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Note

Recycling gel chromatography of phenolic compounds

Recycling gel chromatography has been used for the purification of human ceruloplasmin[1], of the haemoglobin–haptoglobin complex[2] and of lactic dehydrogenase[3] using Sephadex G-100 columns. The separation was based on the molecular sieve properties of dextran gels. With low-molecular-weight aromatic substances, we investigated the possibility of using recycling chromatography for separating some aromatic amines[4]. In this system, the substances were eluted according to their increasing aromatic adsorption on to the gel phase. Different hydrocarbons have been successfully separated on Styragel[5–7].

Resolution by means of recycling gel chromatography

In the course of our preliminary investigations, several comparisons have been made of the behaviour of certain aromatic amino acids and aromatic amines in recycling chromatography.

The number of benzene rings in mono- and bicyclic alkyl amines had a major enhancing effect on their retardation on the column. In addition, acidic, neutral and basic substitutions influenced the mobility and the methylation of hydroxyl and amino groups also had a minor effect. In this paper, we describe some results of the separation of aromatic compounds by recycling chromatography on Bio-Gel P-2 columns. The factors governing resolution on Bio-Gel P-2 are complex[8] and will not be dealt with here. We were especially interested in evaluating the potential of this method in separating very similar substances, having only minor differences in K_d, and also in establishing which of the separation data could be used to determine the number of cycles that affords the optimal separation in each instance. Our experiments were carried out with LKB Produkter Refrigerated ReCyChrom Standard Set at 5° according to the original instructions[1].

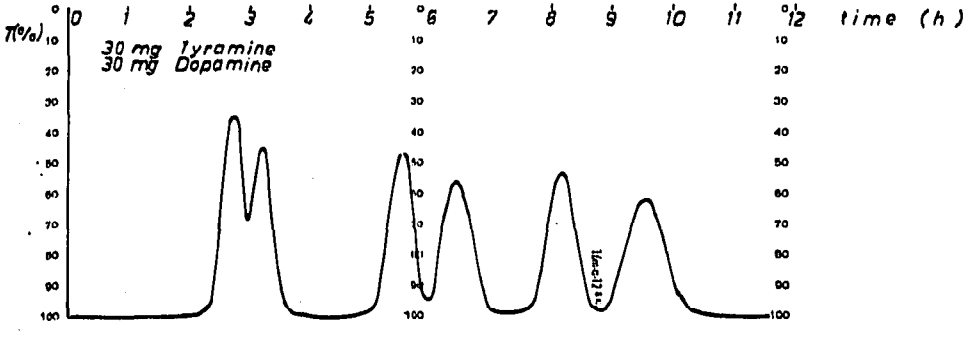

Fig. 1. The separation of tyramine and Dopamine on a Bio-Gel P-2 column (35 × 500 mm). Ultraviolet transmission was measured with a Uvicord II instrument at 254 nm. Elution with 0.2 *N* $CaCl_2$ solution at a flow-rate of 126 ml/h.

With tyramine and dopamine, the difference of the hydroxyl group resulted in a complete separation in three cycles (Fig. 1).

A similar resolution could be obtained with hydroxy-substituted aromatic amino acids (*e.g.*, phenylalanine, tyrosine and dihydroxyphenylalanine (Dopa)), and with phenylethylamine and tyramine, tyramine and octopamine, octopamine and norepinephrine, etc. Methylation of one or more hydroxyl groups may also facilitate

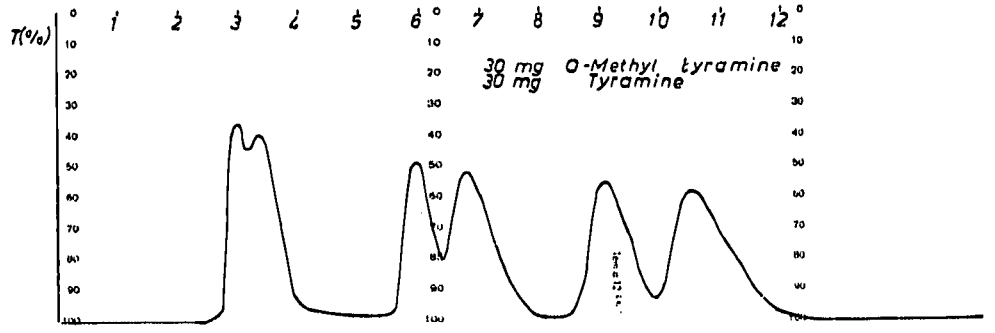

Fig. 2. The separation of O-methyltyramine (*p*-methoxyphenylethylamine) and tyramine on a Bio-Gel P-2 column (35 × 500 mm). Ultraviolet transmission was measured with a Uvicord II instrument at 254 nm. Elution with 0.2 *N* $CaCl_2$ solution at a flow-rate of 68.8 ml/h.

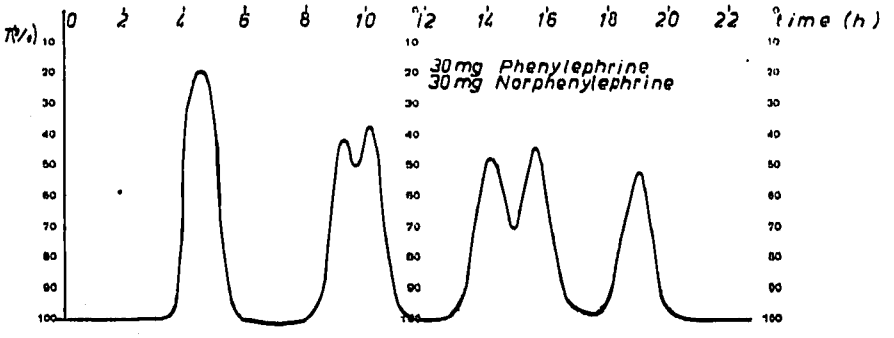

Fig. 3. The separation of phenylephrine and norphenylephrine on a Bio-Gel P-2 column (35 × 500 mm). Ultraviolet transmission was measured with a Uvicord II instrument at 254 nm. Elution with 0.2 *N* $CaCl_2$ solution at a flow-rate of 200 ml/h.

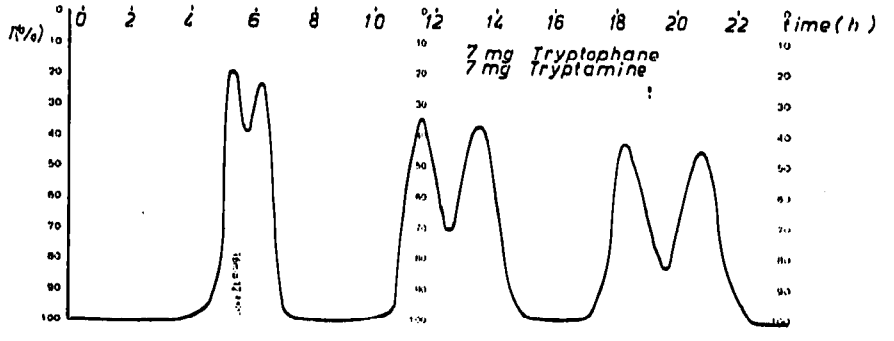

Fig. 4. The separation of tryptophan and tryptamine on a Bio-Gel P-2 column (35 × 500 mm). Ultraviolet transmission was measured with a Uvicord II instrument at 254 nm. Elution with 0.2 *N* $CaCl_2$ solution at a flow-rate of 110 ml/h.

the separation, as demonstrated by the separation of tyramine from O-methyltyramine (Fig. 2). Similarly, N-methylated compounds could be separated from their parent compounds (Fig. 3).

If the difference between two otherwise closely related compounds was the presence or absence of a carboxyl group, as illustrated by the separation of tryptophan from tryptamine in Fig. 4, resolution was generally achieved in 2–4 cycles.
Similar results were obtained in separating the following pairs of related compounds: phenylalanine from phenylethylamine; tyrosine from tyramine and dopa from dopamine.

Numerical method for calculating the optimal cycle number

With each approach to a problem of resolving substances that were eluted partially separated from one another, it was found that the K_d value for a particular substance was not as useful as either the retention time (t_e) or the elution volume (V_e). The latter two values showed a direct relationship to the number of cycles required for optimal resolution. Both values are related through constants for any particular column as follows:

$$\frac{t_e}{t_0} = \frac{V_e}{V_0}$$

where V_0 is the void volume and t_0 the time necessary to collect the void volume. As will be shown, this relationship allows either V_e or t_e to be used in determining the optimal number of cycling steps. Furthermore, it should be pointed out that the K_d can be used to calculate the value V_e/V_0 if certain characteristics of the gel are known:

$$K_d = \frac{V_e - V_0}{V_\infty - V_0}$$

where $V_\infty - V_0$ is the internal volume and is a constant for a particular gel column. In the case of Bio-Gel P-2, for example, one can use the above equation to calculate:

$$\frac{V_e}{V_0} = 1.5K_d + 1$$

In an initial separation on a gel column, one can calculate the mobilities of the substances in terms of either V_e or t_e and subsequently use these values to determine the optimal number of cycles for the best resolution. That there is an optimum number of cycles can be seen by considering the theoretical separation of two substances, one with a mobility x and one with a slower mobility y. As the cycling proceeds, the peak with the faster mobility will at first move away from the slower moving peak but will, at the same time, approach the slower peak from behind and, step by step, overtake it. For this reason, we have found useful an equation that defines the condition where the distance between the two peaks is maximal. For the condition when

$$ax \geqslant (a + \tfrac{1}{2})\, y$$

which can also be written as

$$\frac{a + \frac{1}{2}}{a} \leqq \frac{x}{y}$$

where a is the integer specifying number of cycles and x and y are mobilities expressed in either volume or time. Units for x and y need not be taken into account provided that they are the same, as they will cancel out in the equation.

When one assigns a whole number to a in the above equations, the result is an arithmetic series which yields:

$$\frac{3}{2}, \frac{5}{4}, \frac{7}{6}, \ldots, \frac{2n+1}{2n}, \qquad n \equiv a$$

There will be many values of a that satisfy the equation for any x/y value, but the smallest number to do so will yield the best separation. An example is shown in Fig. 5.

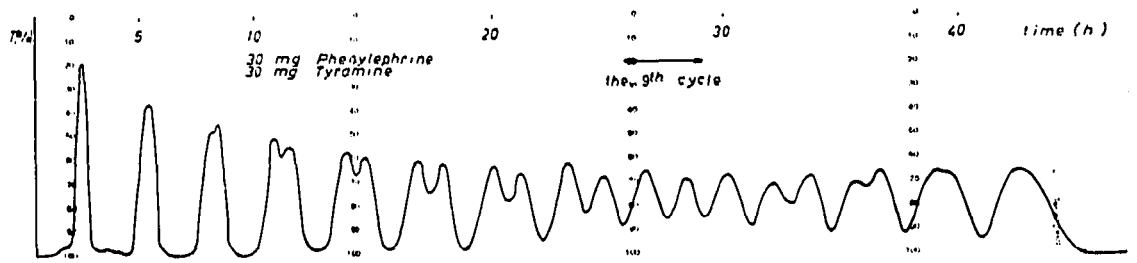

Fig. 5. "Separation" of tyramine and phenylephrine by means of recycling gel chromatography on a Bio-Gel P-2 column at 5°. The x/y value is 1.0607. $(2 \times 9 + 1)/(2 \times 9) = 1.055$ is the first $(2n + 1)/(2n)$ value, which was less than 1.0607, *i.e.*, $a = n = 9$ is the optimal cycle number.

The above results show that mixtures of some aromatic amines and amino acids can be separated by means of recycling gel chromatography through a Bio-Gel P-2 column. It is possible to achieve total separation of compounds with only slight differences in structure in 2–4 cycles, although sometimes 5–15 cycles are necessary. The numerical method has been given here to calculate the optimal cycle number.

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