$A = xhw_y\left(\frac{b}{a}\right)^z \tag{7}$$

where x is a constant factor, depending on y, y is the fraction of peak height where w, b and a are measured ($w = a + b$) and z is an exponent depending on y.

Cases where eqns. 5 and 6 are not valid

As shown above, the given relationship between peak-height ratio and capacity factor is valid if the plate numbers of the two peaks are identical and if they have the same degree, or absence of, asymmetry. Programmed elution is not allowed. Eqns. 5 and 6 are not valid in the following cases:

(1) If $N_1 \neq N_2$. This occurs if the retention mechanisms for the two compounds of the peak pair differ, as is often the case in enantioselective liquid chromatography.

(2) If the detector properties of the two peaks are not equal or if the detector is overloaded. Enantiomers have identical properties if the detector itself is non-chiral (an enzyme reactor is chiral) and if they are not derivatized to give diastereomers. If such derivatization is performed before or after the separation, the detector properties of the diastereomers need to be checked. They can be identical, but there is no guarantee. In the case of detector overload a large peak will give too small a signal.

(3) If a liquid chromatographic mobile phase is used which gives system peaks. Many additives to the liquid mobile phase can give rise to positive or negative, or even invisible, system (extra) peaks. In this event any peak area depends on the separation factor of (or distance between) the system and solute peaks. This effect was described by Schill and Crommen [5].

(4) In liquid chromatography if the composition of the mobile phase is not constant and in gas chromatography if the temperature is not constant. Both cases are an unwanted programmed elution. The effect on peak height is shown below.

All four effects lead to a non-predictable influence on peak heights, and calibration is more needed than ever.

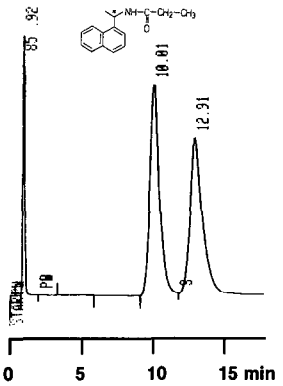

Fig. 1. Isocratic separation of racemic 1-(1-naphthylethyl)propionamide on R-DNBPG. Column, 125 mm × 2.1 mm I.D.; stationary phase, Nucleosil 5 NH_2 with ionically bound N-(3,5-dinitrobenzoyl)-D-phenylglycine; mobile phase, hexane–2-propanol (95:5); flow-rate, 0.5 ml/min; detection, UV (254 nm); integrator, Hewlett-Packard Model 3390 A. The first peak is toluene. Capacity factors: 10.8 for the *R* enantiomer, 14.2 for the *S* enantiomer.

Examples

Fig. 1 shows the liquid chromatographic separation of racemic 1-(1-naphthylethyl)propionamide on a Pirkle-type stationary phase. The peak-height ratio as obtained by the integrator (in the peak-height mode) is 1.35 and as calculated by eqn. 6 it is 1.29. The difference could be due to slightly different plate numbers of the two peaks.

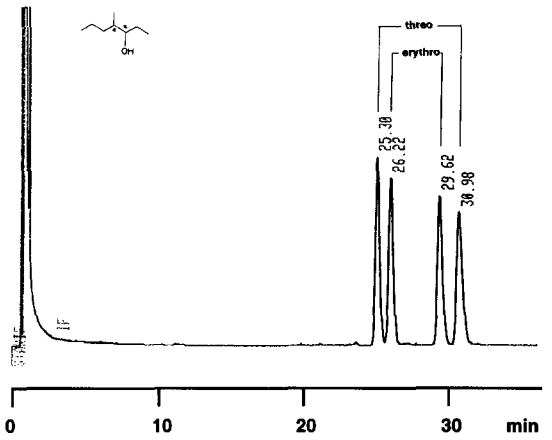

Fig. 2. Isothermal separation of racemic *erythro*- and *threo*-4-methyl-3-heptanol on perpropylated β-cyclodextrin. *Erythro* and *threo* isomers are present in slightly differing amounts, but the respective mixtures are racemic. Column, 10 m × 0.2 mm I.D.; stationary phase, 40% per-*n*-propyl-β-cyclodextrin in OV-1701; film thickness, 0.15 μm; mobile phase, helium, 42 kPa inlet pressure, t_0 was 0.58 min; temperature, 40°C.

Fig. 2 shows the gas chromatographic separation [6] of *erythro*- and *threo*-4-methyl-3-heptanol on perpropylated β-cyclodextrin [7]. The diastereomers are not present in equal amounts, but they are both racemates. The peak-height ratios are as follows: for the *erythro* enantiomers, by integrator 1.12, and by eqn. 6 1.13; and for the *threo* enantiomers, 1.41 and 1.22, respectively.

PEAK-HEIGHT RATIO IN PROGRAMMED CHROMATOGRAPHY

It was mentioned before and it is well known from experience that solvent gradients in liquid chromatography and temperature programmes in gas chromatography compress the peaks and thereby make

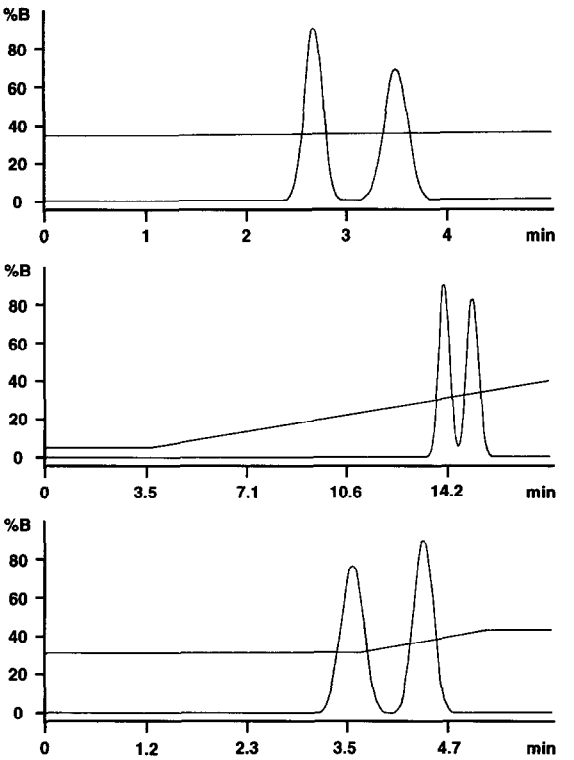

Fig. 3. Computer simulations of the separation of a hypothetical racemate. Software: DryLab G plus. Starting data: increase of the amount of strong solvent from 5% to 100% B in 15 min or 45 min, retention times 8.5 and 9 min or 14 and 15 min. The peak areas are equal in all instances (but the time axes are different), gradient profiles are overlaid. Top, isocratic separation, 35% B, peak-height ratio 1.30; middle, gradient from 5% to 40% B, peak-height ratio 1.10; bottom, gradient from 31% to 43% B, peak-height ratio 0.85.

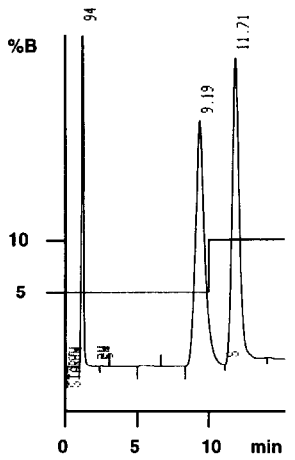

Fig. 4. Programmed separation of racemic 1-(1-naphthylethyl)-propionamide on R-DNBPG. Conditions as in Fig. 1 with the exception of a step gradient from 5 to 10% 2-propanol in hexane after 10 min.

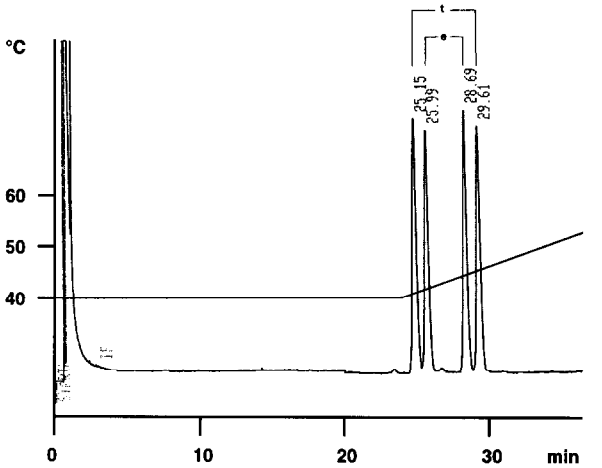

Fig. 5. Programmed separation of racemic *erythro-* and *threo*-4-methyl-3-heptanol on perpropylated β-cyclodextrin. Conditions as in Fig. 2 with the exception of a linear temperature programme of 1°C/min beginning at 24 min.

them narrower and higher. It is difficult to predict these effects from theory. Empirical predictions by using the data obtained by two gradient runs with differing pogramme times are possible [8] and the effects can be studied easily by applying appropriate software (*e.g.*, DryLab from LC Resources, Lafayette, CA, USA). Programmes are available for gas and liquid chromatographic separations and were developed for the computer-assisted optimization of difficult separations. Fig. 3 shows the influence of various gradients on the separation of a hypothetical racemate.

Figs. 4 and 5 show the same separations as in Figs. 1 and 2 but now with solvent and temperature gradients, respectively. Often the effects of (stepwise or continuous) programmed elution are so distinct that the later eluted peaks are higher than the earlier eluted peaks if their aras are equal. Although programmed chromatography could be used to "optimize" peak heights, this is nonsense, but these techniques are a powerful help in improving the resolution of complex sample mixtures. It is clear that with programmed elution peak heights give no quantitative information without calibration.

CONCLUSIONS

It is not possible to use peak-height ratios for quantitative analysis without calibration. In isocratic or isothermal chromatography the second-eluted peak is lower (in absolute or relative terms) than the first-eluted peak; an equation describing this effect has been given. In programmed chromatography the effect on peak heights is not predictable without assumptions or experiments.

ACKNOWLEDGEMENTS

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