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Normal and Reversed-Phase Thin Layer Chromatography of Green Leaf Extracts

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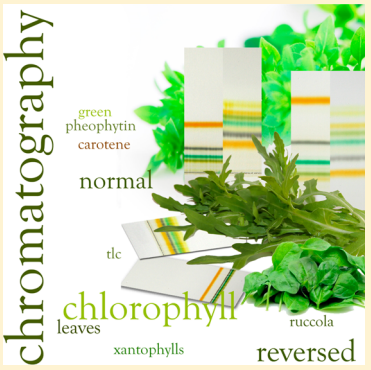
Supporting Information

ABSTRACT: Normal and reversed-phase chromatography can be easily illustrated using thin layer chromatography for the separation of green leaf extracts within a short time and at a low cost.



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INTRODUCTION

Introductory experiments of chromatography are often conducted by separating colored samples, such as inks, dyes, and plant extracts, using filter paper, chalk, or thin layer chromatography (TLC) plates with various solvent systems. Many simple experiments have been reported.[1−6] The relationship between normal chromatography and reversed-phase chromatography is, however, seldom illustrated with experiments. Where such experiments exist, they often utilize column chromatography[7] and, particularly, HPLC,[8,9] although TLC systems[10] and paper chromatography[11] have also been reported.

As is well known, green leaves contain the colored compounds chlorophyll a and b and the main carotenoids such as carotene and xanthophylls (e.g., lutein, violaxanthin, and neoxanthin).[12] These compounds are ideal for demonstrating simple chromatographic principles, as they are easily distinguishable from each other by their retention and their colors. The appearance of pheophytin as a singular grayish spot between the green chlorophylls and the yellow carotenoids provides a particularly valuable reference when the sequence of the migration is to be compared between different separation systems. Previously, we have reported how normal and reversed-phase chromatography can be illustrated using *paper* chromatography of extracts of dandelion leaves (*Taraxacum officinale*), which are rich in pheophytin.[13] In normal-phase chromatography, the stationary phase is relatively hydrophilic, whereas the mobile phase is more hydrophobic. In reversed-phase chromatography, the characteristics of both stationary and mobile phase are the opposite, resulting in a reversed elution order to that obtained when using normal-phase chromatography.

Here, we now report how normal and reversed-phase TLC can be illustrated by separating extracts of spinach (*Spinacia oleracea*) and rocket salad/ruccola (*Eruca sativa*). The novelty of this experiment is that (i) the chromatographic principle is different, that is, TLC (adsorption chromatography) compared to paper chromatography (partition chromatography);[13] (ii) the extract material (ruccola instead of dandelions) is more available but still contains sufficient amount of pheophytin; (iii) the system has been extensively optimized with respect to resolution; and (iv) the quantity of material (TLC plates and solvent) has been reduced substantially, making the experiment inexpensive, time-efficient (elution time reduced from 40 to 8 min), and more in line with health and safety regulations.

Procedures for extraction of plant pigments are well known and can be found in standard books on organic experiments such as Williamson.[14] Simpler extraction processes have been reported[15,16] and, given constrained time limits, these are welcome.

EXPERIMENTAL OVERVIEW

Pedagogical suggestions including student group sizes and time required to undertake these experiments are detailed in the Supporting Information, as there are a varity of options.

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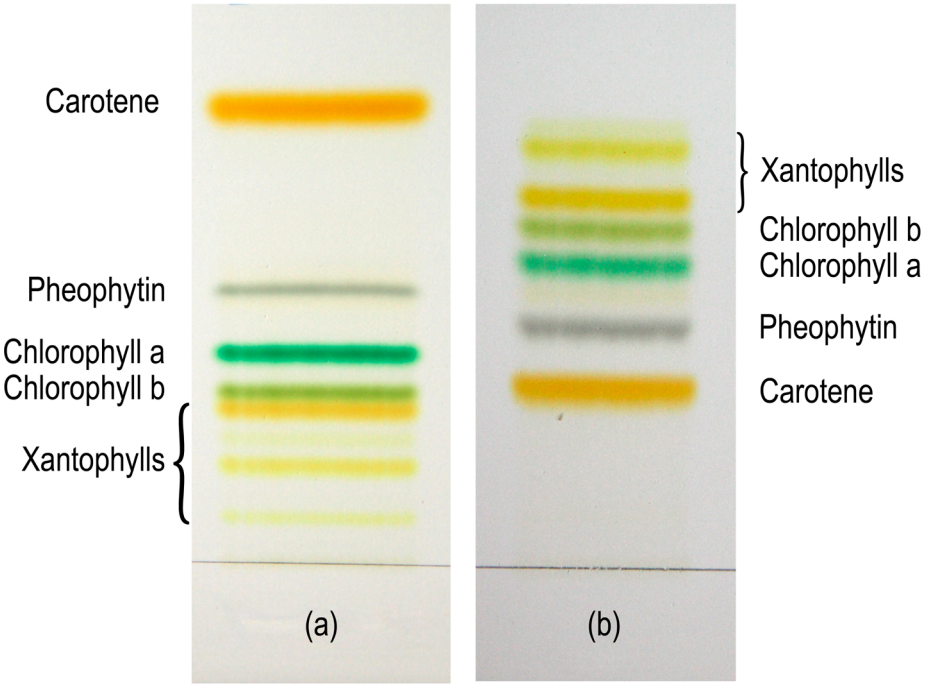

Figure 1. Normal-phase (Silica gel 60, left) and reversed-phase (RP-18 silica, right) chromatography of a *n*-hexane extract of ruccola and spinach on TLC plates developed with *n*-hexane:acetone (7:3 v/v) and *n*-hexane:acetonitrile:ethanol (15:35:50 v/v), respectively.

Two different extracts for TLC analysis are used, an acetone extract and an *n*-hexane extract. A mixture of fresh ruccola and spinach leaves together with some sand and a mild drying agent are soaked in a little acetone and crushed using a mortar and pestle. In the quickest version of the experiments (adaption of references 15 and 16), the acetone extract is decanted and used for TLC analysis as described below.[15,16] In the second version of the extraction, the acetone extract is decanted and discarded, and the wet leaves are dried between filter paper and extracted with *n*-hexane. The extract is dried, filtered, and concentrated using a rotary evaporator. If no rotary evaporator is available, the extract can be concentrated by evaporation from a container with a wide opening at room temperature in a fume hood. This *n*-hexane extract can be stored for several months in a refrigerator. In all cases, *n*-hexane can be substituted with *n*-pentane, cyclohexane, or *n*-heptane.

Chromatography chambers are either small beakers or test tubes with stoppers. The smallest chambers are test tubes (d = 1.5 cm, h = 10 cm) with TLC plates as small as 1.0 cm × 6.5 cm. The inside walls of the beakers are lined with filter paper; however, no lining is used in test tubes.

For normal-phase chromatography on silica, the solvent system is *n*-hexane:acetone (7:3 v/v). The chamber is briefly equilibrated prior to development. The leaf extract is applied with a capillary tube onto a TLC plate (Silica gel 60) and developed until visible separation has been achieved, typically less than 8 min. Immediately after the solvent has evaporated, Scotch tape is applied to the TLC plate to cover the spots, and the chromatogram is documented by photography.

Reversed-phase chromatography is conducted on an RP-18 Silica (octadecyl silica) plate using *n*-hexane:acetonitrile:ethanol (15:35:50 v/v) or *n*-hexane:acetone:ethanol (2:3:5 v/v) as eluent. Sample application and separation time are the same as for normal-phase chromatography.

HAZARDS

Handling organic solvents in open containers should be performed in a fume hood and suitable gloves and eye protection should be used. Acetone, acetonitrile, ethanol, *n*-pentane, *n*-hexane, cyclohexane, and *n*-heptane are highly flammable liquids and vapors, and should be kept away from ignition sources. Acetone, acetonitrile, *n*-pentane, *n*-hexane, cyclohexane and *n*-heptane are harmful if ingested, inhaled or in contact with the skin. Acetone and acetonitrile cause serious eye irritation. Acetone, cyclohexane, *n*-heptane and *n*-hexane vapors may cause drowsiness and dizziness. There is a danger of serious damage to health by prolonged exposure through inhalation of *n*-hexane, including a possible risk of impaired fertility. *n*-Hexane is a neurotoxin. Cyclohexane, *n*-pentane, *n*-hexane and *n*-heptane may be fatal if swallowed and enters airways (do not induce vomiting). Because of chronic aquatic toxicity with long lasting effects they should not be released to the environment.

RESULTS AND DISCUSSION

Extraction of plant material, such as green leaves, is well known but is still subjected to adjustments.[15,16] Therefore, we have described two methods; one quickly accomplished using acetone, and another, less expedient, using acetone and *n*-hexane. As these two solvents differ in polarity, their extracts will also reflect this, leaving the acetone extracts richer in xanthophylls, that is, the more polar compounds and in some cases less rich in β-carotene. It is also possible to mix acetone and *n*-hexane in the extraction process. All types of extracts are best used fresh; however, *n*-hexane extracts have been used for this purpose after months of storage in a refrigerator. In all cases, it is important to avoid residual water in the extracts. After a few days, signs of degradation can appear, more in the acetone extract than in the hexane extract. This degradation was observed in chromatograms as an increase in pheophytin due to

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the limited stability of chlorophyll, and a splitting of the two chlorophyll spots into two spots each due to epimerization of the chlorophyll molecules at the enolizable carbon adjacent to the ketone group, chlorophyll a′ and b′, respectively.[17,18] In the Supporting Information, some example chromatograms of different extracts and their degradation are shown. However, for the purpose of demonstrating normal-phase versus reversed-phase chromatography, some degradation of the extract was acceptable and not in conflict with the chromatographic principles to be demonstrated.

TLC is, in general, a quick analytical method, and this was particularly true for this experiment. Separation was observed almost immediately after the TLC plates were put into the eluent. In under 1 min, most of the compounds were identified, and the separation increased as the elution progressed. The same rapid separation process was observed for reversed-phase chromatography. Thus, the method was convenient under time constraints.

Normal and reversed-phase chromatography on normal and reversed-phase silica TLC plates are shown in Figure 1. Normal-phase chromatography is characterized by the least polar compound moving faster, thus attaining higher R_F values (here: β-carotene) compared to the more polar compounds, which will be adsorbed more strongly (here: xanthophylls). In Figure 1a, an *n*-hexane extract of ruccola and spinach leaves was separated in normal-phase chromatography. In Figure 1b, the same extract was separated in reversed-phase chromatography, where the more polar compounds moved faster than the less polar ones.

In normal phase chromatography, the main spots appeared with increasing R_F values as several yellow spots (often 3, but dependent on the amount of extract applied, and one was partly hidden by the first green spot), two green spots, a grayish one and a yellow/orange one. According to the polarity of the components and literature referring to normal-phase chromatography,[12,14,15] these spots most likely corresponded (from shortest to longest migration) to xanthophylls (yellow) (most likely lutein, violaxanthin and neoxanthin,[14] of which the order is based on their expected polarities), chlorophylls b (yellow green) and a (blue-green), pheophytin (gray), and β-carotene (yellow/orange). In reversed-phase chromatography, the sequence of the spots was reversed. However, identities were difficult to deduce reliably due to the possible interference of various xanthophyll fatty ester derivatives.

Neither teachers nor students had problems with the experiment; the quality of the results varied, but many obtained similar resolution of the components (see Supporting Information for more details). These two TLC experiments were quickly and easily conducted by students in the laboratory. Tank saturation was hardly necessary and the development took 8 min or less. The chromatograms provided an entry for discussing the interconnection of polarity and relative migration rate of the pigments, and the characteristics of normal and reversed-phase systems. In short, these experiments illustrated that polar stationary phases promoted retention of polar compounds, whereas nonpolar stationary phases promoted retention of nonpolar compounds.[11] The difference in polarity for the solvents mixture also reflected the difference between normal and reversed-phase chromatography, the former being a nonpolar (mostly hexane) mobile phase and the latter being a polar one (water-miscible solvents such as acetone, acetonitrile, and ethanol). To which extent one will engage in explanations and discussions depends on the course/level of education in which this experiment is to be used (see Supporting Information).

■ SUMMARY

The separation principles of normal chromatography and reversed-phase chromatography were illustrated by separating leaf extracts by silica based TLC. The method reported was simple, fast, and inexpensive and was easily conducted within constrained time limits.

■ ASSOCIATED CONTENT

Supporting Information

Instructor notes with supplementary experimental details, and student notes. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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