Separation, purification and identification of the components of a mixture

Supplementary Material

Experimental notes

Figures

Experimental notes

Background topics

This experiment does not involve typical chemical reactions of organic compounds, with the exception of acid-base reactions. Its aim is to provide the students the knowledge of fundamental experimental techniques of unitary operations, such as extraction, distillation, filtration, recrystallization and thin layer chromatography.

The work (two laboratory sessions) has a low difficulty level and is adequate to introductory level students, especially engineering students (chemical, biochemical, material sciences, biomedical and related areas) or Chemistry students at introductory level.

This simple pedagogic experiment was already realized by over 500 students of the Faculty of Sciences and Technology, Universidade Nova de Lisboa (in classes of 22 students/class, 11 groups of two), who accomplished the work in two (2x3 hours) laboratory sessions.

The students must be encouraged to rationalise the principles of the unitary operations performed and to understand their application. If this work is a first experiment in an Organic Chemistry laboratory class, every experimental detail must be extensively discussed.

Special attention and care must also be given to collection and treatment of results (calculation of R_f values and recuperation “yields”, measurement of physical properties, e.g. melting points). It is also important to encourage the students to compare the experimental results with those referred in the literature.

Hints for the answers to the proposed questions and topics to discussion:

1. Deprotonation of the carboxylic group of benzoic acid and protonation of the amino group of *p*-toluidine.
2. Chemically inert; not mixable with the solution to extract; possible low boiling point; …
3. Removal of traces of acid / base.
4. Assure complete precipitation.
5. Removal of traces of water.

Experimental details

Experimentally the work is simple, with low difficulty and hazard levels.

In the “separation” step explain to the students how to recognize and distinguish an organic layer from an aqueous layer and how to separate them correctly. Ask them how to increase the rate of an extraction.

In the “recovery” step it is important to assure the complete reversal of the pH of the solutions for complete precipitation of the compound to be isolated. Ask the students how to test if this has occurred and how to proceed if the result is not satisfactory (use indicator paper and add more acid or base if necessary). Concerning the naphthalene solution in methylene chloride left after extraction with aqueous solutions, teach the students how to dry a liquid (use of a good drying agent, clearness of the liquid, efficient filtration).

The “TLC” part is very important to verify the “success” of the experiment. Show the students how to apply the samples, to prepare the camera, to develop the elution, to reveal the spots. Encourage them to compare the spots from the initial mixture with the spot(s) from each separated compound. Show them how to calculate correctly the R_f values. Average R_f values (CH_2Cl_2 / silica gel plates): benzoic acid = 0.37; *p*-toluidine = 0.47; naphthalene = 0.88.

The results of the TLC test show clearly which of the three isolated compound is less pure. Usually it is naphthalene; ask the students the reason (it is the last one remaining after the sequential separation steps and accumulates all the impurities). Naphthalene is therefore the better candidate to be purified by recrystallization. However, it should be interesting to suggest other groups in the laboratory classroom to recrystallize also *p*-toluidine and benzoic acid, and encourage all the students to share the results obtained by each group. Recommended solvents for recrystallization are: commercial ethanol for naphthalene; distilled water for benzoic acid; petroleum ether (40-60°) for *p*-toluidine.

For every compound the students must weigh the amount of the compound before (crude) and after (purified) recrystallization. The same applies to the measurement of the melting points. In both cases, the results should be compared and interpreted. Discuss with the students the melting point value as a “criterion” of purity of a compound (comparison with values from the literature).

Some experimental results obtained by the students in the laboratory are presented in Table 1.1.1.

Table SM 1.1.1. Typical experimental results obtained in the Laboratory

Compound	Recuperation (%)	Melting point (°C)
p-toluidine	40 - 48	40 - 43
benzoic acid	40 - 67	110 - 115
naphthalene (crude)	72 - 80	74 - 77
naphthalene (recrystallized)	40 - 50	78 - 79

Finally, an additional characterization can be made by recording the IR spectra of the purified compounds. For this purpose a further 3 hours session may be needed. Students will be familiarized with the technique of preparing a solid transparent disc for IR spectroscopy, by using a small amount of a dried sample of the purified compound and KBr as the adequate support material. The assignment of the absorption bands characteristic of the functional groups of the compounds will be an excellent complement of the work.

Figures

Photos of the experiment

Fig. SM 1.1.1. Acid/base extraction

Fig. SM 1.1.2. Recovery of naphthalene

Fig. SM 1.1.3. Aqueous extracts A and B and recovered naphthalene

Fig. SM 1.1.4. Precipitation of *p*-toluidine and benzoic acid

Fig. SM 1.1.5. TLC plates (under UV light)

Left: too concentrates samples

Right: correct samples

Fig. SM 1.1.6. Recrystallization of naphthalene

Spectra

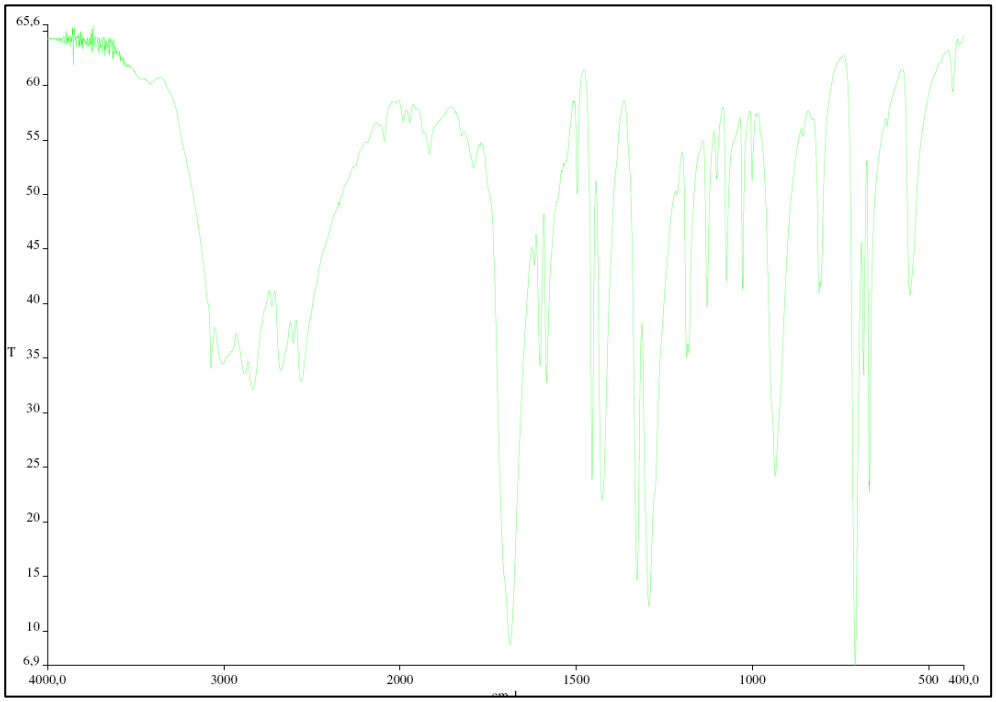

Fig. SM 1.1.7. IR spectrum of benzoic acid (in KBr disk)

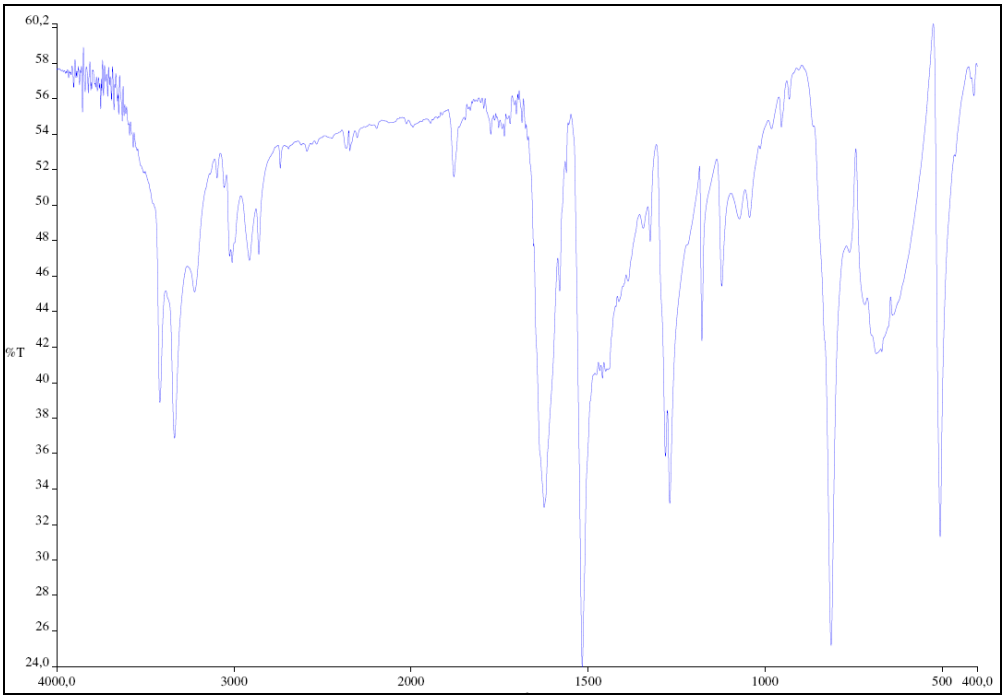

Fig. SM 1.1.8. IR spectrum of *p*-toluidine (in KBr disk)

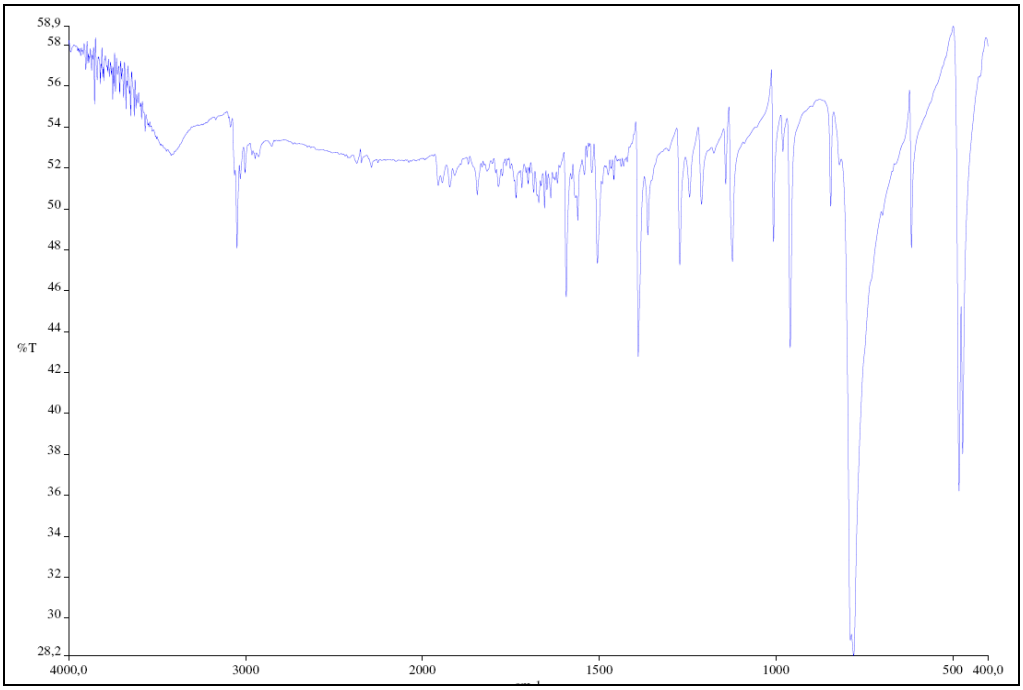

Fig. SM 1.1.9. IR spectrum of naphthalene (in KBr disk)

Isolation of (+)-Limonene from orange oil

Supplementary Material

The extraction of (+)-Limonene from orange oil is a popular experiment done in our laboratory with students from the first years of the graduation in Chemistry and Chemical Engineering, but also by students from other degrees. It is used to exemplify the use of the common techniques of steam distillation, vacuum fractional distillation and liquid-liquid extraction. It is also useful for introducing the students on the usual methods to assess the purity of a liquid product by comparing the refraction index, boiling point and specific rotation of the product with the literature values. It is a good example to introduce the study of chirality and optical rotation.

The orange oil used in our laboratory classes was a kind gift from SUMOL+COMPAL Company (previously SUMOLIS). According to the literature, it can be also extracted from orange peel with an average yield of 0.3%.[1] However, when we performed the extraction the yield was so low that was unpractical for students. Figure 1 shows a steam distillation apparatus.

Figure SM 1.2.1: Steam distillation of orange oil.

In the absence of material or conditions to perform the steam distillation, an alternative method (Method B) is suggested where (+)-Limonene can be isolated only by fractional distillation of orange oil. This affords the product with a level of purity very similar to the one obtained from Method A. The average yield of both methods is 70-80%. The refraction index obtained is normally in the range 1.474-1.476, and the specific rotation in the range +111º to +125º.

The boiling point of pure Limonene obeys the formula $\log P = - 2296/T_{eb} + 8.0163$, where P is the pressure in mmHg and T_{eb} the boiling point in K.[2] Note the use of common logarithms (base 10). This formula can be used to assess the purity of the product distilled, together with the refraction index (n_D^{20}=1.4730) and specific rotation ($[a]_D^{20}$=+125.6º) of pure limonene.

[1] O. S. Rothenberger, S. B. Krasnof and R. B. Rollins, *J. Chem. Ed.* 1980, **57**, 741.
[2] R. Weast, CRC Handbook of Chemistry and Physics, 1st Student Ed. **1988** Florida.

Supplementary information for *Comprehensive Organic Chemistry Experiments for the Laboratory Classroom*

Isolation of plant pigments from green and red leaves

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Supplementary Material

1. Introduction

The traditional separation of plant pigments from extracts of green leaves (usually spinaches) by column chromatography has been used, during the last twenty years, in the practical classes of Organic Chemistry at the University o Minho (Braga). The separation of yellow and green bands into silica columns has delighted the 1st year students of Chemistry, Biology, Biochemistry degrees, and also by Biological Engineer students. This experiment is part of a set of activities, usually including recrystallization, extraction and distillation experiments, designed to introduce the principles and practice of purification and separation techniques in Organic Chemistry. At the end of each semester, all these students were asked what experiment they enjoyed most. Every year, the chromatography of plant pigments was the most often identified experiment. Aiming to improve the attractiveness and the learning methods provided by this experiment, a novel experiment was developed replacing the green leaves by green and red leaves (A. M. Dias, M. L. Ferreira, *J. Chem. Educ.,* 2015, 92, 189–192). A more colourful and challenging column resulted incorporating the hydrophilic anthocyanins. An additional red band can be visualized on the top of the column that can be successfully eluted by the use of fairly polar organic solvents. Interest in green chemistry principles led to the search for safer and inexpensive adsorbents leading to selection of potato starch as alternative adsorbent to the more hazardous silica stationary phase.

2. Additional Notes on the extract preparation

The most experimentally challenging aspect of the separation of carotenoids, chlorophylls and flavonoids by column chromatography, in a unique and green experiment, is the preparation of a suitable extract from green and red leaves.

Diverse red- or purple-leaf plants are suitable sources of both anthocyanins and chlorophylls. Plants with multi-coloured leaves may be used, provided that they have considerable amounts of red colour. We selected the *Stromanthe sanguinea* species (Fig. SM 1.3.1a), because their leaves have a green upper face and the lower face is dark red.

Maceration of the well-crushed (Fig. SM 1.3.1b) leaves with acetone gives good results. Acetone has the intermediate polarity required to extract both hydrophilic and hydrophobic pigments from green and red leaves. Direct application of this crude extract onto the column is not convenient, due to the presence of acetone mixed with residual water released from the vegetal tissues. The presence of these polar solvents in the extract will dramatically increase the polarity of petroleum ether, the first solvent of the eluent series, preventing the separation of the nonpolar carotenoids from the more polar chlorophylls. Traditional green leaf extracts are usually obtained by elimination of water and acetone from the crude extract by liquid-liquid extraction in a water/petroleum ether system. In this case, this method is impracticable due to the consequent elimination of the hydrophilic anthocyanins in the aqueous layer. At this stage, students must realize that the pigment molecules show distinct physical and chemical properties, like colour, solubility and acid-base character. Even so, they may be challenged to find solutions to remove the water from the extract maintaining all the pigments together.

(a) (b) (c)

Figure SM 1.3.1 – (a) *Stromanthre sanguinea* leaves; (b) Crushed leaves; (c) Crude extract under anhydrous sodium sulphate.

The elimination of water can be accomplished by treatment of the crude extract with a desiccating agent, such as kitchen salt or anhydrous sodium sulphate (Fig. SM 1.3.1c). The solid may be easily removed from the extract by gravity filtration. It is very important to use sufficient amount of desiccating agent, because the column may be clogged if a significant amount of water enters the packing of the column. Thus, an efficient desiccating is essential. During this period, the students must analyse the extract composition by paper chromatography (Fig. SM 1.3.2).

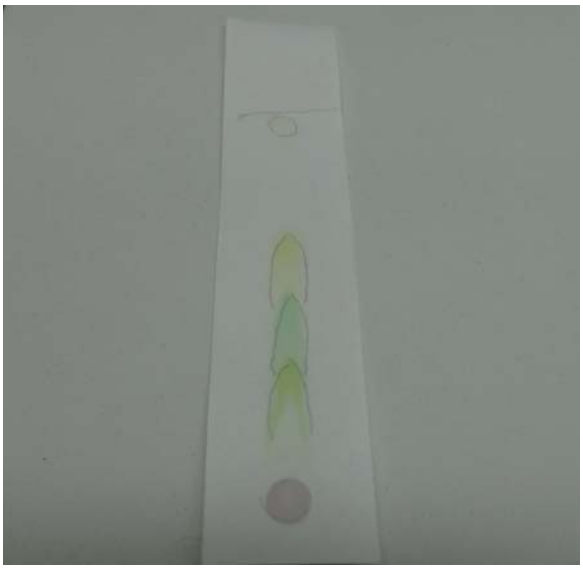


Figure SM 1.3.2 – Paper chromatography (petroleum ether 40 - 60 °C / acetone 90:10) liquid extract (Fig SM 1.3.1c) with the following R_f values:

β-carotene, R_f=0.87; xanthophyll, R_f=0.87; chlorophyll *a*, R_f=0.87; chlorophyll *b*, R_f=0.87; anthocyanins, R_f=1.0.

The pigments can be identified on the basis of characteristic colours and relative affinities to mobile and stationary phases. The results can be interpreted and discussed relating molecular structures with polarity, intermolecular bonds, adsorption and solubility concepts. Principles of green chemistry should be also introduced at this time.

At this stage, the students must understand the principles of chromatography and should be stimulated to envisage the elution process into the column chromatography, using the concept of an eluotropic series. They should realize that the use of nonpolar eluents is required to obtain elution of the nonpolar beta-carotene and, eventually, the slightly more polar xanthophylls. This will help to understand the need to remove the acetone from the crude extract. Students may be encouraged to propose alternative solvents to transfer the mixture of the three classes of pigments onto the column. The concept of solid solvent must be introduced/remembered. Since cellulose

afforded a good separation of pigments (paper chromatography), the use of the closely related starches is a good alternative as they are good adsorbents from our daily life and agree with principles of green chemistry. The use of the alternative corn starch is not recommended in this experiment, it led the column packing to clog.

After this discussion period, students can be instructed to prepare the solid extract by adsorption of the liquid extract on the surface of potato starch (Fig. SM 1.3.3).

Figure SM 1.3.3 – Solid extract obtained by adsorption of the liquid extract (Fig SM 1.3.1c) on the surface of potato starch.

They will observe a fast evaporation of the acetone by stirring the liquid with the finely divided solid. After 10 min the mixture is free of acetone leading to recognize that further procedures to evaporate acetone are not be necessary. The effect of the surface area on the rate of evaporation should be mentioned, making clear that this procedure avoids less sustainable heating or vacuum processes and prevents pigment degradation. Moreover, the hydration capacity of starches may be useful in retain traces of moisture on the surface of starch.

3. Additional notes on the column packing

Disposable syringes of 10 mL or 20 mL are selected to support the packing of the column, because they are inexpensive items that may be freely reused avoiding the risks associated with glass materials. The use of thinner columns is not advisable, because the percolation rate through the column is too slow due to the small particle size of starch granules. The column packing is the most variable factor of this experiment and may cause, sometimes, lack in the reproducibility. The use of

20 mL syringe gives more reproducible results. The use of a slurry, made up with potato starch and petroleum ether, also gives more reproducible results, when compared with the dry method. This column experiment was repeated a few dozen times and it can be stated that the reproducibility of this column should not be inferior, when compared with the silica packing of columns. It should also be noted that the normal use of a layer of sand at the top of the column is not recommended, since this would mask the visual effect of the pinkish band due to the anthocyanins on the top of the column. The top of the packing of this column is particularly fragile and the eluents must be added carefully to the column to avoid irregular bands.

After loading the column with petroleum ether, an appropriated amount of the solid extract is carefully applied on the top of the packing (Fig. SM 1.3.4). No more than the recommended amount of solid extract should be used.

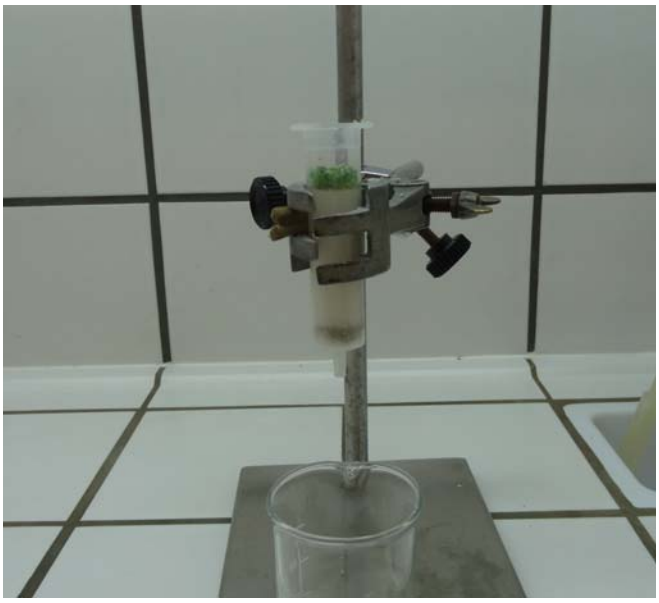


Figure SM 1.3.4 – Potato starch column chromatography immediately before elution (0 min).

4. Additional notes on the column elution

The results of the elution are strongly related to the packing process and the eventual presence of traces of acetone and/or water in the extract. Typically, the elution of the column with petroleum ether 40-60 ºC led to the development of a colourful column within 5-10 min, showing two well-separated yellow bands, two fairly-well defined green bands and one pink band on the top of the column (Fig. 5). The first yellow band was easily isolated with petroleum ether and was composed

only by carotenes. The second yellow band, detected quite high up in the column, is retained during elution with this solvent. Elution with a mixture of petroleum ether/acetone 90:10 afforded a new colour pattern in the column, showing one yellow band at the bottom of the column that is followed by two green bands, one bluish green band and one yellowish green band, with the pink band retained on the top of the column (Fig. SM 1.3.6). Based on their characteristic colours and polarity, these bands could be easily assigned to xanthophylls, chlorophylls *a* and *b*, and anthocyanins, respectively. Thus, in an elution period of 30-45 min, an attractive column could be achieved revealing the major components of the extract obtained from green and red leaves. It should be mentioned that different plant species might lead to different results. However, differences in the content of pigments may be easily predicted by early paper chromatography analysis of the extract. Sometimes, the beta-carotene is too faint to be identified on the column. In this case, elution by petroleum ether should be maintained for 10 min, before changing to the next eluent.

The elution of xanthophylls and chlorophylls from the column takes about 30 min more (Fig. SM 1.3.7 and 1.3.8). By elution with a petroleum ether/acetone mixture 90:10 two fractions were collected and evaluated by paper chromatography: the first one showed an intense yellow colour and was mostly composed of xanthophylls, but was slightly contaminated with chlorophyll *a*; the second fraction, with a bluish green colour, was composed only by chlorophyll *a.* Petroleum ether/acetone mixture 75:25 afforded a yellowish green fraction with a unique spot of chlorophyll *b*. Acetone can be used to elute the flavonoids from the column, which take further 20 min. An acetone wash of the column gave one last crop that blushed red by addition of acid demonstrating the presence of anthocyanins.

We can state that these simple, accessible and safe experimental conditions provide an attractive and efficient separation of pigments from red and green leaves. A colourful column is obtained with yellow, blue-green, yellow-green and red bands. The composition the corresponding extracts (Fig. SM 1.3.9) was evaluated by paper chromatography demonstrating an efficient separation of beta-carotene, xanthophylls, chlorophyll *a* and *b*, and anthocyanins (Fig. SM 1.3.10).

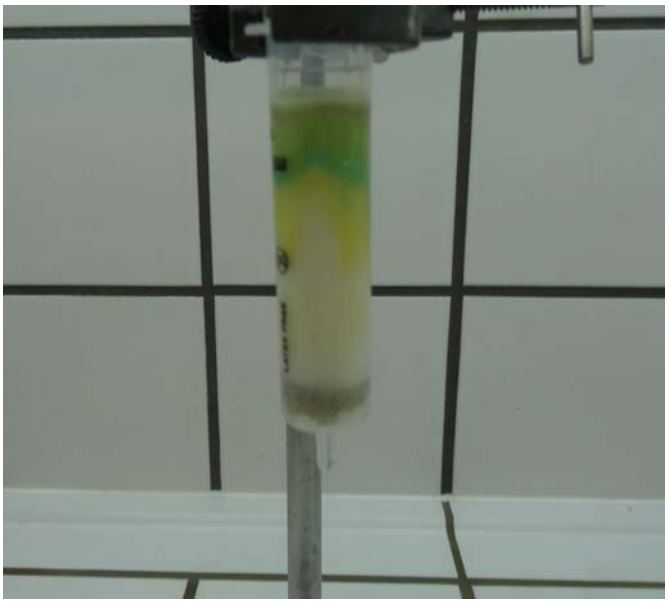


Figure SM 1.3.5 – Potato starch column chromatography by elution with petroleum ether 40-60 °C (5 min).

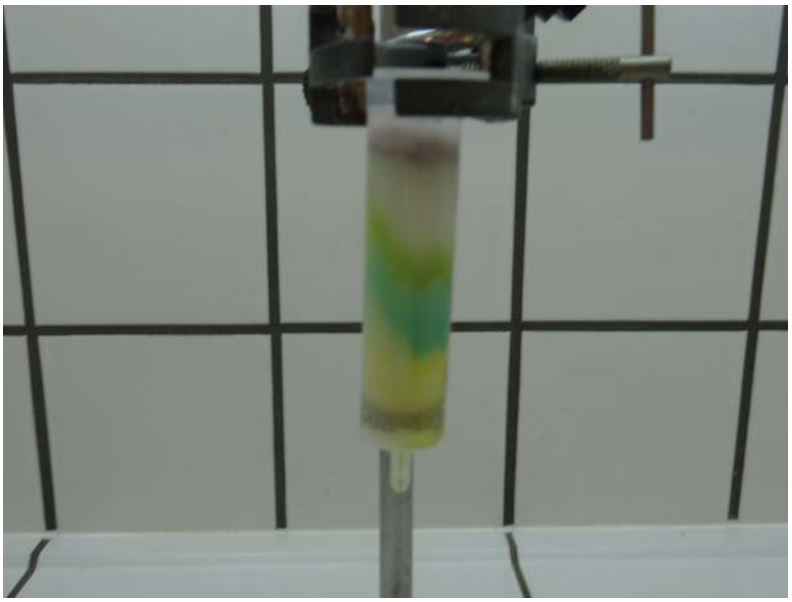


Figure SM 1.3.6 – Potato starch column chromatography by elution with the mixture of petroleum ether 40 - 60 °C / acetone 90:10 (23 min).

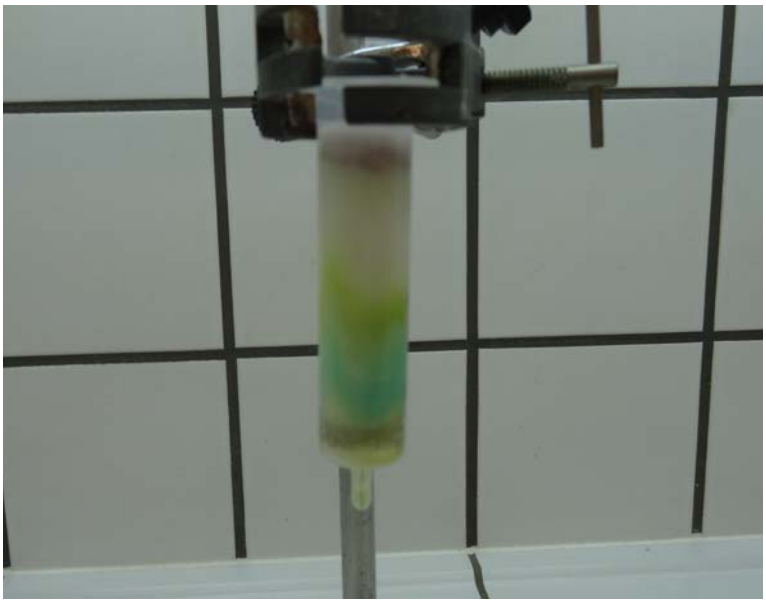


Figure SM 1.3.7 – Potato starch column chromatography by elution with the mixture of petroleum ether 40 - 60 °C / acetone 90:10 (28 min).

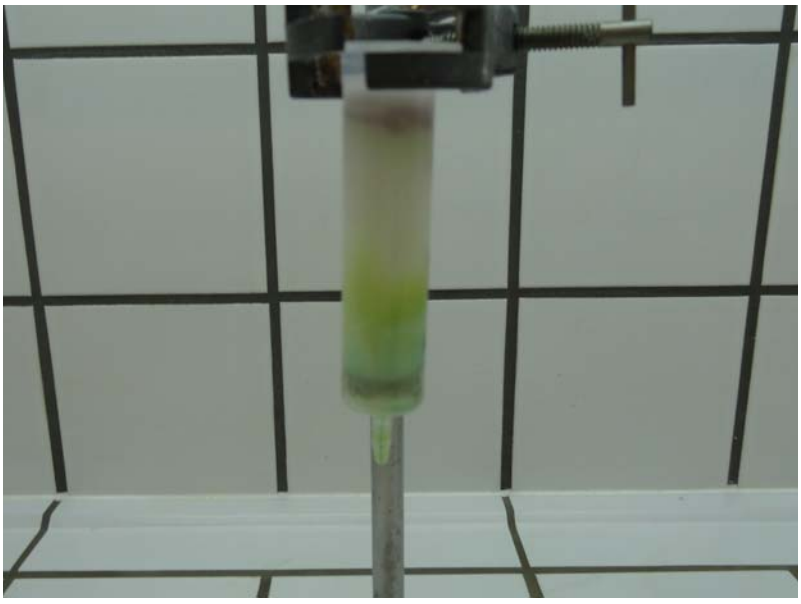


Figure SM 1.3.8 – Potato starch column chromatography by elution with the mixture of petroleum ether 40 - 60 °C / acetone 90:10 (32 min).

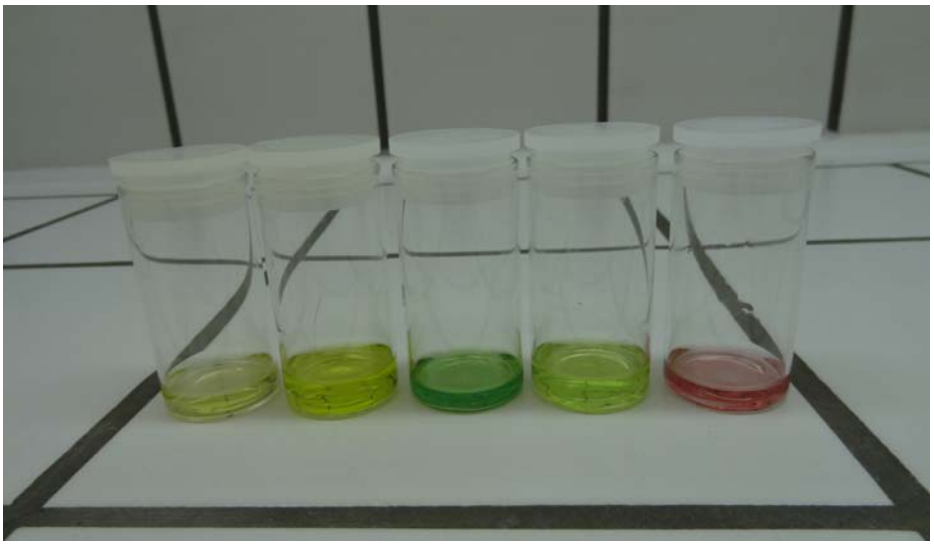


Figure SM 1.3.9 – Main fractions eluted from potato starch column chromatography.

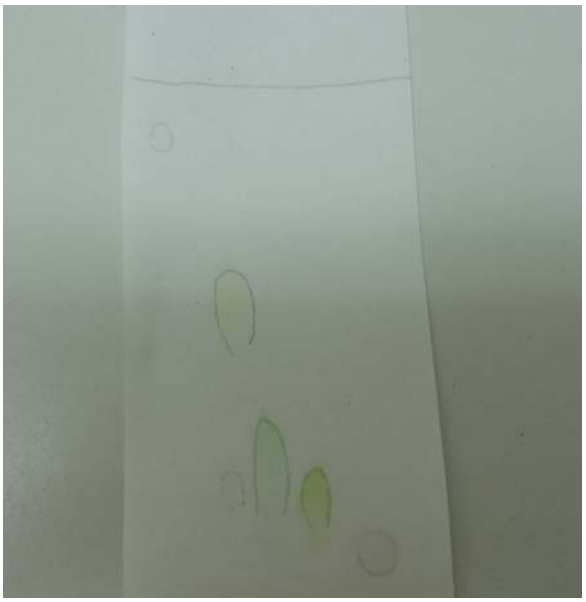


Figure SM 1.3.10 – Paper chromatography (petroleum ether 40 - 60 °C / acetone 90:10) of the main eluted fractions (same order as in Fig. 9).

Extraction of Usnic Acid from Lichen
Supplementary Materials

Students in our teaching laboratories use a PolyScience SR-6 Polarimeter to carry out their measurements. However, the instructions in this experiment have been written more generally so that other polarimeter models can be readily employed by just adding further directions on instrument operation. Each set of measurements (unknown and then the usnic acid) takes 5-10 minutes each. Most of the learner's laboratory time is spent on the bench work extracting, isolating and recrystallizing the usnic acid. It is recommended that at the recrystallization stage that students proceed to measuring the melting point and optical rotation of their unknown compound (*L*-tartaric acid). Table SM 1.4.1 gives a sense of the range of results students obtain in this experiment.

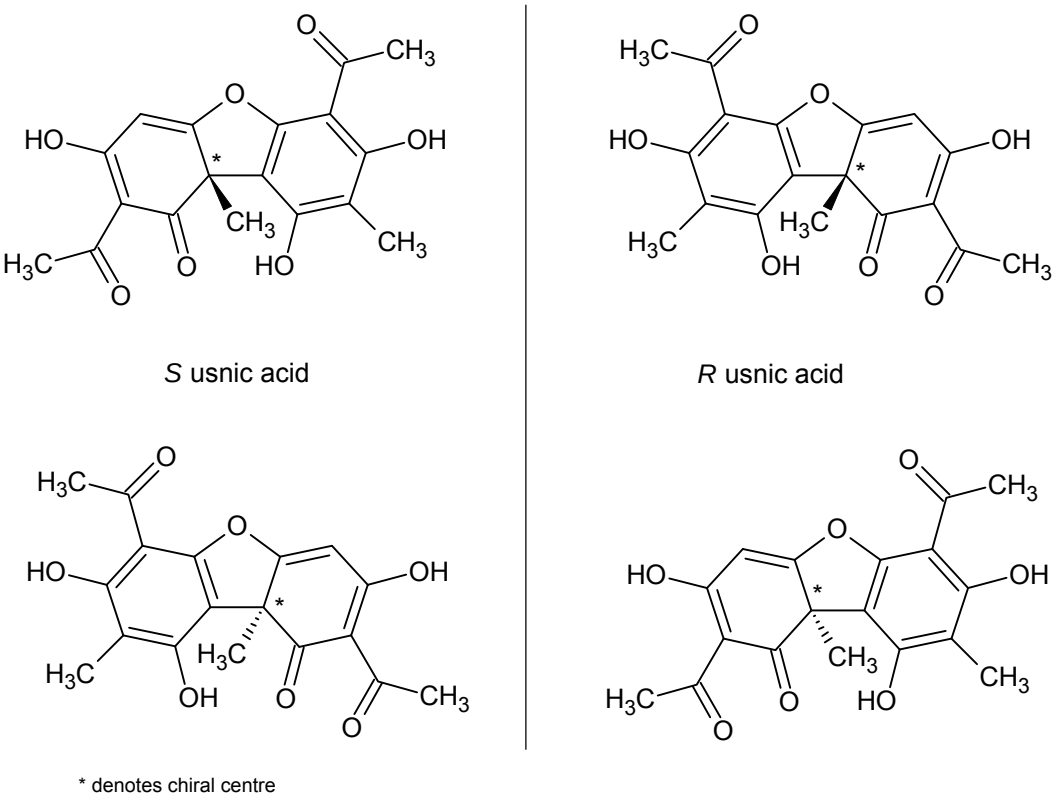


Figure SM 1.4.1 – *R* and *S* Usnic Acid Showing Two Views of Each Enantiomer

Table SM 1.4.1 – Sample Usnic Acid Yields and Polarimetry Results

1st Crop (g)	2nd Crop (g)	mp (° C)	$[\alpha]_D^{\sim 20}$	Yield[a] (% Wt Lichen)
0.5160	–	204-205	+493	4.30
0.2028	0.2545	202-203	+458	3.80
0.3971	–	195-196	+469	3.31
0.3840	–	198-199	+482.6	3.20
0.3766	–	191-192	+500	3.14
0.3626	–	200-202	+370	3.02
0.3480	–	203-204	+487	2.90
0.3380	–	191-192	+428.3	2.82
0.5491[b]	–	195-196	+457.2	2.77
0.3264	–	193-194	+484.4	2.72
0.3180	–	199-200	+466	2.65
0.3082	–	195-196	+482	2.57
0.3067	–	195-196	+481	2.56
0.2477	–	200-202	+473	2.06
0.2436	–	205-206	+453.1	2.30
0.2160	–	202-204	+473	1.80
0.0559[c]	0.0186[c]	201-202	+469.8	1.57
0.1339	–	197-199	+444.1	1.11
0.1080	–	204-206	+440	0.90
0.0541	–	195-197	+352	0.45

a) Percent pure usnic acid by weight from total biomass of the lichen. **b)** Approximately 20 g lichen used. **c)** 4.75 g lichen used.

Table SM 1.4.2 – Pertinent Physical Values

Sample	mp (° C)	Sample Concentration (g/mL)	$[\alpha]_D^{\sim 20}$ (°)
Water (blank)	0	neat	+0.01
L-(+)-Tartaric acid (unknown)	171-174	0.16-0.20	+12.7
THF (blank)	−108.5	neat	+0.01
R-(+)-Usnic acid	201-203	~ 4×10^{-3}	+464[a]

a) Student average. Literature values $[\alpha]_D^{\sim 20}$ = +466° (*c* = 0.02, THF)[1] and $[\alpha]_D^{\sim 20}$ = +469° (*c* = 0.7, $CHCl_3$)[2]

Sample Calculations:

Yield

Recovered 0.4789 g of pure usnic acid from 12.123 g lichen

$$\%\ \text{yield by weight} = \frac{mass\ product}{mass\ of\ starting\ material} \times 100$$

$$= \frac{0.4789\ \text{g}}{12.123\ \text{g}} \times 100 = 3.95\ \%$$

Observed Rotation

Polarimeter reading of neat distilled water was +0.02 and the aqueous unknown sample was +2.47.

$$\alpha = \alpha(\text{solution}) - \alpha(\text{blank}) = (+2.47) - (+0.02) = +2.45$$

Specific Rotation

The observed rotation is +3.79º for a 0.00402 g mL^{-1} solution in a polarimeter employing a sample cell that is 200 mm long.

$$[\alpha]_D^{20} = \frac{\alpha}{L \times c} = \frac{+3.79°}{2\ \mathrm{dm} \times 0.00402\ \mathrm{g/mL}} = +471°$$

[Formally the units for specific rotation are deg mL g^{-1} dm^{-1}, but it is normally reported just in units of degrees.]

[1] D. W. Mayo, R. M. Pike, and D. C. Forbes, *Microscale Organic Laboratory: With Multistep and Multiscale Syntheses*, John Wiley & Sons, Toronto, 5th ed., 2010, Instructor's Notes for MOL, Chapter 6, 13.
[2] *CRC Handbook of Chemistry and Physics*, ed. R. C. Weast, CRC Press, Boca Raton, 67th ed., 1986, C-541.

Thin layer chromatography of plants pigments

Supplementary Material

Experiment notes

The main goal of this experiment is the extraction, separation and identification of the major pigments of spinaches. This experiment is much appreciated by students because they easily recognized the raw materials and the pigments involved. The experimental work was projected to be developed in one three hour session and can be carried out with several vegetable samples (fresh, frozen or canned spinaches, carrots, kale etc.). Depending on the vegetable sample (fresh or frozen), conditions of the experiment and amount of sample spotted on the TLC plate, the TLC pattern can change. It is very common to observe other pigments on samples of frozen spinaches resulting from air oxidation, hydrolysis and other chemical reactions as mentioned in the “Background”.

In spite of being a quite simple experiment and easy to perform, several precautions must be taken to have good pigments extraction yields.

- If frozen samples are used, the vegetable must be squeezed to eliminate as much freezing water as possible.
- If necessary, a small amount of sea sand can be added to the mortar to help the grinding and extraction process.

- During the grinding of the spinaches with acetone, usually a major quantity of solvent will evaporate. At the end of step 4 (Experimental procedure) usually more acetone is need to be added to compensate the evaporation.
- The acetone extract withdrawal may cause some problems. A pipette with a small piece of cotton swirled at the end (see Fig SM 1.5.3) might be useful.
- Since no dry agent is added during the grinding process, after the addition of the *n*-hexane and shaking, a lower water phase can be formed and a three phase system can appear. This phenomenon does not interfere with the experiment success.
- For TLC, use flexible precoated silica gel F_{254} plates with fluorescent indicator. If the plates have not been purchased recently, place them in an oven at 100 ºC for 30 min.

This experiment has been assayed by four groups of two students from the 2nd year of the Integrated Masters Degree in Pharmaceutical Sciences (Faculty of Pharmacy, University of Lisbon). All groups were able to reach the end of the experiment with a very nice and well developed TLC plate, similar to the one presented in figure SM 1.5.7.

Photos of the experiment

Fig. SM 1.5.1. Material and reagents.

Fig. SM 1.5.2. Outlook of the solid-liquid extraction.

Fig.SM 1.5.3. Pipette adapted for acetone withdrawal.

Fig.SM 1.5.4. Outlook of the liquid-liquid extraction.

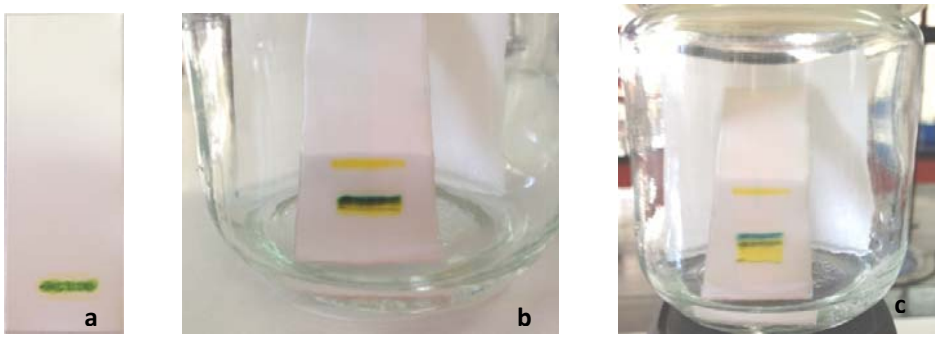


Fig.SM 1.5.5. Different stages of TLC experiment: (a) sampling; (b) at the beginning; (c) after a few minutes;

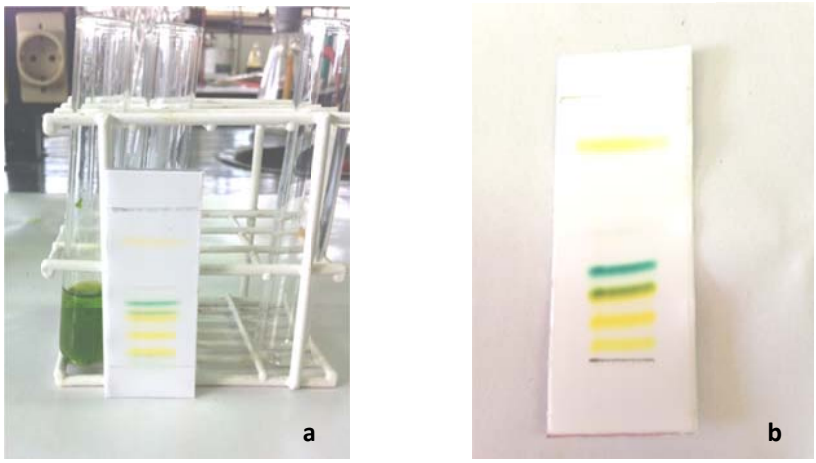


Fig. SM 1.5.6. TLC outlook. (a) frozen leaves; (b) fresh leaves.

TLC interpretation

In the spinach leaves crude extract the following pigments can be present:

β-Carotene	1 spot; yellow orange; >>>Rf
Pheophytin **a**	1 spot; gray
Pheophytin **b**	1 spot; gray (may be absent)
Chlorophyll **a**	1 spot; light-green
Chlorophyll **b**	1 spot; green
Xanthophylls	3 possible spots; yellow; <<<Rf

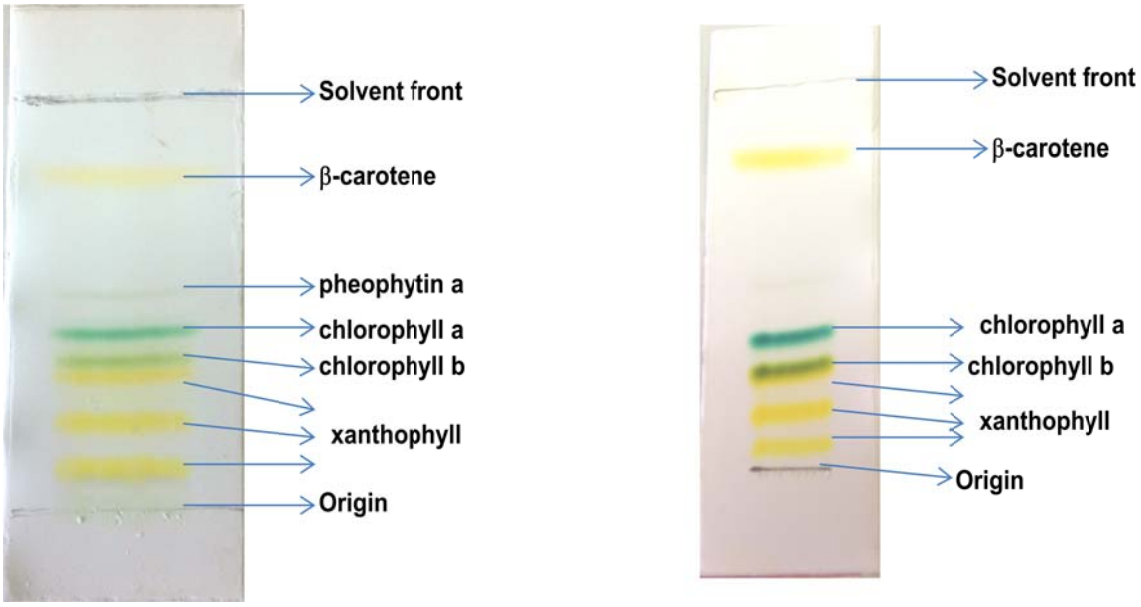


Fig. SM 1.5.7. TLC of frozen (a) and fresh (b) spinach leaves.

Isolation of cinnamaldehyde from cinnamon

Supplementary Material

This experiment was performed by several students with different backgrounds. Last year the experiment was reproduced by 140 students (two students per group) of Introductory Organic Chemistry Laboratories. It is an elementary work that will allow the student to consolidate different separation processes namely steam distillation, extraction with *soxhlet* and liquid-liquid extraction as well as to elucidate structurally the isolated compounds by infrared (IR) and ^{1}H NMR. Additionally, this experiment will allow the students to associate basic organic chemistry operations with nature as an important source of organic molecules.

The students used one individual commercial powdered (14 g/each) for the steam distillation. They obtained 0.15-0.27 (1.1-1.9 %(w/w)) of cinnamaldehyde in high purity grade as can be seen in the attached ^{1}H NMR spectra. Since the students had easily access to the refractive index (n_D) facility, they obtained n_D = 1.6060-1.6108 (reported 1.6195). In case of last 140 students execution, since the extraction with *soxhlet* requires more glassware, one extraction set-up was installed by the teacher while the students were performing the steam distillation procedure. The product obtained by extraction with *soxhlet* is much less pure including the presence of some solid residue which is due to the extraction of other less polar natural products. In case of steam distillation those impurities are not present due their lack of volatility. The extraction can also be performed using petroleum ether instead of dichloromethane which allows a more selective extraction however in less considerable yields. The fact that the obtained isolated yield for both processes is usually low (inferior to 2%) highlights the inherent problems associated with isolation of natural products.

In Figures SM 1.6.1-1.6.4 it is presented the infrared (IR) and ^{1}H NMR spectra obtained for cinnamaldehyde isolated by steam distillation and the IR obtained by extraction with *soxhlet*. The typical chemical shifts and IR bands for conjugated aldehyde can be clearly assigned. In addition, the students can interpret the observed splitting for the alpha carbonyl proton which shows the observed coupling with the aldehyde proton and the *trans* coupling with the other olefinic proton. In Figure 5 is presented photos of the used apparatus for both extraction procedures.

The question 4 on the air stability of the cinnamaldehyde and the correspondent reduced product (cinnamic alcohol) allows the students to consolidate the reactivity of functional groups, namely alcohol *vs* aldehyde, in which the aldehyde is much prone to oxidation by oxygen present in the air at room temperature than the corresponding alcohol.

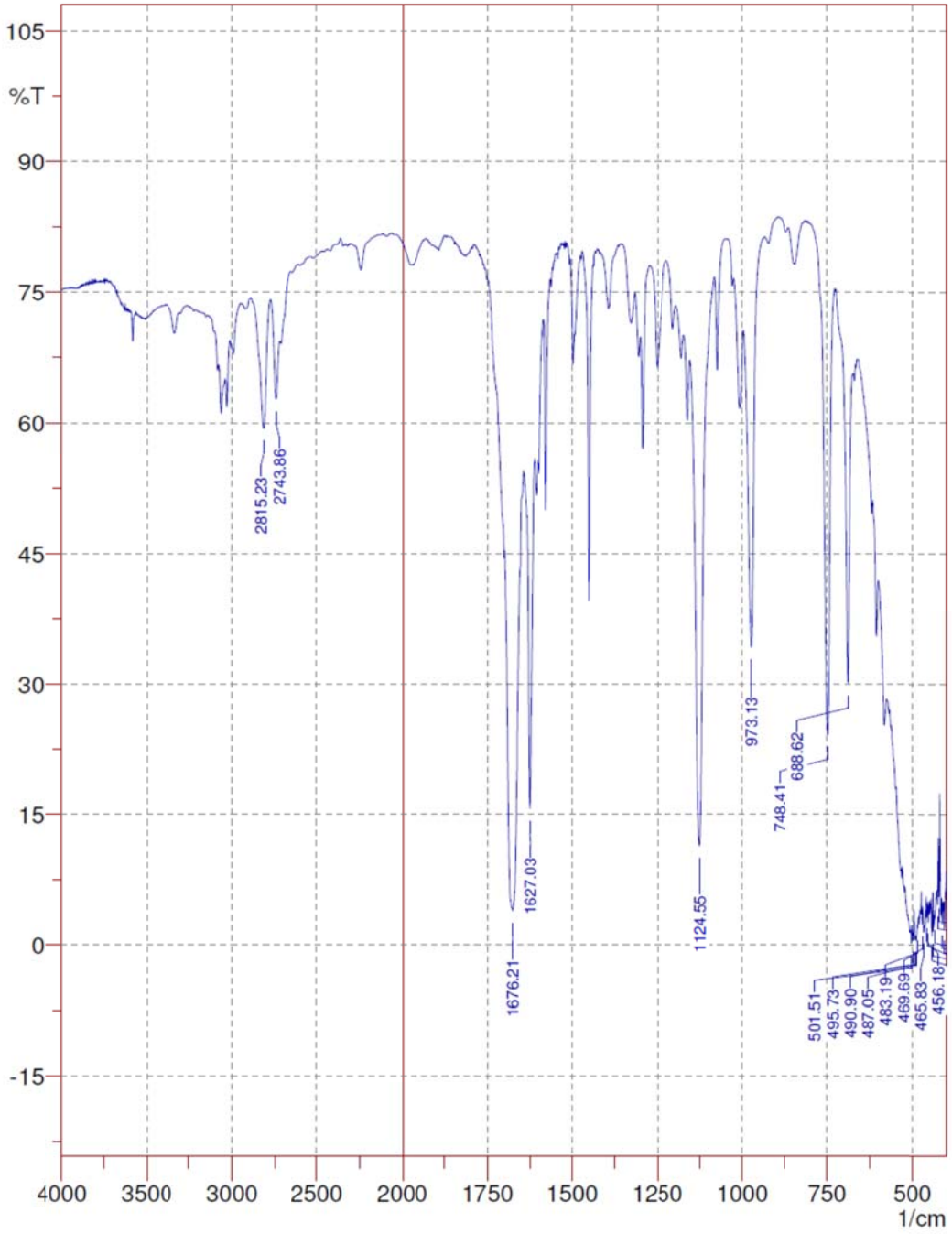


Figure SM 1.6.1. Obtained IR spectra of the *trans*-cinnamaldehyde (film) from commercial sample.

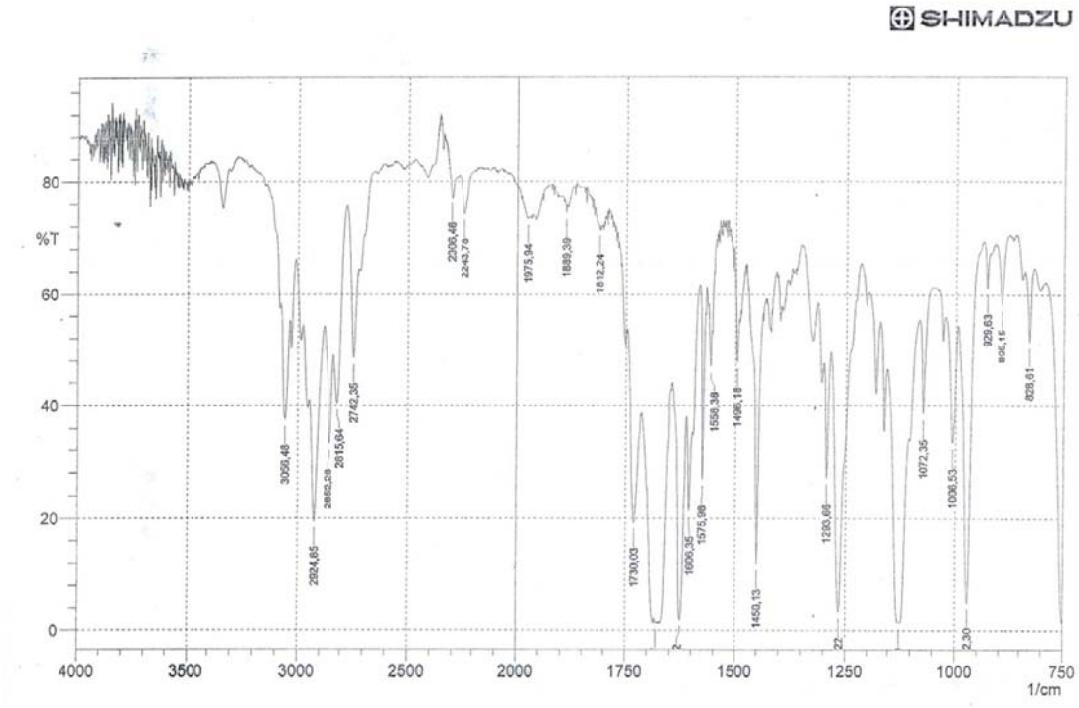


Figure SM 1.6.2. IR spectra (film) obtained from the isolated product resultant from the steam distillation.

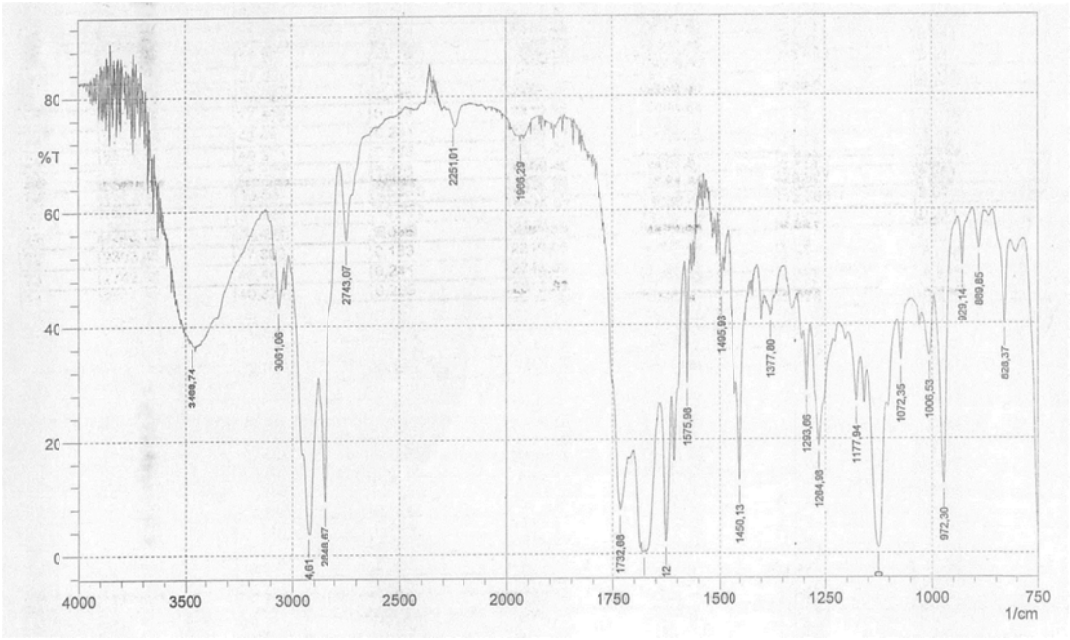


Figure SM 1.6.3. IR spectra (film) obtained from the isolated product resultant from extraction with *soxhlet*.

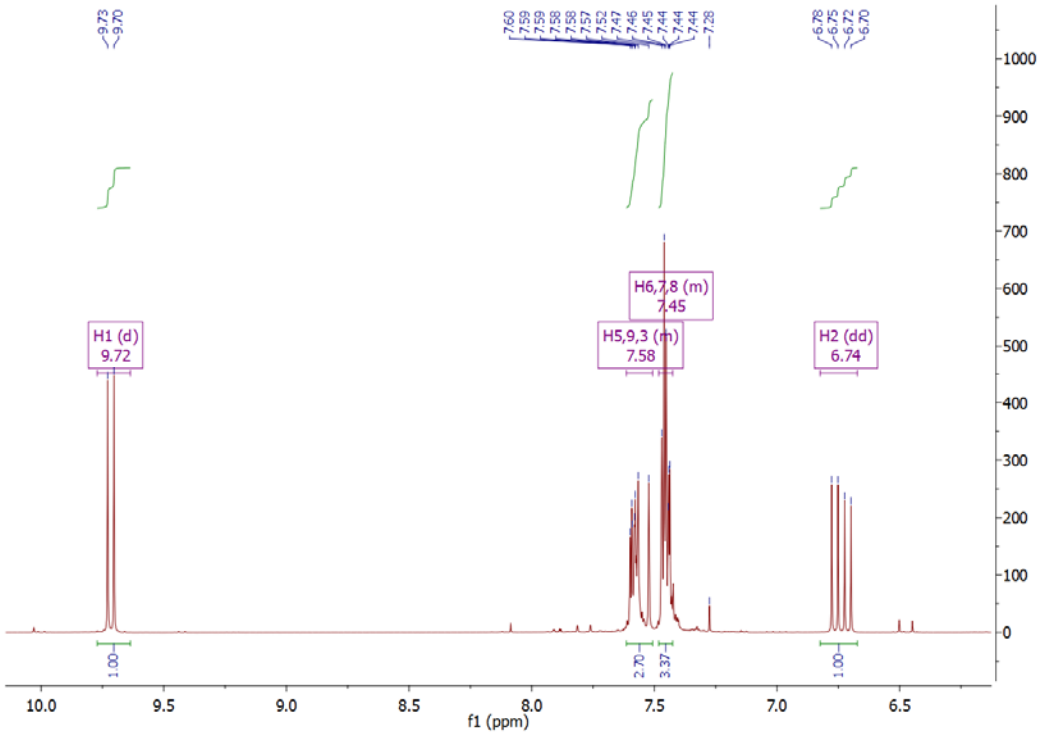


Figure SM 1.6.4. Described ^{1}H NMR ($CDCl_3$) spectra of the *trans*-cinnamaldehyde.

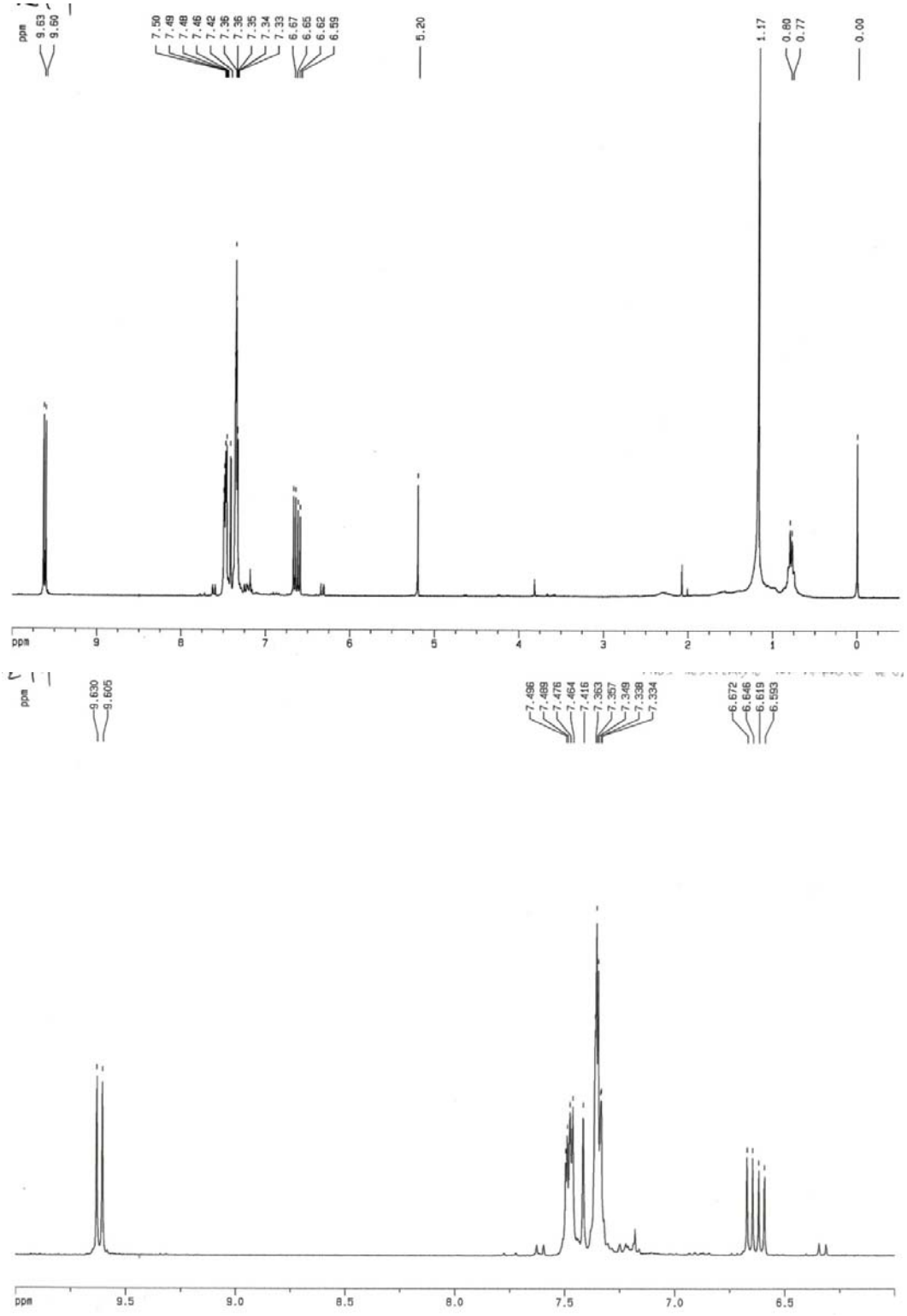


Figure SM 1.6.5. ^{1}H NMR ($CDCl_3$) spectra obtained from the isolated product resultant from the steam distillation.

Figure 1.6.6. Photos of the steam distillation (left) *soxhlet* extraction (right) apparatus.

Isolation and structural identification of piperine, the major alkaloid of black pepper

Supplementary material

Experimental notes

This experiment aims to isolate and identify piperine, the major alkaloid from black pepper. It is a good way to introduce natural products chemistry to students. It uses several basic organic chemistry techniques like extraction (solid-liquid), reflux, recrystallization and thin-layer chromatography. Furthermore, the piperine structure is a good example to practice structural identification through the analysis of IR, UV, MS, ^{1}H and ^{13}C NMR spectra. The experimental procedure is easy and it can be carried out in one session (3/4h).

The amount of piperine present in commercial black pepper depends on the plant species, the plant origin and the manipulation and storage practices of suppliers. For this reason, isolated student yields may vary.

To perform this experiment safely and successfully, follow the precautions below.

- Dicloromethane is a very volatile solvent; therefore reflux and solvent evaporation should be performed carefully, with tight control of bath temperatures and pressure of the rotary evaporator;
- During reflux, the extraction volume should be controlled to assure the minimum quantity of dicloromethane necessary to the success of this step;
- The pepper ground after initial filtration should be washed several times to improve experimental yield;
- Ether manipulation requires additional safety. It is extremely flammable, especially in the presence of enriched oxygen mixtures, so its manipulation should be performed in a fume hood, away from the heating plates and another heat sources;
- Recrystalization of piperine should be performed using a minimum volume of solvent. Major quantities of acetone/hexane solution will not allow crystals formation.

Photos of the experiment

Figure SM 1.7.1. Ground black pepper, 50-mL round-bottom flask, magnetic stir and 10 mL of dichloromethane.

Figure SM 1.7.2. Reflux apparatus: water condenser attached to the top of the flask. Reflux temperature around 50ºC for 20 minutes.

Figure SM 1.7.3. *Vacuum* filtration of pepper ground.

Figure SM 1.7.4. Solvent removal using a rotary evaporator.

Figure SM 1.7.5. Piperine precipitation, using cold ether in an ice bath.

Figure SM 1.7.6. Crystals formed after recrystallization, (melting point 128-129º C).

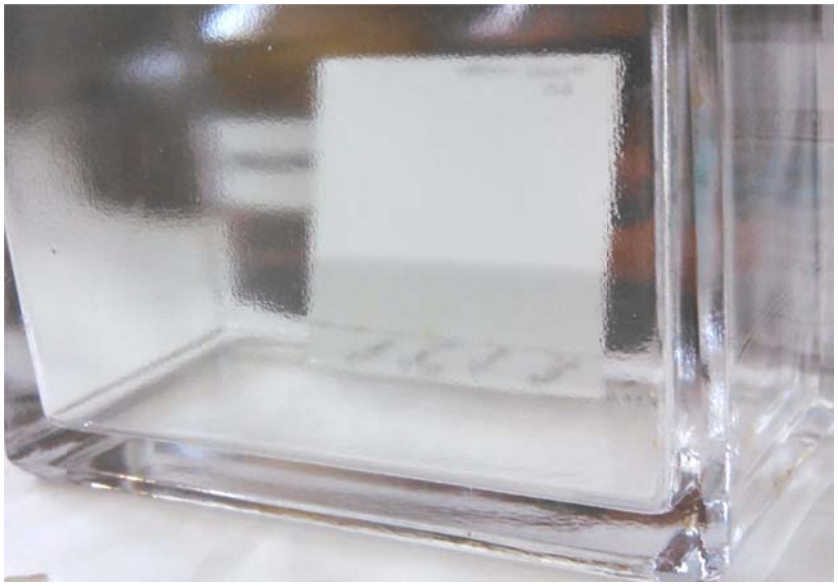


Figure SM 1.7.7. Thin-layer chromatography, using a silica-gel plate and a hexane/ethyl acetate (1:1) solution as mobile phase.

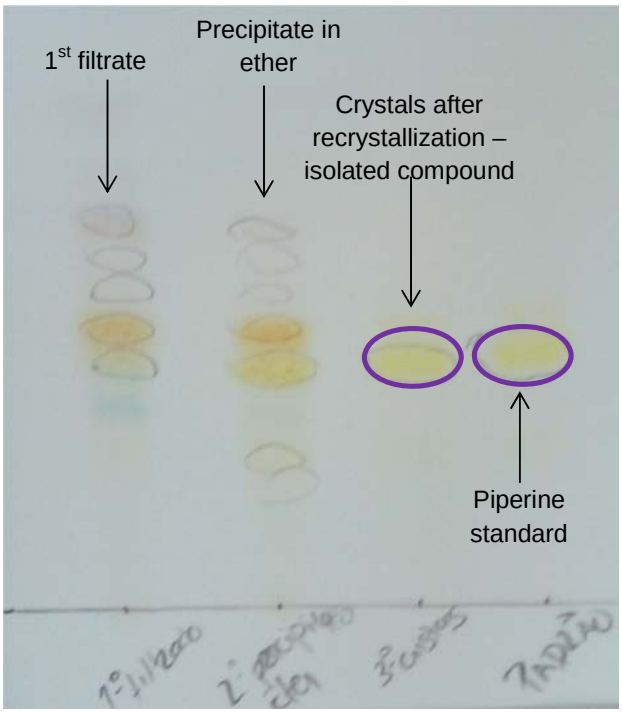


Figure SM 1.7.8. Silica-gel plates sprayed with an H_2SO_4/MeOH 1:1 solution, followed by heating. Piperine has a retention factor (R_f) value of 0.48.

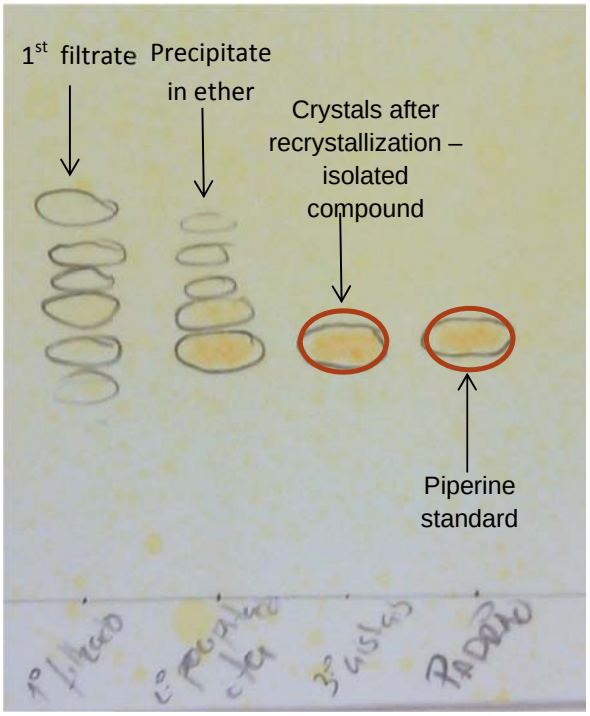


Figure SM 1.7.9. Silica-gel plate sprayed with Dragendorff's reagent, (alkaloids appear as orange spots). Piperine has a R_f value of 0.48.

Spectra

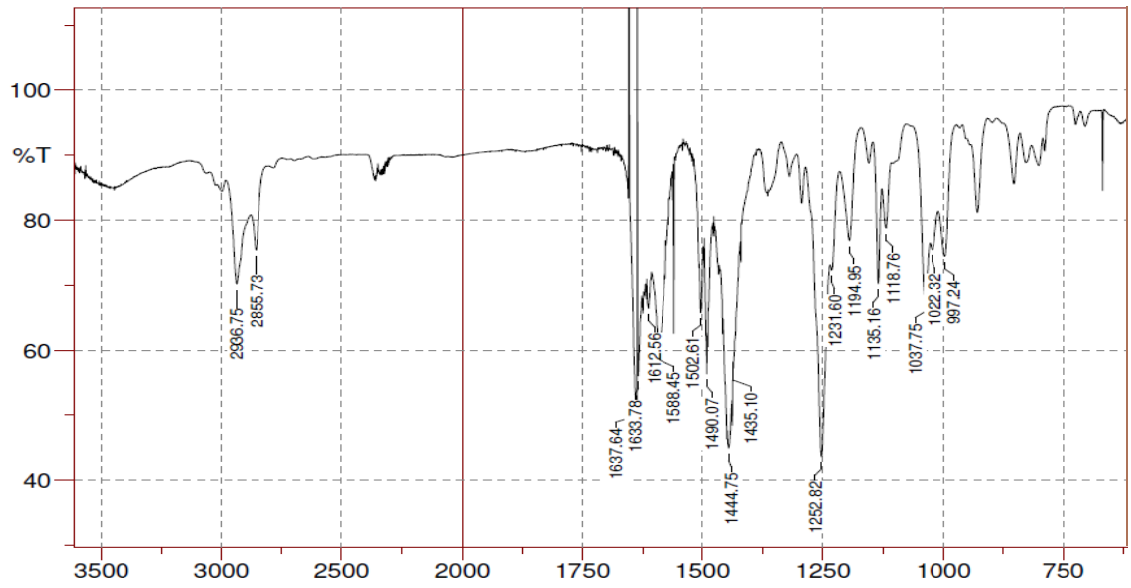
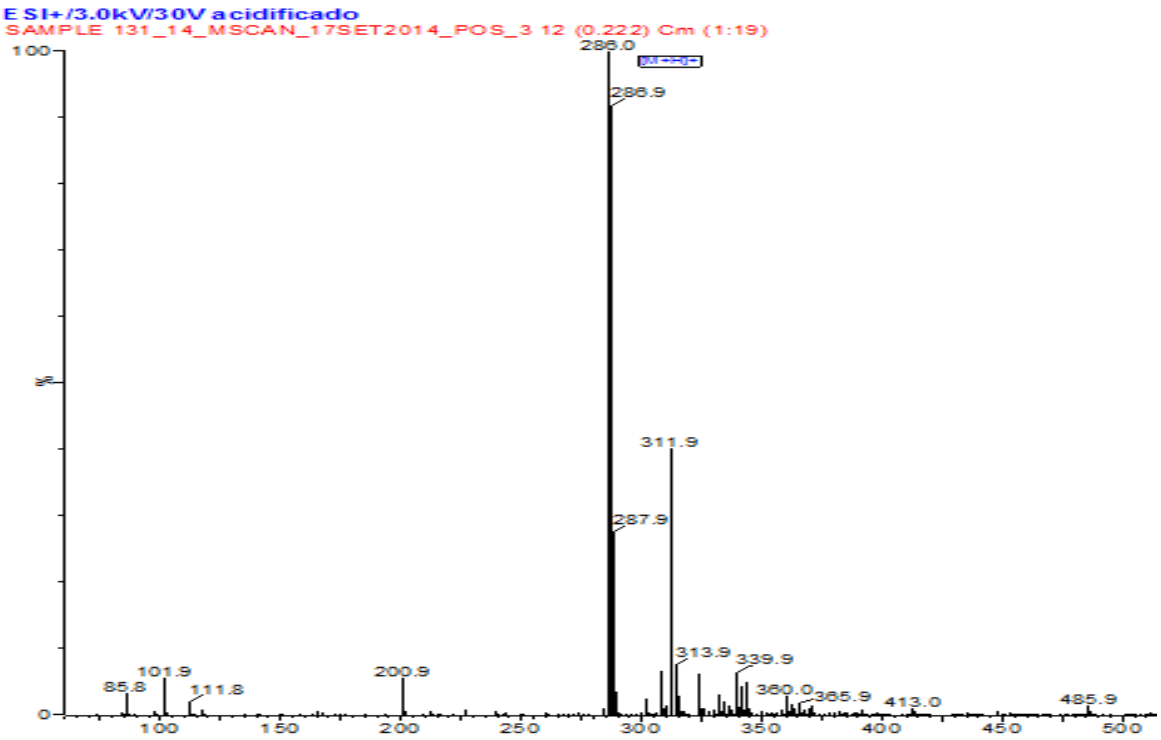


Figure SM 1.7.10. Infrared spectrum of piperine.

-- PEAK --	
λ	ABS
345.0	1.337
253.5	0.387
243.5	0.385
205.0	0.425



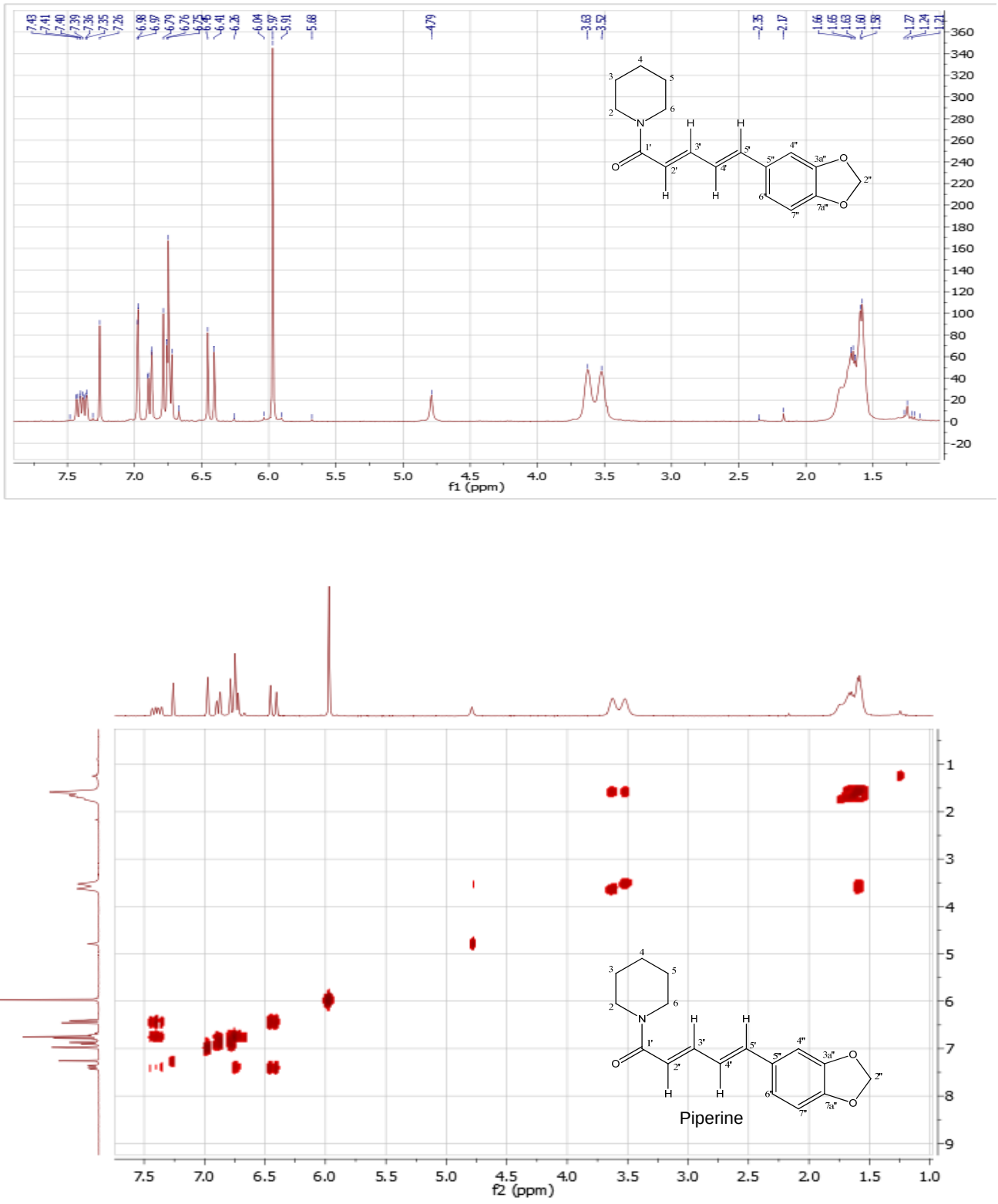
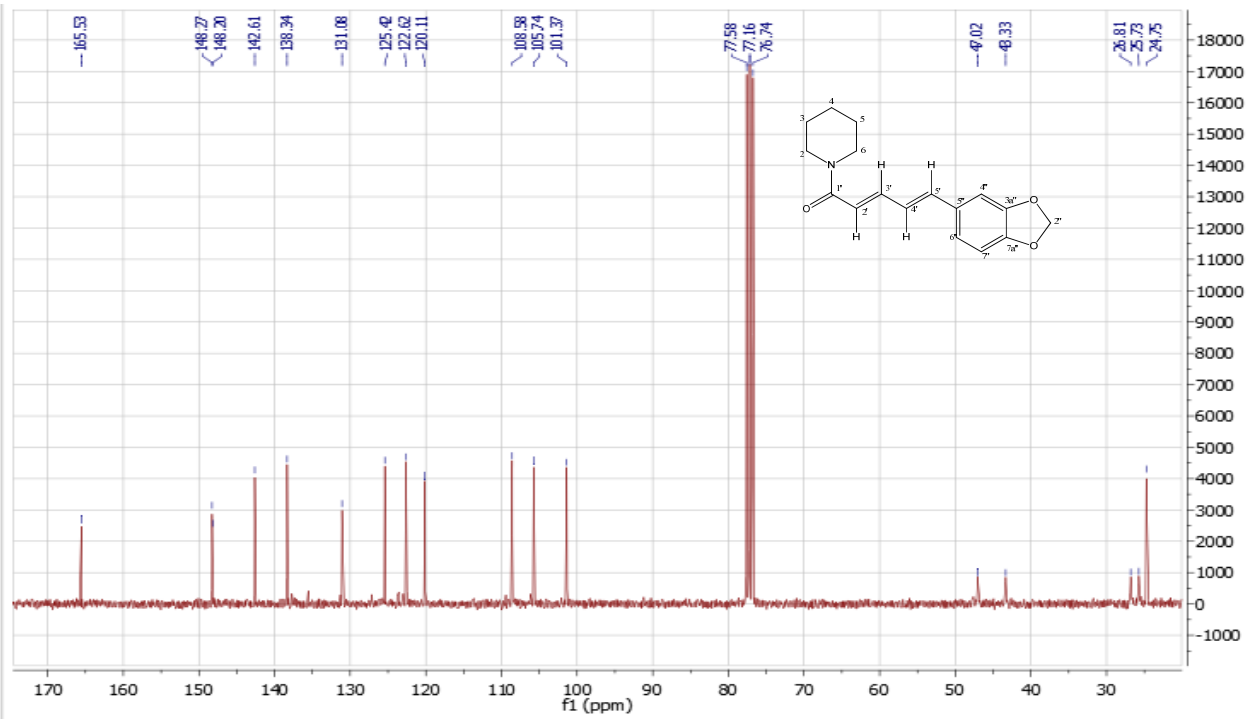
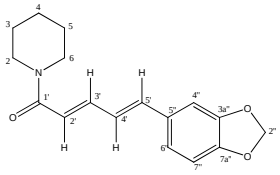


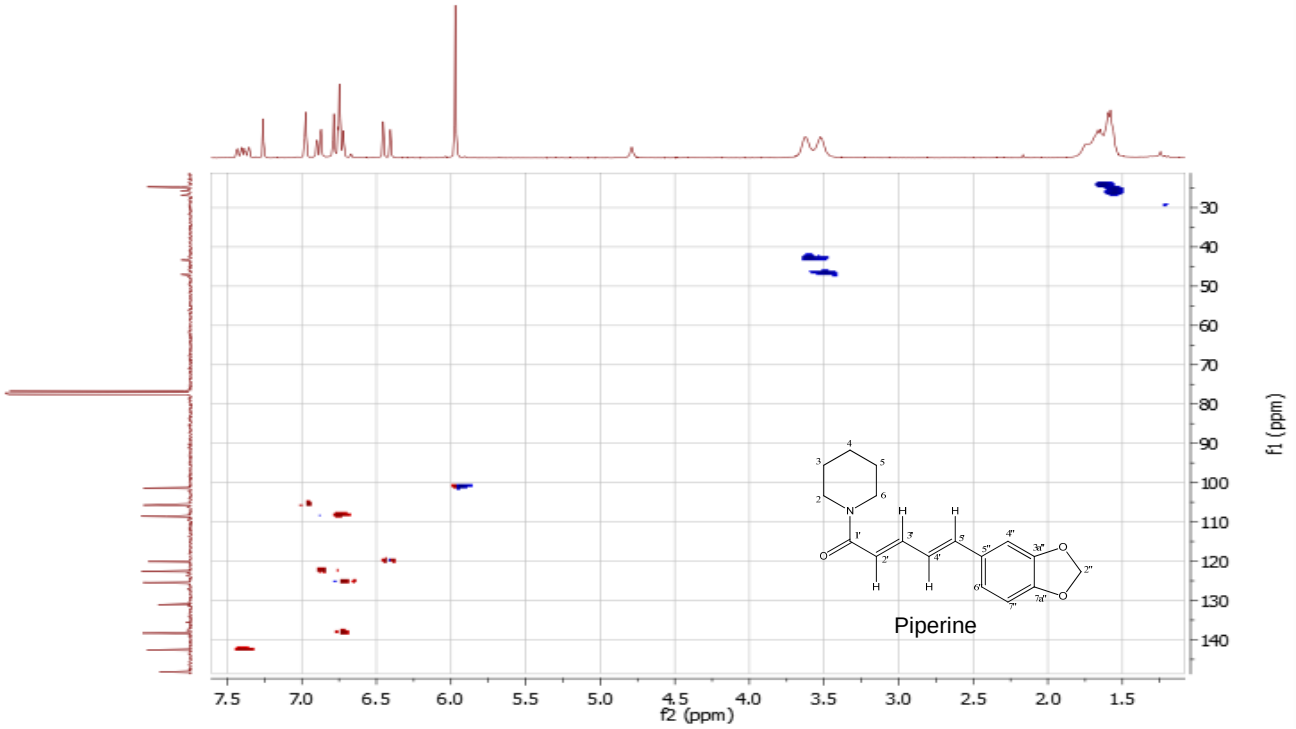
Figure SM 1.7.14. COSY spectrum of piperine.

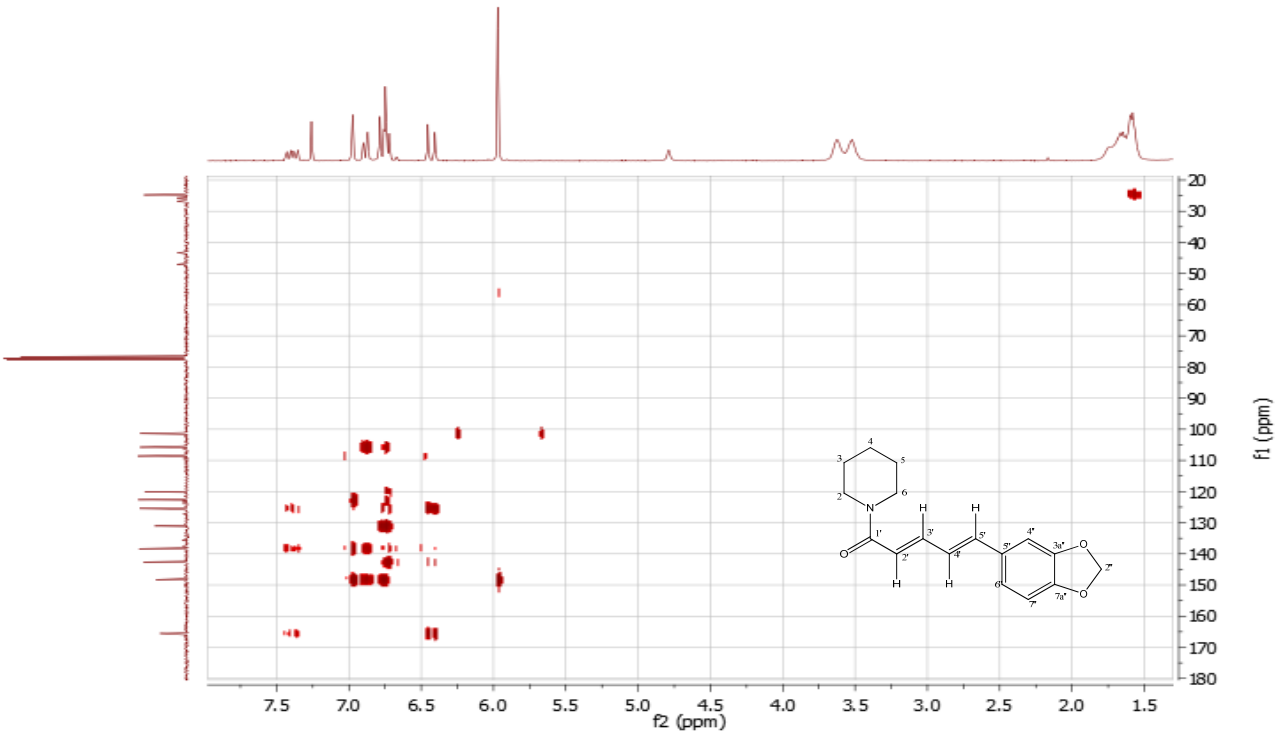


CH



CH_2


f2 (ppm)
f1 (ppm)
Piperine


f2 (ppm)
f1 (ppm)

Caffeine extraction from tea and coffee

Supplementary Material

Experiment notes

Background topics

The main goal of this experiment is the isolation of pure caffeine from tea/coffee. It can be performed in one 4 h session (student will realize only points I and II described in the experimental procedure) or in two sessions of 3 h each as fully described in the experimental session. The class might be divided in two; one half of the students will purify caffeine by sublimation, while the other half will perform recrystallization. At the end the students will compare both the purity (by the analysis of the NMR data) and the yields of caffeine obtained. Sublimation illustrates a less used (but important and efficient in special cases) procedure for purification of solids.

This experiment was realized by students of the Faculty of Pharmacy, University of Lisbon (12 classes of 16 students/class, 8 groups of two), who extracted caffeine from soluble coffee and by students of the Faculty of Sciences and Technology, Universidade Nova de Lisboa (8 classes of 22 students/class, 11 groups of two), who performed the same experiment using tea leaves as raw material. The experiment was

very well accepted by the students who enjoyed the isolation of a drug from a day-to-day product, with good yields and very nice crystals at the end.

Experimental details

The raw material can be tea or soluble coffee. The use of tea requires longer contact time between water/Na_2CO_3 and the bags (20 min), to assure the efficient extraction of caffeine. Students should be careful in this manipulation to avoid breaking the bags.

As has been pointed out in the additional safety item, dichloromethane easily forms emulsions with the water. To prevent these emulsions, the separatory funnel should be gently shaken. Dichloromethane is a low boiling point liquid, so additional precaution must be taken during the solvent evaporation: tight control of bath temperature and/or pressure of the apparatus must be achieved.

Recrystallization of the crude caffeine should be performed using the minimum volume of boiling ethanol. Major quantities of ethanol will not allow the crystals formation.

In Table SM 1.8.1. average values of predictable yields are displayed.

Using the phase diagram of caffeine, the students will be able to understand the temperature/pressure dependent regions of solid, liquid and gas states, and how easily caffeine undergoes sublimation under moderate (temperature/pressure) conditions.

Table SM 1.8.1. Experimental yields

	Soluble coffee (10 g)	Black tea leaves (15 g)
Original product	≅ 0.325 g of caffeine/10g	≅ 0.350 g of caffeine
Crude extract	0.7-1 g /10 g of coffee	0.45 -0.9 g/ 15g of leavesg
Caffeine recrystallized	90-120 mg /10 g of coffee	130-180 mg

Caffeine phase diagram

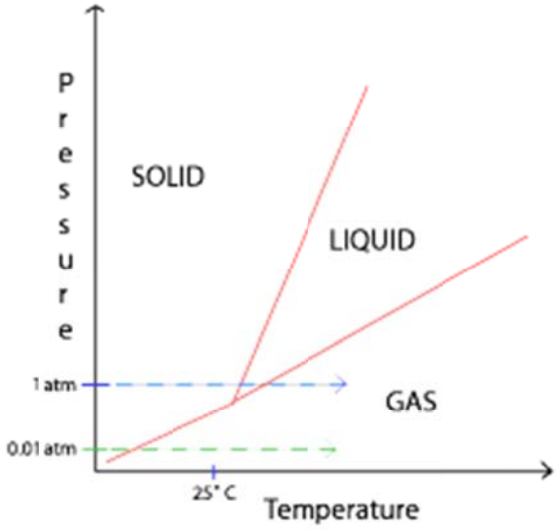


Fig.SM 1.8.1. Caffeine phase diagram.

Photos of the experiment

Fig.SM 1.8.2. Material and reagents.

Fig.SM 1.8.3. Reaction with Na_2CO_3

Fig.SM 1.8.4. Liquid-liquid extraction

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Fig.SM 1.8.5 Elimination of the drying agent.

Fig.SM 1.8.6. Crude caffeine outlook.

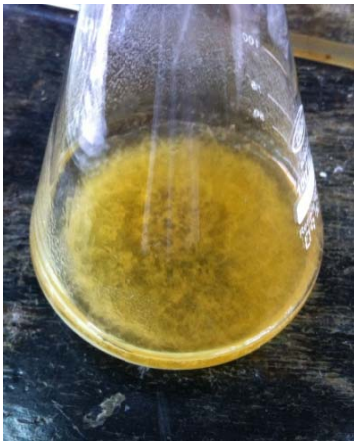


Fig.SM 1.8.7. Crystals formation.

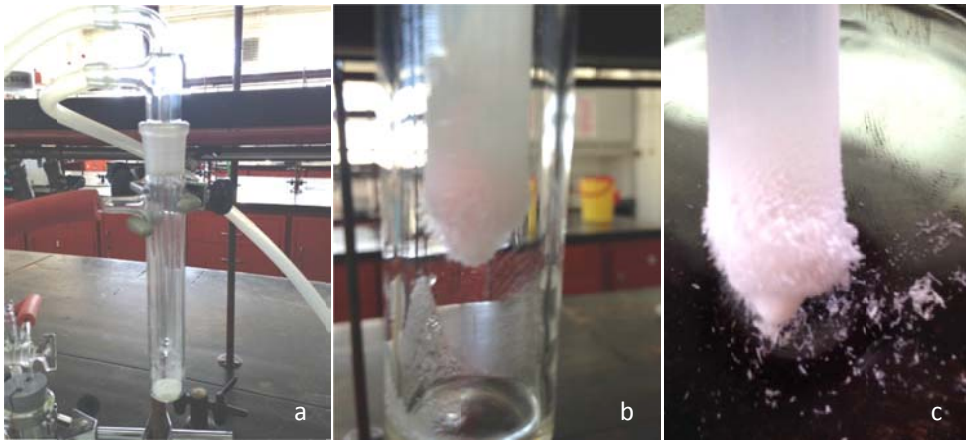


Fig.SM 1.8.8. Sublimation. a) apparatus; b) sublimation process; c) caffeine crystals obtained

Spectra

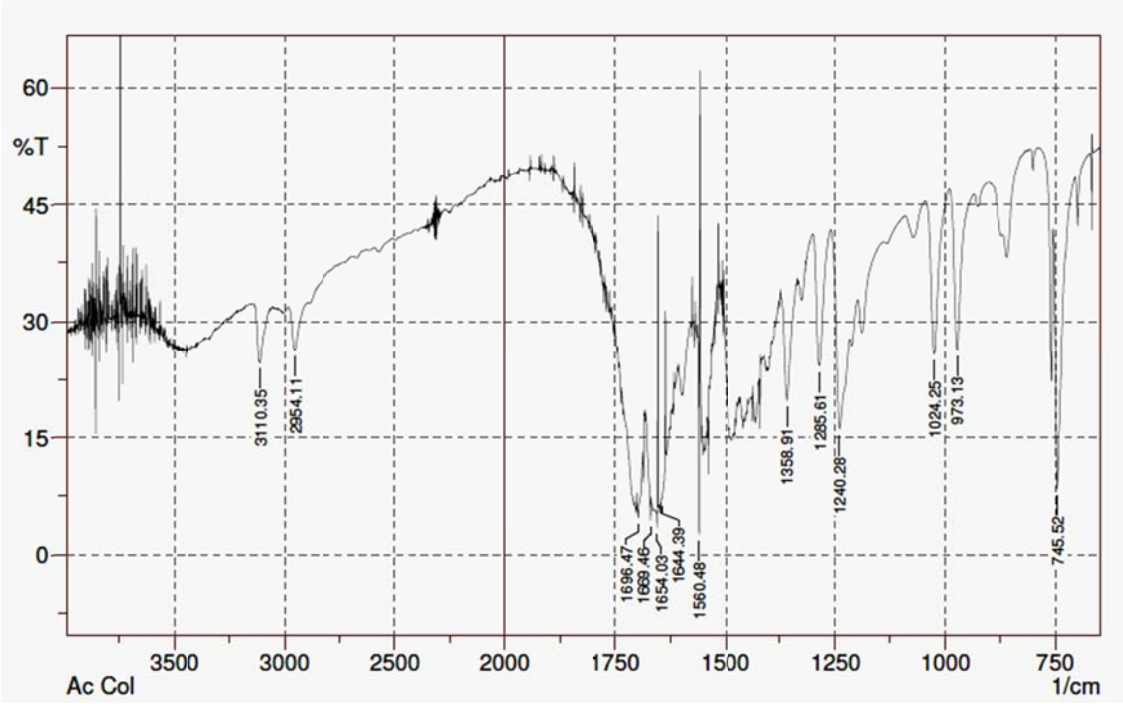


Fig.SM 1.8.9. IR spectrum of pure caffeine.

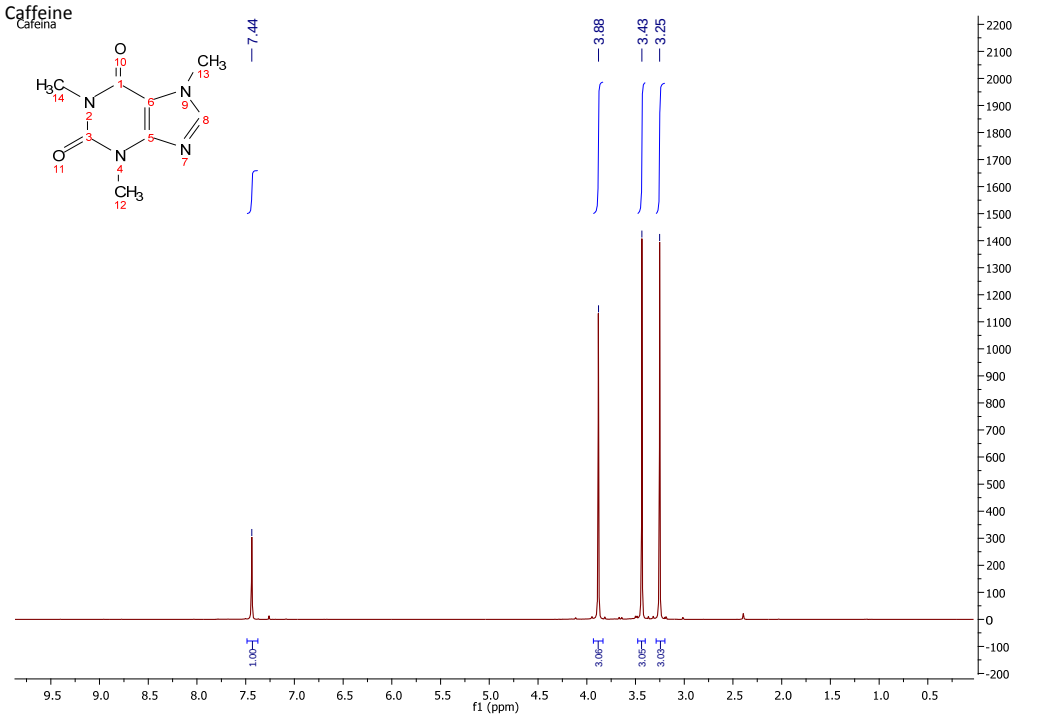


Fig.SM 1.8.10. ^{1}H NMR spectrum of pure caffeine (300 MHz, $CDCl_3$).

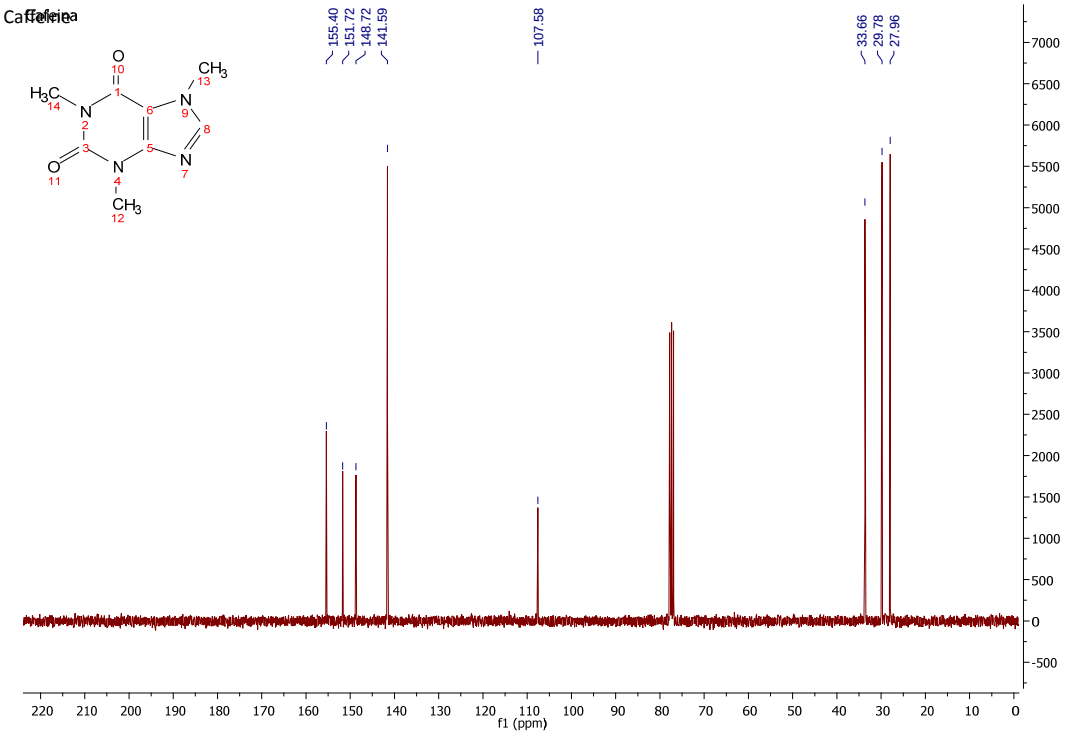


Fig.SM 1.8.11. ^{13}C NMR spectrum of pure caffeine (300 MHz, $CDCl_3$).

Isolation of the alkaloid lupanine from *Lupinus albus* seeds

Supplementary Material

Nature gives us the main valuable compounds that have direct applications in human life and is the inspiration for new synthetic or semisynthetic structures that are crucial for improvement in quality of life and for technological development. Vegetal material is the most explored source of natural compounds due to its accessibility. The extraction procedures are essential steps to separate compounds from the organism that produce them and the optimization of these processes is justified by its economic relevance.

In this experimental work students carry out a process of solid-liquid extraction to obtain lupanine, a quinolizidine alkaloid (QA) from the seeds of *Lupinus albus* (Fig. SM 1.9.1). The role of these alkaloids in the plant species and the usefulness of the major compound lupanine are briefly introduced in the experimental. The scope of this work covers other important issues in organic chemistry namely chromatography and NMR data interpretation.

o 1) information about the student body to whom the experiment was previously given to;

The technics employed in this experimental work are of easy implementation (filtration, column chromatography, evaporation under reduced pressure). These procedures are included in the basic laboratory technics and hence accessible to any student. Nevertheless the interpretation of spectral data for lupanine structure assignment must be performed after a spectroscopy course. The students that performed this experimental work were of the last year of the bachelor degree.

o 2) contextualization of the experiment in the organic chemistry subject at the proper level;

Considering the above arguments it is adequate to perform this work with students of the 3rd grade of the bachelor degree.

At this stage students have properly assimilated knowledge of spectroscopy that allows them to interpret spectra without great difficulty and make comparisons with data from literature. Moreover the current experimental techniques are at this stage performed effortlessly by the students so they can concentrate better in the interpretation of results (chromatography and spectroscopy).

o 3) the tricks needed to successfully perform the experiment in the classroom;

The present work does not rely on particular procedures acquired by experience or any key step brought forward during experimental processes. Everything is done by the book. The experimental procedures were performed several times with different students of 3rd degree and are optimized so that the best results can be achieved relaying on current laboratory practices.

o 4) parameters that can be changed in order to adapt the experiment to lab sessions of different duration;

The period of extraction should not be changed. Less time of extraction leads to smaller efficiency of the process, although if necessary some adjustments may be done in further steps.

If the experimental classes must have a shorter duration the sessions of the recovery of the extract and purification of the QA lupanine may be divided into two separated classes. The process can be carried out by extraction in basic medium, drying the residue and adsorption on silica gel. In the last section the dry mixture of the extract and silica gel would be separated by column chromatography to afford the purified lupanine.

o 5) experimental results obtain by students that performed this work;

The mass of ethanol extract disposable for chromatography ranges from 0.90 to 1.5 g.

The ethanol extract of *Lupinus albus* is analysed by TLC with two eluents, dichloromethane:ethyl ether (1:1) and [dichloromethane:ethyl ether (1:1)]:ammonia 1%. The R_f values are quite different in each case. The use of ammonia decreases the acidity of the silica gel plate. Lupanine has basic character

so the affinity for the plate decreases as it turns less acidic and the R_f value increases. It is worth increasing the lupanine R_f value to be able to better analyse its purity. With dichloromethane:ethyl ether as eluent the lupanine Rf is 0.25 and in 1% ammonia the R_f goes to 0.43.

The spots are visualised in the chromatographic plate by spraying with ninidrine. Lupanine turns dark purple after heating. Draggendorff reagent is appropriated for alkaloids and can also be used. Spots turn immediately orange after spraying.

After purification the lupanine can be recovered between 0.16 and 0.43% yield. This value depends on the experience of who performs the experimental procedure and much from the origin of the Lupinus seeds. The quantities of metabolites present in different specimens can vary significantly.

o 6) photos of the setup apparatus needed to perform the experiment.

Fig. SM 1.9.1. Lupinus seeds

Fig. SM 1.9.2. Ground Lupinus seeds.

Fig. SM 1.9.3. Ground seeds macerated with a KOH solution (25%).

Fig. SM 1.9.4. Above mixture after addition of celite.

Regarding the questions that students should answer the main issues that should be considered are as follows.

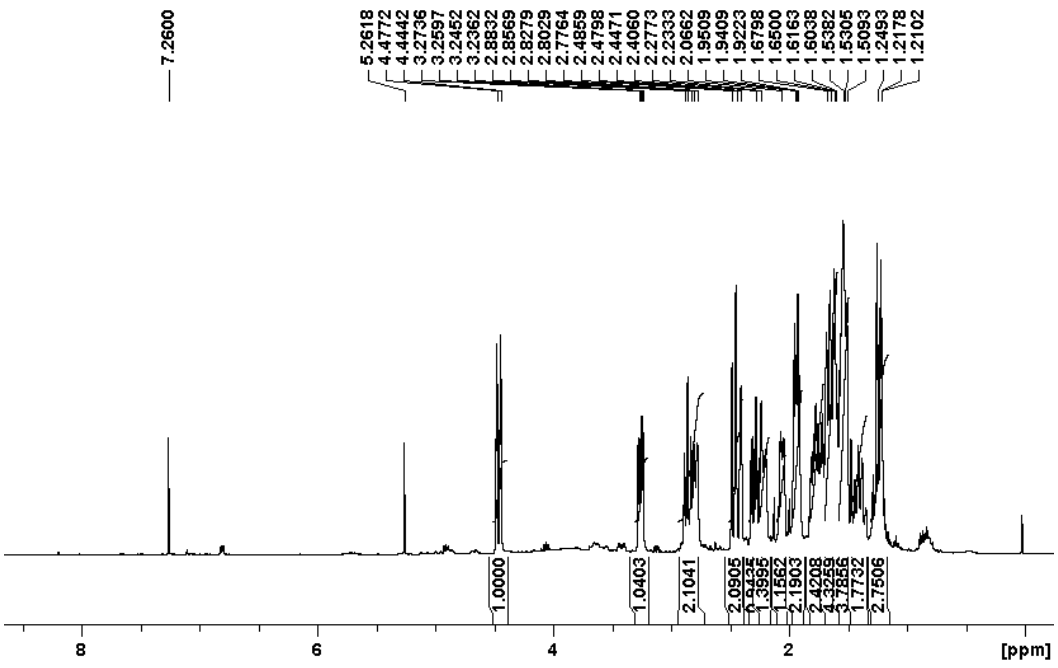
The explanation about the solubility of lupanine in water that is the traditional method of extraction, and with ethanol should be presented on the bases of the electronegativity of the heteroatoms, and the interaction of the nitrogen atom and the amide function that take place with the solvents. The lipophilic character of the other moieties should not be forgotten in this discussion.

The second question about the efficiency of lupanine extraction does not only concern the final quantity obtained. The answer must include a balance between all the parameters related with the experimental work. Subjects to be discussed are time consuming, reagents consumption, purity of the final product based on TLC and NMR analyses, risks involved in handling during the experimental work. Students must discuss if the method is convenient for lupanine recover from *Lupinus albus*.

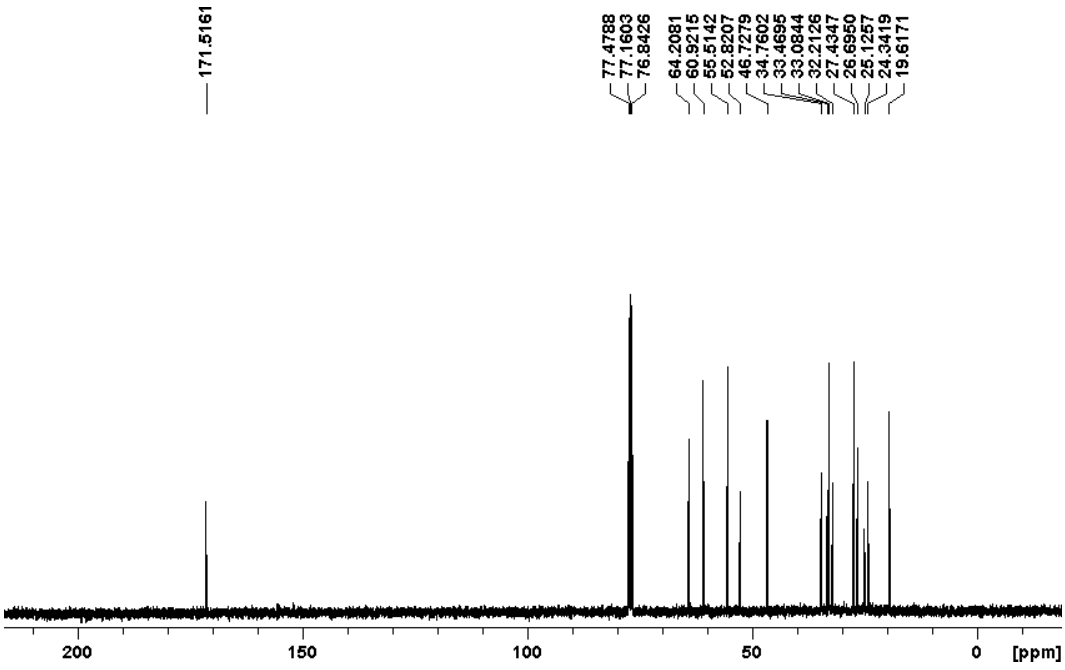
Potassium hydroxide is essential to guaranty that the nitrogen atom of the quinolizidine nucleus is not protonated due to the presence of an acidic species in the extract mixture. The quantity of potassium hydroxide is sufficient to maintain a basic medium during the extraction with ethanol.

The selection of the best chromatographic conditions for a good separation process is of great importance. The adjustment of the eluent composition must be done with care and in some cases simple changes can significantly improve the final separation. In the present case lupanine is a compound with basic character and silica gel has acid character. In such a system the elution of lupanine can be facilitated with the addition of a basic component in the solvent that reduces the acidic character of the stationary phase by partial neutralization. This is a common procedure and in most cases for alkaloids ammonia is the chosen agent for this purpose.

Fig. SM 1.9.5. NMR spectra of Lupanine



^{1}H NMR of lupanine after column purification (400 MHz; $CDCl_3$).



^{13}C NMR of lupanine after column purification (100,5 MHz, $CDCl_3$).

Isolation and purification of atropine, a tropane alkaloid obtained from *Atropa belladonna* L. (*Solanaceae*)

Supplementary Material

Background on the experiment topic

This experiment aims at the isolation of atropine a tropane alkaloid extracted from *Atropa belladonna* L. dried leaves (*Belladonnae folium*).

Alkaloids are a group of naturally occurring *N*-containing heterocycles, consisting of secondary and tertiary amines. These compounds are known for their basic properties, as a result of the presence of an endocyclic nitrogen atom. The presence of a nitrogen atom capable of protonation facilitates the extraction of these alkaloids.

According to the dissociation constants and depending on the purpose of the operation, two classical methods can be considered for the extraction of alkaloids:

Method A. The powdered material is extracted in an acidic diluted solution. The free alkaloids are then precipitated by the addition of a basic diluted solution and subsequently unwanted materials (*e.g.* pigments, organic acids, tannins, phenolic constituents) are removed by liquid-liquid extraction with an organic solvent.

Method B. The powdered material is extracted in a basic diluted solution, which sets free the alkaloids (if they exist in the plant as salts). The liquid-liquid extraction is carried out with an organic solvent and the concentrated organic layer is then shaken with an acidic diluted solution and allowed to separate. Alkaloid salts are retained now in the aqueous layer, while many impurities remain in the organic layer.

Tropane alkaloids are usually unstable and sensitive to strong acid and basic conditions and consequently the method of extraction and the choice of a suitable solvent should be carefully selected. Atropine and other tropane alkaloids are more efficiently extracted in an acidic solution (Method A), at pH 2-3. During the extraction of tropane alkaloids from *Belladonna folium*, partial racemization of L-hyoscyamine occurs and atropine is obtained, as a racemic mixture (DL-hyoscyamine).

This experiment was executed and reported by 77 groups and 3rd year undergraduate Pharmaceutical Sciences students. The experiment is intended to allow the students to be familiar with pH manipulation in the context of liquid-liquid extraction, chromatographic control and chromatic reactions of tropane alkaloids. Basic chemistry concepts as pK_a value, and usual organic chemistry techniques as TLC control and liquid-liquid extraction should be previously acquainted by the student body..

Additional notes

Alkaloid-directed extraction

In this experiment, ultrasound assisted extraction followed by liquid-liquid partitioning is given as a method for the isolation of atropine, without further purification techniques. The existence of minor coextractive compounds, does not interfere with the remaining steps for the identification and detection of atropine.

Atropine is a weak base (pK_a = 9.9, 20 ºC), commonly extracted in acidic media from solanaceous plants. Sonication improves the yield of the extraction, without needing to increase the temperature of the bath. The total alkaloid extract consists mainly of atropine (*ca*. 80%), which discourages further purification and isolation techniques.

TLC analysis

Thin-layer chromatography (TLC) is a valuable analytical tool for the qualitative determination of small amounts of plant-derived products, being an effective, easy-to-perform and inexpensive technique. However, during the execution of this technique some details should be taken into consideration.

Saturation of the chromatographic chamber:

To achieve saturation of the chromatographic chamber, line at least half of the total area of the inside walls of the chamber with filter-paper, pour into the chamber a sufficient quantity of the mobile phase to saturate the filter paper and form a layer about 5 mm deep. Close the chamber and allow it to stand for at least an hour at room temperature.

Application:

Using a micro capillary pipette, place the spots of the alkaloid extract (A), reference (H) and the alkaloid extract and reference (A+H), onto the starting line (Figure SM 1.10.1). All the spots should be parallel to and about 15 mm above the lower edge, 15 mm from each side of the plate and at least 15 mm apart. The spots should be carefully applied to be as small as possible, preferably not exceeding a diameter of 4 mm. If necessary, apply the solutions in portions and dry between applications.

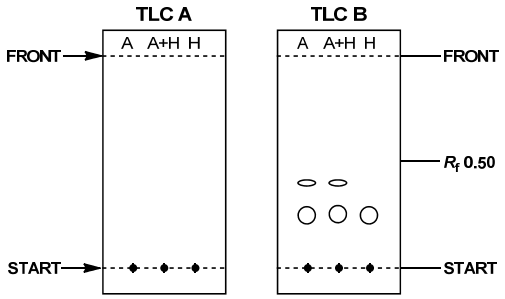


Figure SM 1.10.1 TLC plate before elution and final chromatogram

Development of the chromatograms:

After application, allow the spots to dry, place the plate as nearly vertical as possible into the chromatographic chamber, ensuring that the points of application are above the surface of the mobile phase. Close the chromatographic chamber and develop the chromatogram at room temperature, allowing the solvent to ascend the specified distance. Remove the plate from the chromatographic chamber and allow the solvent to evaporate at room temperature, or in a stream of cool air, as specified.

Observation and interpretation of chromatograms:

Observe the spots in a long wavelenght emitting ultraviolet light source (Wood's lamp) and carefully mark the plate with pencil. Spray the plate according to the described procedure (with Dragendorff reagent and then with $NaNO_2$), observe the differences at daylight, mark the plate with pencil, measure and record the distance from the center of each spot to the point of application and calculate the R_f of atropine and scopolamine (minor constituent).

Vitali Morin reaction

The presence of tropane alkaloids can be easily confirmed by simple colorimetric reactions. In the context of organic chemistry, this method consist of an example of a nitration reaction used exclusively for detection purposes, instead of the common derivatization steps in synthetic routes. The quantity of the added base in this reaction determines the colour of the nitrated products.

Analysis of the results

Part 1 - Alkaloid-directed extraction

- In the above-mentioned extraction conditions, atropine is yielded in 0.3 – 0.5 % (m/m). For quality control purposes the minimal contend in atropine expected in *Belladonnae folium* is 0.3 % in the European Pharmacopoeia and 0.35 % in the United States Pharmacopoeia (with small differences in the extraction procedure).

Part 2 – TLC analysis

Detection a) 366nm:

- The total alkaloid extract (Lane A) shows two bands, corresponding to atropine and scopolamine, whereas the reference standard (Lane H) appears as a single band. The mixture of the total alkaloid extract with the reference standard (Lane A+H) reveals two bands, corresponding to scopolamine and atropine bands, however only the atropine band is intensified. A TLC chromatogram is represented in Figure SM 1.10.2.

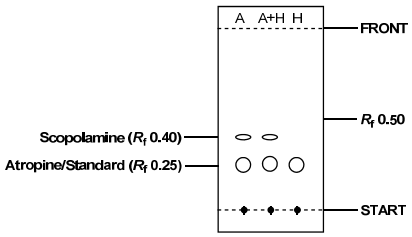
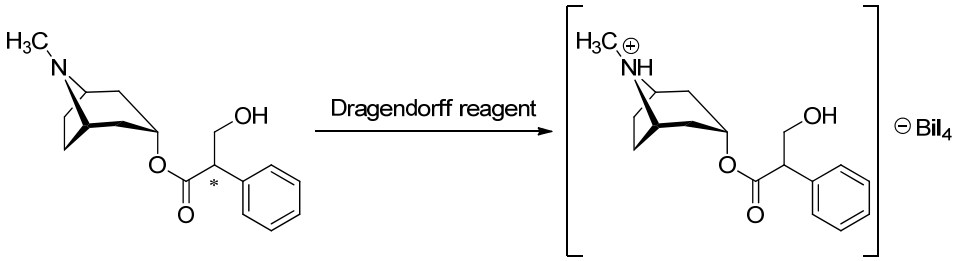


Figure SM 1.10.2

In this chromatographic system, atropine reveals R_f values between 0.25 and 0.40 and scopolamine reveals upper values, between 0.40 and 0.51.

Detection b) Dragendorff/$NaNO_2$:

- Previously detected bands respond to Dragendorff reagent with orange, unstable colour. Dragendorff reagent is a mixture of basic bismuth(III) nitrate, potassium iodide and glacial acetic acid. Due to the acid contained in the spraying solution, the tertiary amine (in the protonated form) is capable of forming a complex with the ionic pair $[BiI_4]^-$. The formation of this complex is revealed in Scheme SM 1.10.1.

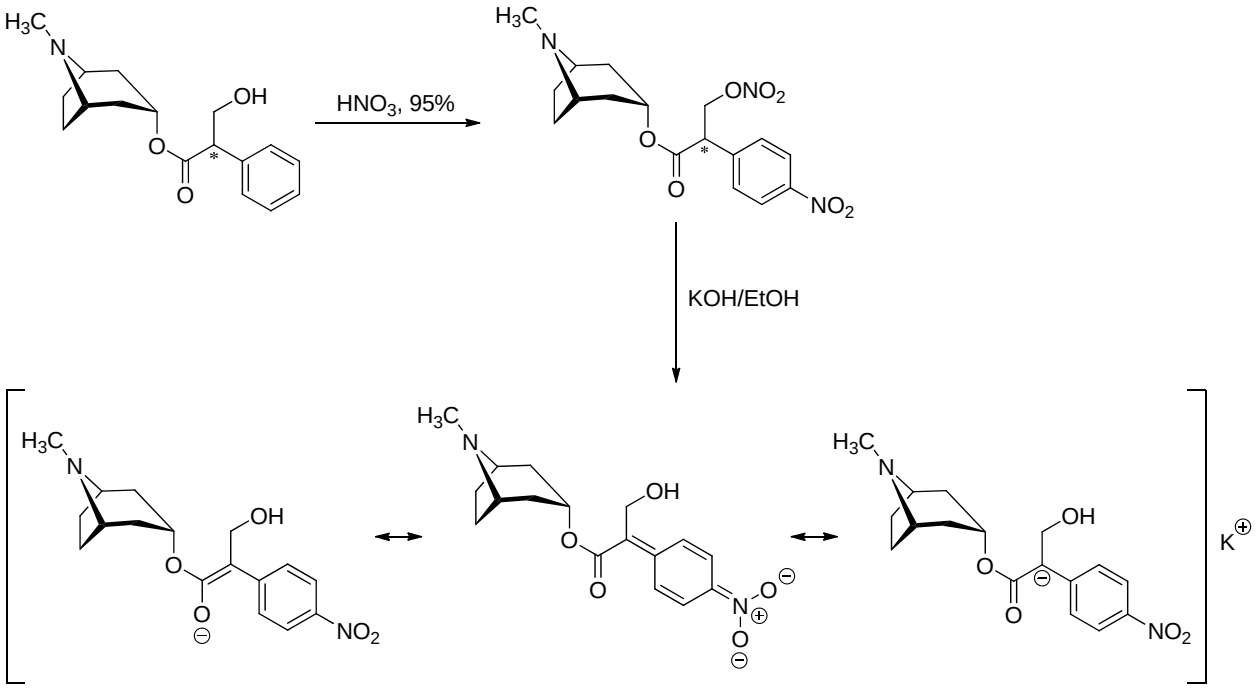


Scheme SM 1.10.1

- Treatment with $NaNO_2$, modifies and increases the colour stability of tropane alkaloids, after Dragendorff revelation. This phenomena is probably due to the *in situ* generation of $[NO_2]^+$ and subsequent nitration of the aromatic ring.

Part 3 – Vitali-Morin reaction

Tropane alkaloids can be easily detected by colorimetric reactions. The non-substituted tropane moiety is nitrated, which is suspended in acetone and then treated with potassium hydroxide in methanol. Holmes's proposal for the Vitali-Morin reaction is illustrated in Scheme SM 1.10.2 (Manske, 1950).



Scheme 1.10.2

In the course of Vitali-Morin reaction, the formation of unstable purple/bluish colored salts allows the colorimetic detection of tropane derivatives. This method consists of a variation of a previous reaction developed by Vitali and Gerrard, conferring more sensitivity to the method.

Acknowledgements

The author gratefully acknowledges the students who participated in the photographic register of this experiment.

Further reading

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Photos of the experiment

Ultrasound-assisted extraction (Figure SM 1.10.3, A-D)

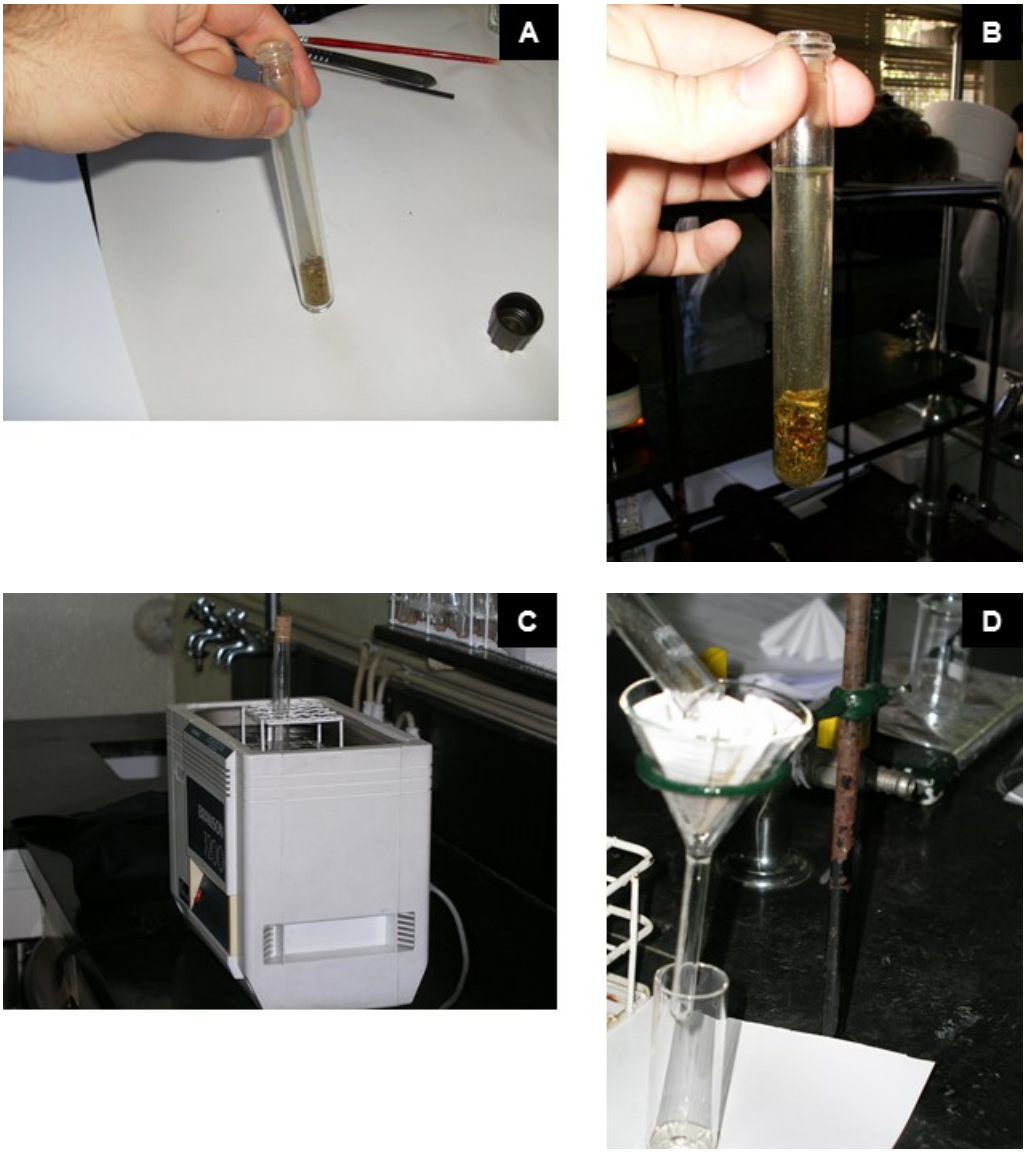

Figure SM 1.10.3.

TLC analysis (Figure SM 1.10.4, A-D)

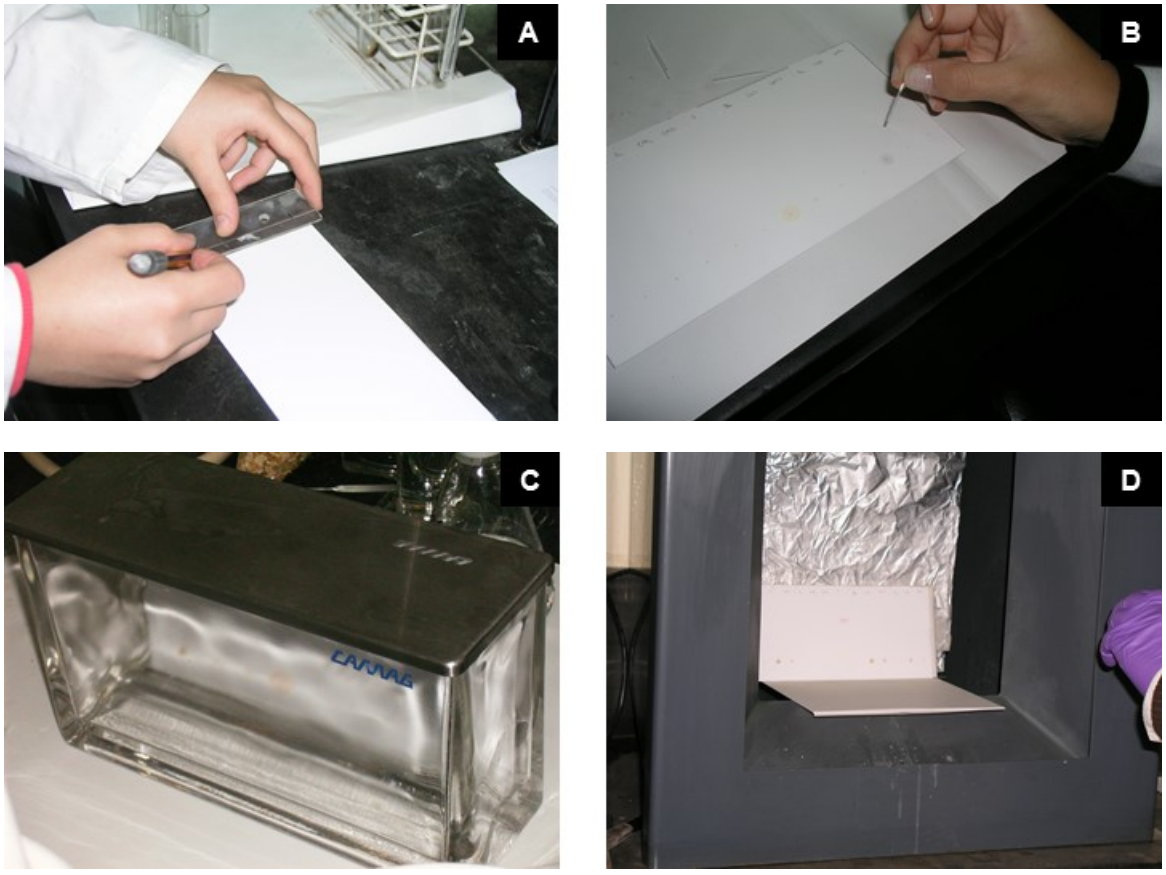

Figure SM 1.10.4.

Vitali-Morin reaction (Figure SM 1.10.5, A)

Figure SM 1.10.5.

Isolation and purification of carnosol from *Salvia officinalis*

Supplementary Material

This experiment "Isolation and purification of carnosol from *Salvia officinalis*" is proposed for under graduated students with organic chemistry laboratorial skills. Dried leaves of *S. officinalis* L. are commonly found in the markets at a relatively low price. Alternatively, it is also possible to carry this experiment with *Rosmarinus officinalis* (rosemary) where carnosic acid and carnosol are also both the major constituents.

Salvia officinalis (sage) is a common plant used as a kitchen herb since ancient times and belongs to the mint (Lamiaceae) family. Sage name is derived from the Latin, ''Salvia'' means to cure and ''Officinalis'' means medicinal, and has a long history of use as a medicinal herb for the promotion of health and treatment of ailments. In the last decades, sage became the subject of intensive study for its phenolic antioxidant components. In a laboratory scale, the most reported extractive methods were the conventional solvent extraction using acetone, hexane, methanol or ethanol.[1]

The antioxidant compounds isolated from rosemary were mostly the phenolic diterpenes carnosic acid and carnosol (approximately 5% of the dry weight of rosemary leaves). Several related phenolic diterpenes, such as rosmanol and epirosmanol were also identified. Carnosic acid has been reported as the main metabolite substrate that leads to diverse oxidated phenolic diterpenes such as carnosol.[2]

The conventional extraction techniques are time and energy-consuming, may degradate metabolites and involve large volumes of toxic organic solvents.[3] The ultrasound method was used in this work to increase the extraction efficiency of carnosol from *S. officinalis* L. using ethanol as solvent. Sonication improved the yield of carnosol and shortened the extraction time.[4] The most probable mechanism for the ultrasonic enhancement of extraction is the intensification of mass transfer and the easiest access of the solvent to the cell material.[5,6]

The plant must be powdered to increase extraction efficiency (Figure SM 1.11.1).

Figure SM 1.11.1: Powdering leaves of commercial *Salvia officinalis*.

The first session of 4h is mostly dedicated to the ultrasound plant extraction (Figure SM 1.11.2) and results are presented in Table SM 1.11.1.

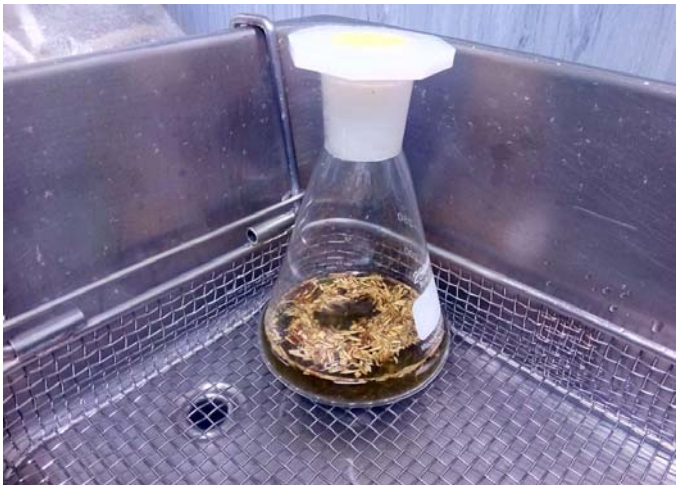

Figure SM 1.11.2: Ethanol extraction using a ultrasonic bath.

Table SM 1.11.1: Results (weight and yield) obtained by the students during the experiment.

Powdered plant (g)	Extract (g)	Extraction yield (%)	Fractions containing carnosol (mg)	Pure carnosol (mg)	Yield (%)
20	0.5 - 0.8	2.5 - 4.0	40 - 70	5 - 9	0.025 – 0.045

The filtration was used to remove insoluble materials (Figure SM 1.11.3A). The centrifugation step is important to remove the water-soluble extract components thus simplify the chromatography step (Figure SM 1.11.3B).

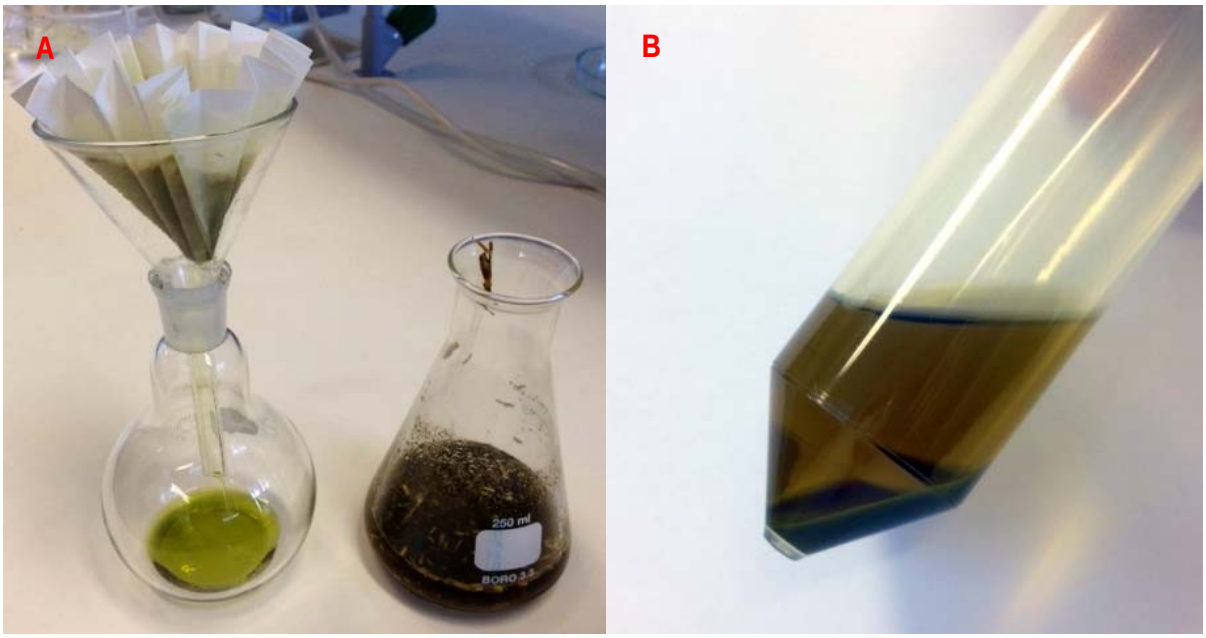

Figure SM 1.11.3: (A) Extract filtration; (B) Precipitate formed after centrifugation.

The too small particle size of the precipitate requires the use of the centrifugation, alternatively it's possible to do a filtration (vacuum or gravimetric) with poor separation efficient. The resulting precipitate dissolved in ethyl acetate, must be analysed by TLC (Figure SM 1.11.4), where carnosol is identified as a band with a R_f value of ≈ 0.3 (Silica gel; *n*-hexane:ethyl acetate, 7:3) above the most intense yellow band.

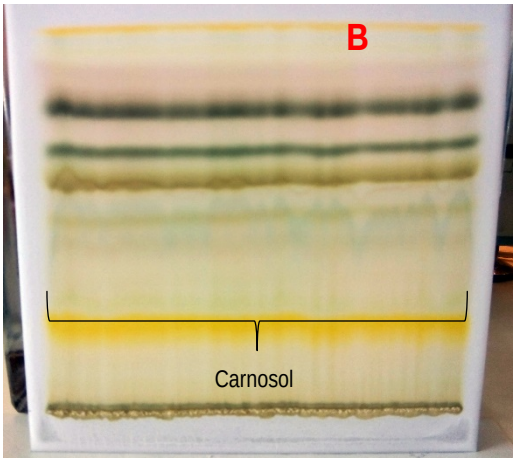

Figure SM 1.11.4: TLC plate of *S. officinalis* extract with *n*-hexane:ethyl acetate (7:3).

The second session (4h) should be performed by the column chromatography purification of carnosol and is the most time-consume step (Figure SM 1.11.5).

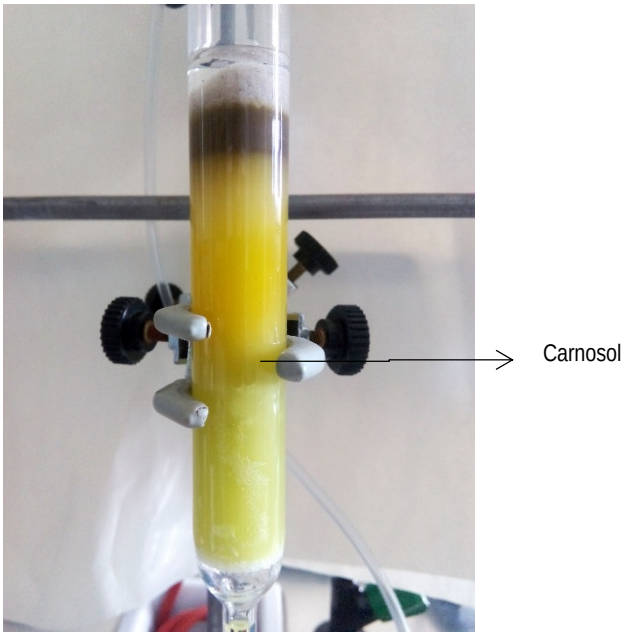

Figure SM 1.11.5: Purification of carnosol using column chromatography.

Alternatively the column chromatography can be packed in advance in the first session. The column chromatography elution should be performed with a solvent gradient from 100% *n*-hexane to 100% of ethyl acetate (Table SM 1.11.2). By our experience it is not possible to replace hexane by other solvent. The column should have a reduce diameter (lower than 20 mm) to increase theoretical plates and efficiency for the isolation of pure carnosol (≈ 9 mg, 0.045% on dry plant material).

Table SM 1.11.2: Chromatography column eluents and volumes.

Eluent	Volume (mL)
***n*-hexane**	20
***n*-hexane: ethyl acetate (9:1)**	20
***n*-hexane: ethyl acetate (8:2)**	20
***n*-hexane: ethyl acetate (7:3)**	60
***n*-hexane: ethyl acetate (5:5)**	20
ethyl acetate	20

During the column chromatography the first eluted band is yellow, followed by the green chlorophylls bands. Carnosol is contained in the next band eluted just before the last intense yellow band, which is eluted with *n*-hexane: ethyl acetate 7:3 (Figure SM 1.11.5). The fractions collected during the elution should have small volumes (4-6 mL).

Carnosol and carnosic acid are the major compounds of *S. officinallis* with similar *Rf* values. Carnosol is the product of oxidative degradation of carnosic acid. Considering the carnosol recrystallization from *n*-hexane, is possible to obtain the crystals of carnosic acid as contaminant.

Pure carnosol isolated in this experiment can be identified by its ^{1}H-NMR spectrum (Figure SM 1.11.6; Table SM 1.11.3), due to the characteristic protons H-7α (δ 5.25), H-14 (δ 6.56), and H-15 (δ 3.22; Figure SM 1.11.6).

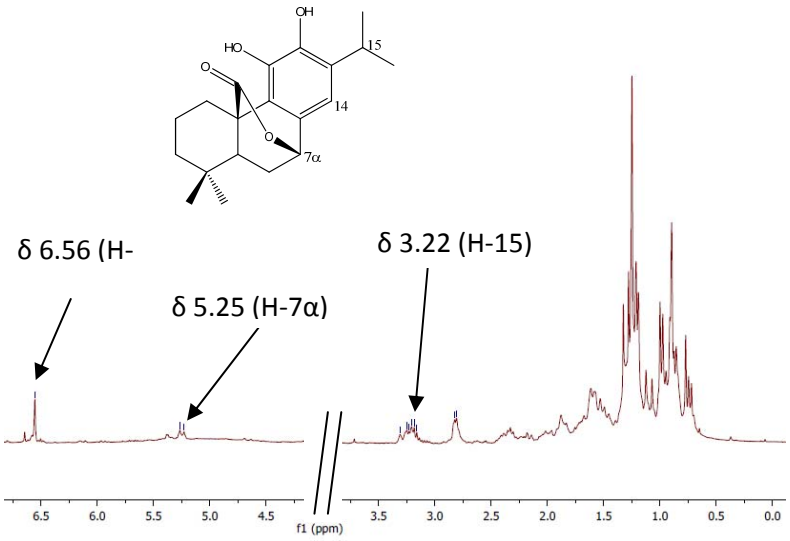

Figure SM 1.11.6: ^{1}H-NMR spectrum of carnosol.

Table SM 1.11.3: ^{1}H-NMR (300 MHz; $CDCl_3$) data of isolated carnosol and obtained from literature[7].

Position	δ_H (ppm)	δ_H (ppm)[7]
1α	2.82 d (*J* = 4.8 Hz)	2.80 *dd* (*J* = 14.0; 3.0 Hz)
1β	2.32 m	2.56 *ddd* (*J* = 14.3; 4.5; 4.5 Hz)
2α	1.99 m	1.91 *m*
2β	1.53 m	1.59 *m*
3α	1.49 m	1.52 *m*
3β	1.33 m	1.30 *m*
5	1.60 m	1.69 *dd* (*J* = 4.2;4.3 Hz)
6α	1.86 *m*	1.83 m
6β	2.18 m	2.19 m
7α	**5.25 d (*J* = 10.9 Hz)**	5.41dd (*J* = 7.3; 7.5 Hz)
7β	-	-
14	**6.56 *s***	6.69 *s*
15	**3.22 m**	3.25 *m*
16	0.99 d (*J* = 7.3 Hz)	1.18 *d* (*J*= 6.7 Hz)
17	1.20 d (*J* = 6.2 Hz)	1.20 *d* (*J*= 6.9 Hz)
18	0.86 s	0.89 *s*
19	0.90 s	0.90 *s*

Additional references

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Analysis of racemic and (*S*)-ibuprofen

Supplementary Material

Experiment Notes

Schemes and Figures

Chirality is the property of a molecular entity of being non-superposable on its mirror image. An equimolar mixture of a pair of enantiomers is a racemate. Ibuprofen is a chiral non-steroidal anti-inflammatory drug widely used for the treatment of inflammatory diseases commercialized in most pharmaceuticals as a racemate. Nevertheless, the enantiomer with therapeutic interest is the (*S*)-ibuprofen (Scheme SM 1.12.1). Because the enantiomers of ibuprofen differ greatly in their pharmacological and pharmacokinetic properties, it is important to adopt stereospecific assay methodologies in ibuprofen analysis. This experiment aims to analyse the racemic mixture and the (*S*)-enantiomer of ibuprofen by two different methods: the direct and the indirect methods.

Analysis of racemic and (*S*)-ibuprofen by chiral HPLC (direct method)

Prior to the experimental, a section of tips for practical HPLC analysis could be performed including aspects concerning solvent handling, mobile phase preparation, priming the HPLC, column handling and

equilibration, and system performance checks.[1] Before the experimental takes place, the instructor should prime the HPLC system, connect the column to the solvent delivery system according to the HPLC system requirements and turn on the detector (warm-up at least 20 minutes)

The students should start by preparing the mobile phase that should be filtered, preferably through a 0.45 μm porosity filter, and degassed before use (by stirring under vacuum or in an ultrasonic bath, membrane degassers, or helium purges), equilibrate the column, and record the detector response until it reaches a stable response. This procedure assures reproducible results. Students should not pressure shock the column while equilibrating with the mobile phase.

During the elution, the instructor can discuss with the students theoretical aspects of chirality,[2] practical tips for HPLC,[3] as well as expected results with other columns (e.g. C-18) or detectors (e.g. polarimeter).

By chiral HPLC, transient diastereomeric interactions between the enantiomers of ibuprofen and the chiral selector of the stationary phase lead to different retention times and thus, to the separation of the enantiomers. Considering the racemic ibuprofen, two chromatographic bands are observed with retention factors (k) of 1.37 ± 0.12 and 1.77 ± 0.15; on the other hand, only one chromatographic band is observed for the (*S*)-ibuprofen with k = 1.77 ± 0.15 (Figure SM 1.12.1). These k values are obtained using the chiral column (*S*,*S*)-Whelk-O1 (250 × 4.6 mm internal diameter, 5 μm particle size, 100 Å pore size) commercially available from Regis Technologies, Inc. (Morton Grove, IL, USA); and the HPLC-UV apparatus consisted of a Finnigan Surveyor (Thermo Electron Corporation, USA) equipped with an autosampler (AutoSampler Plus) and a diode array detector TSP UV6000LP (Thermo Separation Products, USA). Some differences in the k values can be observed taking in account the column supplier and the HPLC-UV apparatus used to performed this experiment.

Analysis of racemic and (*S*)-ibuprofen by diastereomers synthesis (indirect method)

By the indirect method, the racemic and (*S*)-ibuprofen are first converted into diastereomeric amides by reaction with the enantiomerically pure (*S*)-(–)-α-methylbenzylamine in the presence of the coupling

reagent TBTU (Scheme SM 1.12.2). The products resulting from the synthesis are then isolated by liquid-liquid extraction, and analysed by TLC.

The reaction is performed at small scale (50 mg, 0.24 mmol of ibuprofen). The formation of the amide bond can also be achieved by the use of other coupling reagents, such as 1,1'-carbonyldiimidazole. However, the synthetic methodology using TBTU demonstrates to be very efficient, and presents various advantages including stability, long shelf-life, solubility in common organic solvents, and generation of 1-hydroxybenzotriazole (Scheme SM 1.12.2), a water-soluble by-product.

After the addition of TBTU, it is essential to keep the stirring for 5 minutes in order to achieve the activation of the carboxyl group of ibuprofen before the addition of the enantiomerically pure amine.

Is not necessary to monitor the reaction in order to observe the disappearance of the starting material, since the aim of this experiment is only to distinguish the (*rac*)-ibuprofen from the (*S*)-ibuprofen, and the reaction does not reach completion in 1 hour. The reaction time parameter can be decrease in 30 minutes in order to adapt the experiment to a shorter lab session without affecting the quality of the analysis. After 1 hour, the reaction is stopped and the products formed are efficiently isolated by liquid-liquid extraction. Is not necessary to evaporate the reaction solvent (DMF), just transfer each reaction mixture into the separatory funnel and wash the organic layer (diethyl ether) with the aqueous solutions. If the two immiscible layers are difficult to observe due to a higher volume of DMF, 1 mL of diethyl ether should be added. The sodium hydrogen carbonate (5% in water) solution is efficientlin extracting the ibuprofen that remained in the reaction mixture. Is not necessary to eliminate the excess of (*S*)-(–)-α-methylbenzylamine since it does not interfere with the analysis (will not elute).

The isolated products are then analyzed by TLC: the (*rac*)-ibuprofen generates spots of (*S,S*) and (*R,S*) diastereomeric amides with Rf values of 0.45 and 0.59, respectively, while (*S*)-ibuprofen generates a spot of (*S,S*) amide with Rf value of 0.45 (Figure SM 1.12.2). With variation of the proportion of the ionization suppressor (acetic acid) in the mobile phase, alteration in the Rf value of ibuprofen or tailing can compromise the analysis. The resolution of the samples of the diastereomeric amides depends on their concentration and purity on the final extract.

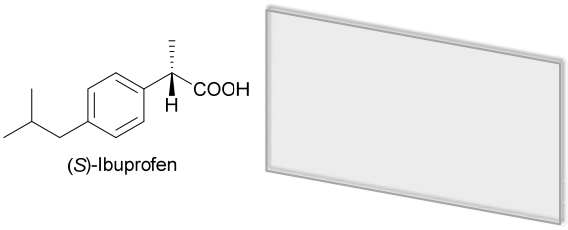
It is emphasized that by TLC technique it is not possible to distinguish both enantiomers of (*rac*)-ibuprofen (only one spot is observed with the Rf value of 0.64) since they have identical properties in an achiral environment.

After the experiments, the students should discuss the advantages and disadvantages of both direct and indirect methods and, additionally, their scope in preparative applications. For example, the indirect method presented some drawbacks such as: is a time-consuming and labor-intensive procedure; requires optically pure reagents, since only with 100% of purity the results can be directly representative of the enantiomer composition; is applicable only to enantiomers presenting a single (or more, but selectively addressable) functional group suitable for derivatization; considering preparative applications, if the reactions are not complete or are associated with racemization or epimerization, differences in product yields may result in large errors; and, needs a subsequent chemical treatment to the recovery of the enantiomers. However, the advantage of this method over the direct method is that it does not require a chiral stationary phase (CSP). In fact, the students should be alerted for the limitations of the CSPs, i.e., one CSP can only separate a limited number of chiral compounds, and in many cases the choice of the CSP may became a difficult task. The direct method offers many advantages, both for preparative as analytical separations, since there is no need of previous derivatization, much less sample manipulation is required, and more rapid results are obtained after the chiral chromatography. Additionally, the advantages of HPLC over TLC methodology should be emphasized, such as being automated, the sensitivity of detection and the possibility to determine the enantiomer excess (e.e.). The e.e. is an indicator of the enantiomeric purity. For a mixture of (*S*)- and (*R*)-ibuprofen, with composition given as the mole or weight fractions $F_{(S)}$ and $F_{(R)}$, the e.e. is defined as $|F_{(S)} - F_{(R)}|$ (and the percent enantiomer excess by $100|F_{(S)} - F_{(R)}|$) by the equation X.1.[2] The scope of this work can be increased if the students use random enantioenriched mixture of ibuprofen, thus allowing them to work with the peaks areas and calculate e.e.

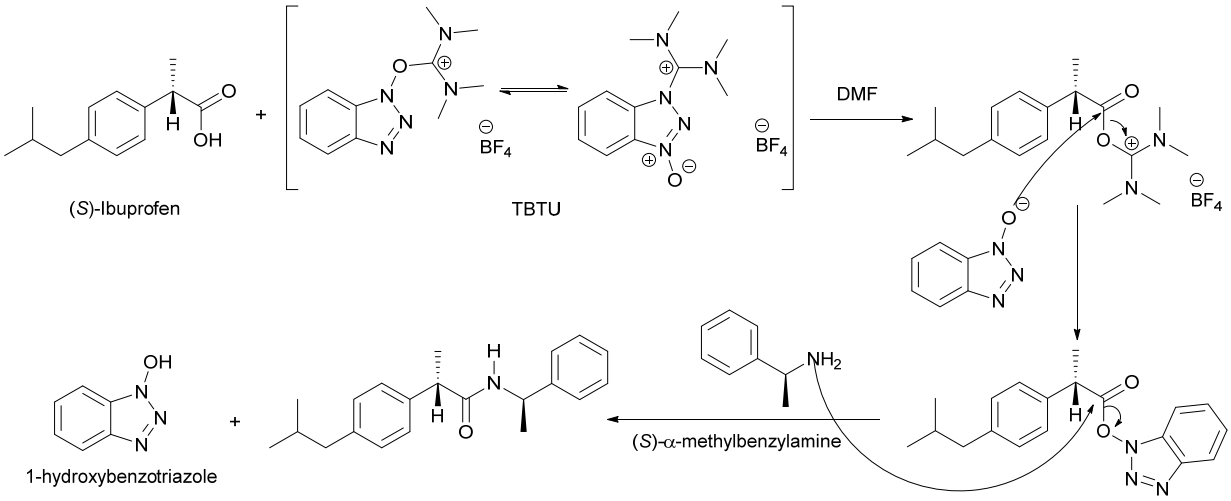
$$\boldsymbol{e.e.} = \frac{\mathrm{F}(S) + \mathrm{F}(R)}{|\mathrm{F}(S) - \mathrm{F}(R)|} \mathrm{X}\ 100 \quad \textbf{(X.1)}$$

This experimental work takes a lab session with a medium hazard level and targets advanced master students. The reproducibility of the experiment was assessed by testing a large number of times in laboratory classes, namely by 1st year Medicinal Chemistry M.Sc. students from Faculty of Pharmacy, University of Porto.

Schemes and Figures



Scheme SM 1.12.1 – Chemical structure and configuration of both enantiomers of ibuprofen.



Scheme SM 1.12.2 – Reaction mechanism for the synthesis of the (*S*,*S*) diastereomeric amide through TBTU activation.

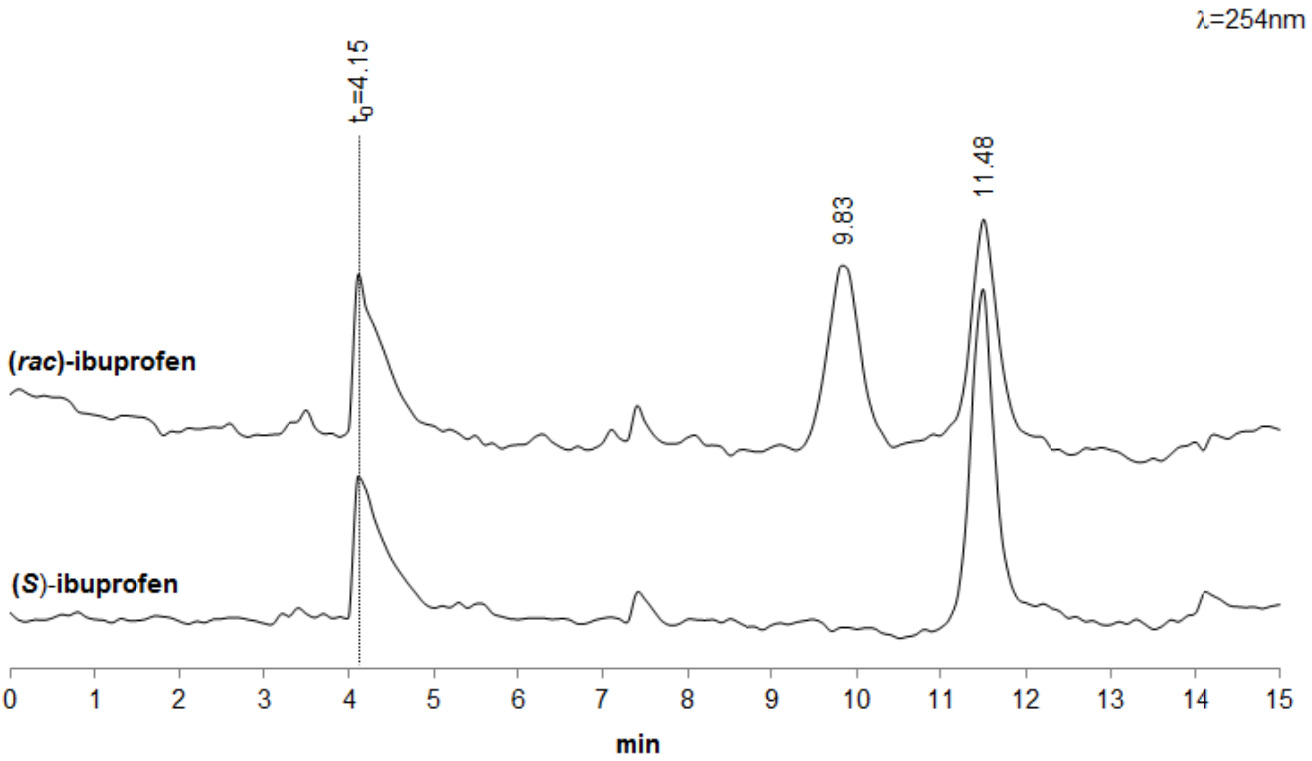


Figure SM 1.12.1 - HPLC chromatograms of (*rac*)-ibuprofen and (*S*)-ibuprofen. Column, (*S,S*)-Whelk-O1 (250 × 4.6 mm); mobile phase, *n*-hexane/2-propanol/acetic acid (98:2:0.05 v/v/v); flow rate, 0.9 mL/min; detection, UV 254 nm.

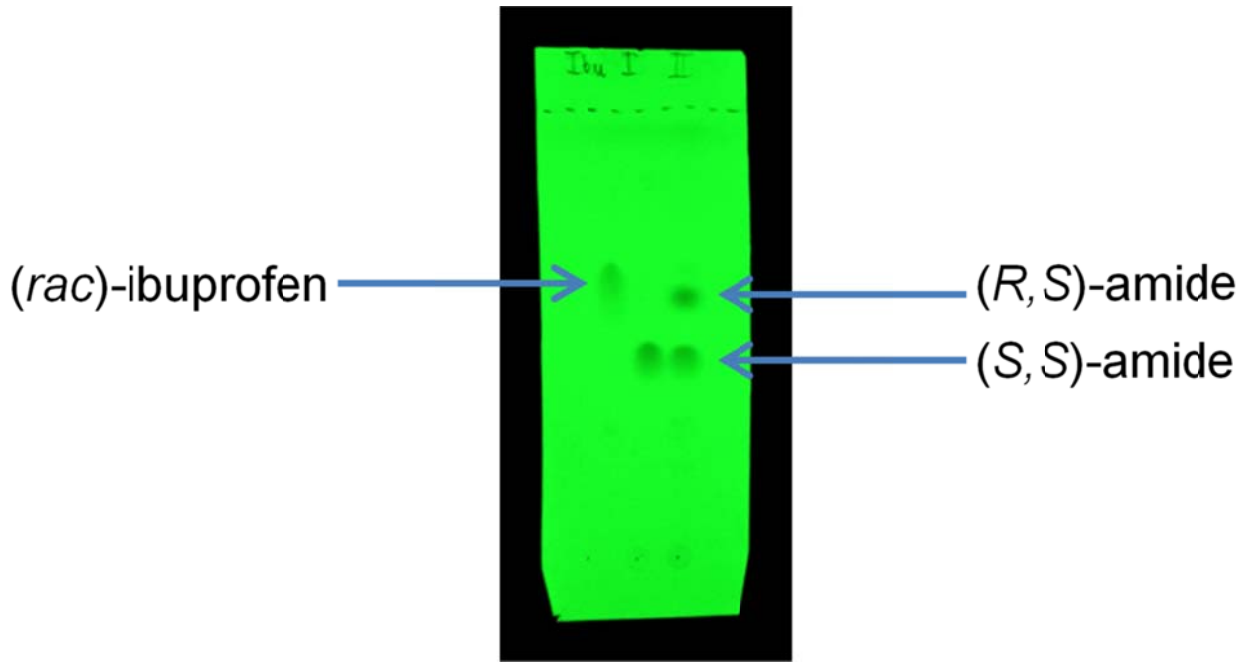


Figure SM 1.12.2 - TLC chromatogram (under UV light 254 nm) of (*rac*)-ibuprofen (**Ibu**),

diastereomeric amide(s) of (*S*)-ibuprofen (**I**) and (*rac*)-ibuprofen (**II**). Silica gel HF_{254}; diethyl ether/petroleum ether 60-80°C/acetic acid (5:5:0.01 v/v/v).

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[1] M. C. McMaster, *HPLC – A Practical User's Guide*, 2nd ed. Wiley-Interscience, New Jersey, 2007.
[2] G. P. Moss, *Pure & Appl. Chem*., 1996, **68**, 2193.
[3] Y. Kazakevich and R. Lobrutto, *HPLC for Pharmaceutical Scientists*, Wiley-Interscience, New Jersey, 2007, **22**, 987-1041.

Determining partition coefficients of sulfonamides by reversed-phase chromatography

Supplementary Material

This experiment aims determining the logarithm of the partition coefficients (log P) of three sulfonamides - sulfanilamide, sulfathiazole and sulfamethazine - by two different chromatographic methods.

Determining the log P of sulfonamides by RP-HPLC

By RP-HPLC, the analyses are carried out using octadecylsilica as stationary phase and 0.1% aqueous acetic acid /methanol: 85/15 (v/v) as mobile phase. The HPLC-UV apparatus consisted of a Finnigan Surveyor (Thermo Electron Corporation, USA) equipped with an autosampler (AutoSampler Plus) and a diode array detector TSP UV6000LP (Thermo Separation Products, USA) (**Figure SM 1.13.1**)

Prior to the experimental, a section of tips for practical HPLC analysis could be performed including aspects of solvent handling, mobile phase preparation, priming the HPLC, column handling and equilibration, and system performance checks.[1] Before the experimental takes place, the instructor should prime the HPLC system with the column installed, turn on the detector (warm-up at least 20 minutes), equilibrate the column, and record the detector response until it reaches a stable response. This procedure assures reproducible results.

It is important to emphasize that varying the proportion of the eluents or ionization suppressor (acetic acid) in the mobile phase, the results can be compromised. Measurements should be made on ionisable substances only in their non-ionised form (free acid or free base) produced by the use of an appropriate buffer. In fact, the lipophility of sulfonamides is pH dependent.[2] Moreover, the pH of the mobile phase should be within the operating range of the column, i.e. usually between 2 and 8.

The students should start by preparing the mobile phase that should be filtered, preferably through a 0.5 µm porosity filter, and degassed before use (stirring under vacuum or in an ultrasonic bath, membrane degassers, or helium purges). Students should not pressure shock the column while equilibrating with the mobile phase.

During the elution, the instructor can discuss with the students theoretical aspects of log P, practical tips for HPLC,[1] and specifically for determining log P,[2] as well as expected results considering the chemical structure of the sulfonamides. It is expected that based on the different substituents in sulfonamides, students can predict the order of retention times.

By RP-HPLC, the retention factors (expressed as log k) are used to estimate the log P of each sulfonamide since there is a correlation between these two parameters. In order to correlate the measured HPLC data of the sulfonamides with their log P, a calibration graph of log P *versus* log k is needed for a number (typically between 5 and 10) of reference compounds (preferentially related to a test compound) to define the straight-line graph over the desired range.[2] To plot the calibration graph of log P *versus* chromatographic data, solutions of seven sulfonamides, namely sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, sulfaguanidine, sulfacetamide, and sulfanilamide (**Scheme SM 1.13.1**) were injected and the retention times were evaluated in duplicate (**Figure SM 1.13.2**). The corresponding retention factors were calculated (**Table SM 1.13.1**) and plotted as a function of the experimental log P obtained for this series of sulfonamides in similar pH conditions.[3] A linear relationship between log k and experimental log P for the seven sulfonamides was obtained (**Figure SM 1.13.3**).

In this experiment, the three solutions test samples of sulfonamides (sulfanilamide, sulfathiazole, and sulfamethazine) are injected separately and their retention times evaluated in order to calculate the log k. From the correlation graph of the reference sulfonamides, the log P of each test compound can be interpolated.

<u>Determining the log P of sulfonamides by RP-TLC</u>

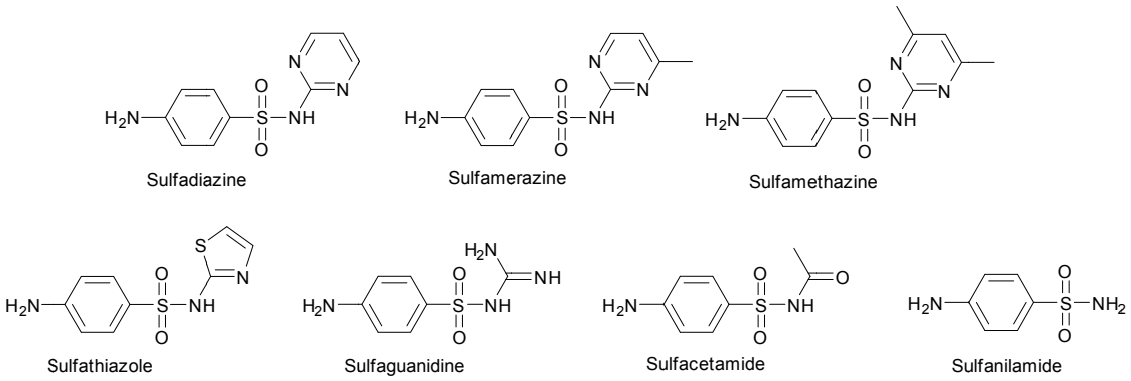
By RP-TLC, a silica gel HF_{254} plate impregnated with 1-octanol as stationary phase and an aqueous buffer at pH 7.4 as mobile phase are used to perform the chromatographic analyses.

The impregnation of the silica gel plate (home-made or precoated) can be carried out by spraying uniformly the plate with a solution of 1-octanol (5% in diethyl ether) or, alternatively, by immersion of the plate in the referred solution. Commercially precoated plates of RP-18 are also operative.

The students need to measure and, if necessary, adjust the pH of the mobile phase: potassium dihydrogen phosphate solution (acid) or sodium hydroxide solution (base) is added in order to get a pH of 7.4. A pH meter is recommended for accurate, reproducible, precise, and continuous measurements.
During the elution, the instructor can discuss with the students theoretical aspects of log P, differences between normal and reversed-phase TLC, as well as expected results considering the chemical structure of the sulfonamides being tested.
After development, the plate is dried with a stream of hot air, and yellow spots will appear by spraying the plate with a solution of vanillin hydrochloride: only one spot is observed for each sulfonamide (Rf values of 0.80, 0.53, and 0.38 for sulfanilamide, sulfathiazole, and sulfamethazine, respectively) (**Figure SM 1.13.4**). From the relationship between the derived Rm values and the 1-octanol/water partition coefficient for reference substance sulfanilamide, the partition coefficients of sulfathiazole and sulfamethazine are calculated. **Table SM 1.13.2** describes the Rm, π, and log P values obtained for each sulfonamide. Estimation of log P values is also calculated by ChemBioDraw® to support any conclusions drawn from the experimental data.
These experiments offer an opportunity to show multidisciplinary applications with two important chromatographic techniques, HPLC and TLC, involving concepts of physicochemical properties and *in silico* calculations.
After the experiments, the students should be able to conclude that reversed-phase chromatography is a rapid and accurate method for the measurement of the lipophilicity, compared to the classical "shake-flask" method. Also, the students should be aware of the range of applicability and pitfalls of each method, HPLC being automated, more reproducible and sensitive and TLC being faster and cheaper.[3]
This experimental work takes a lab section with a low hazard level and targets advanced master students. The reproducibility of the experiment was assessed by its repetitive execution, namely by 1st year Medicinal Chemistry M.Sc. students from Faculty of Pharmacy, University of Porto.

Schemes and Figures

Figure SM 1.13.1 – Photo of HPLC equipment (Finnigan Surveyor (Thermo Electron Corporation, USA) equipped with an autosampler (AutoSampler Plus) and a diode array detector TSP UV6000LP (Thermo Separation Products, USA)).



Scheme SM 1.13.1 – Chemical structures of the seven sulfonamides used to construct a correlation plot of log k *versus* log P.

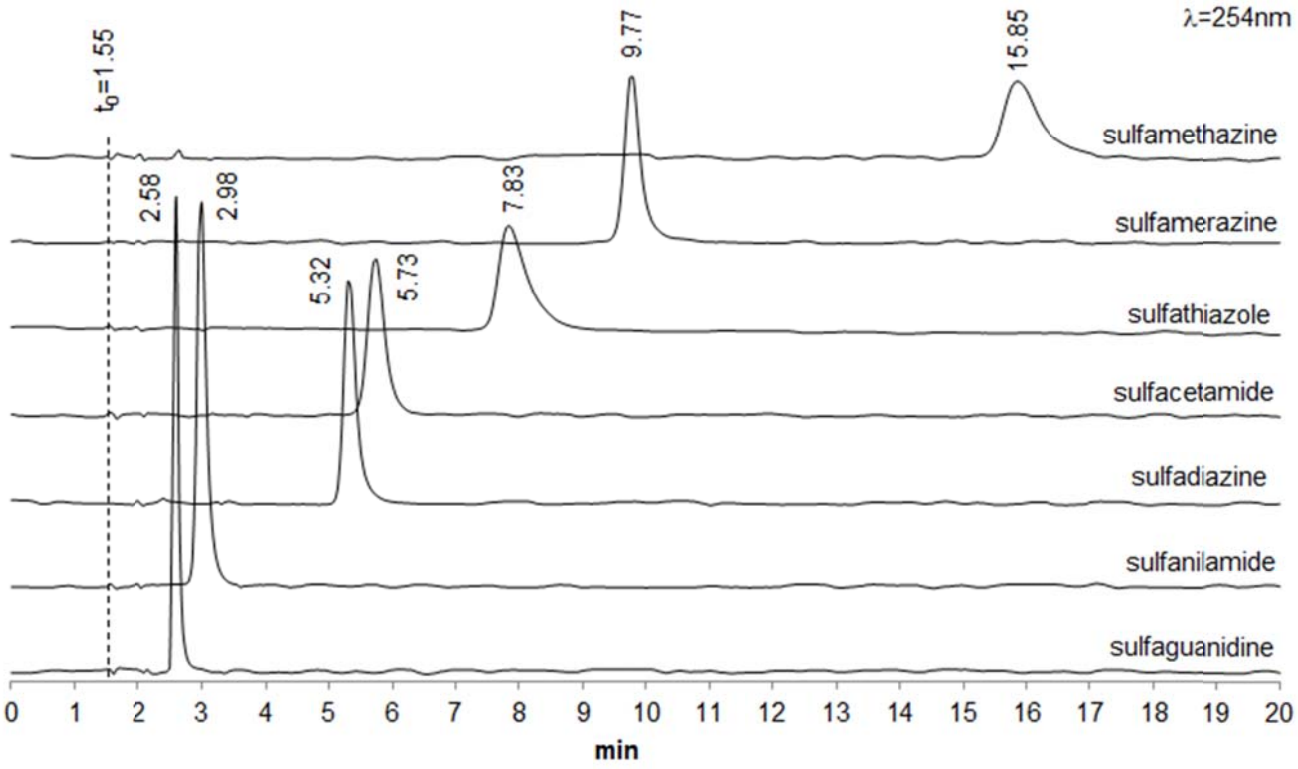


Figure SM 1.13.2 – HPLC chromatograms of the sulfonamides. Column, C18 (150 × 4.6 mm internal diameter, 5 µm particle size); mobile phase, 0.1% aqueous acetic acid/methanol: 85/15 (v/v); flow rate, 1 mL/min; detection, UV 254 nm.

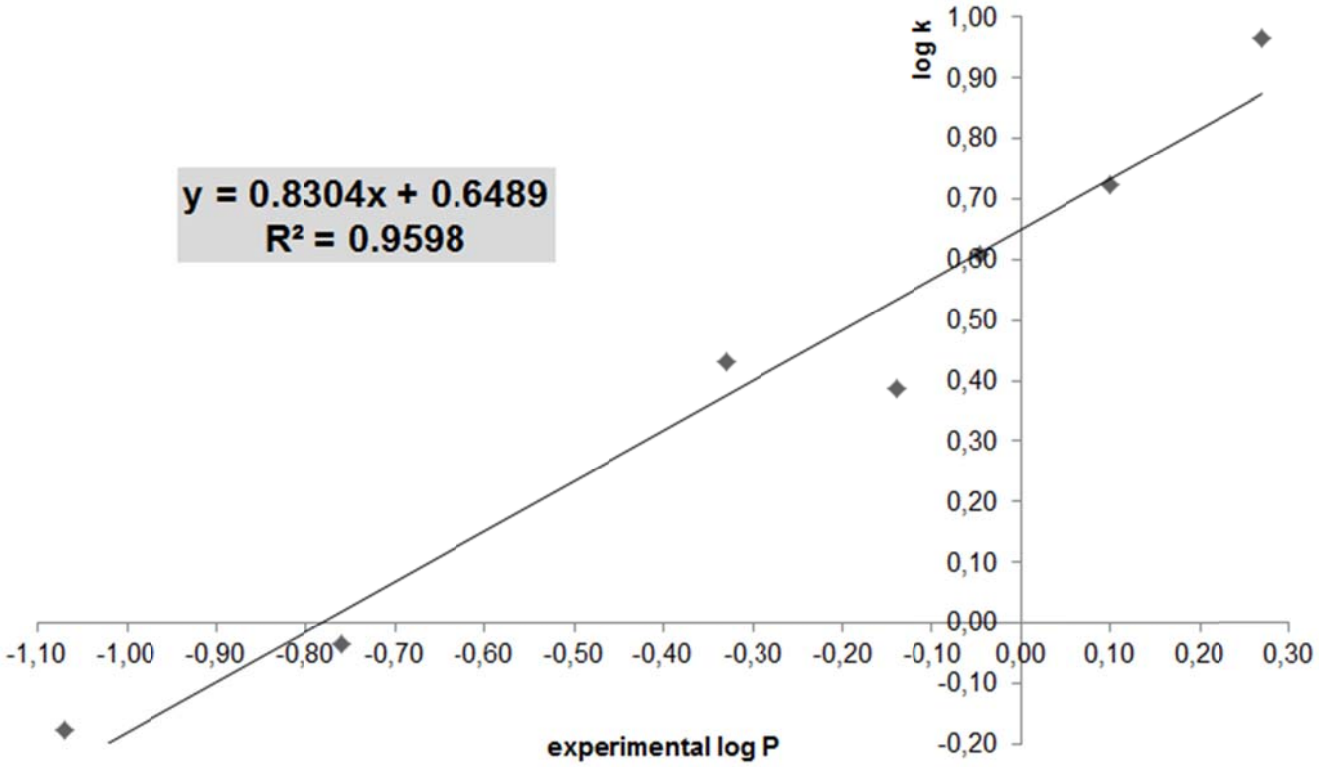


Figure SM 1.13.3 – Correlation between retention factors values (expressed as log k) and experimental log P[3] for the seven sulfonamides.

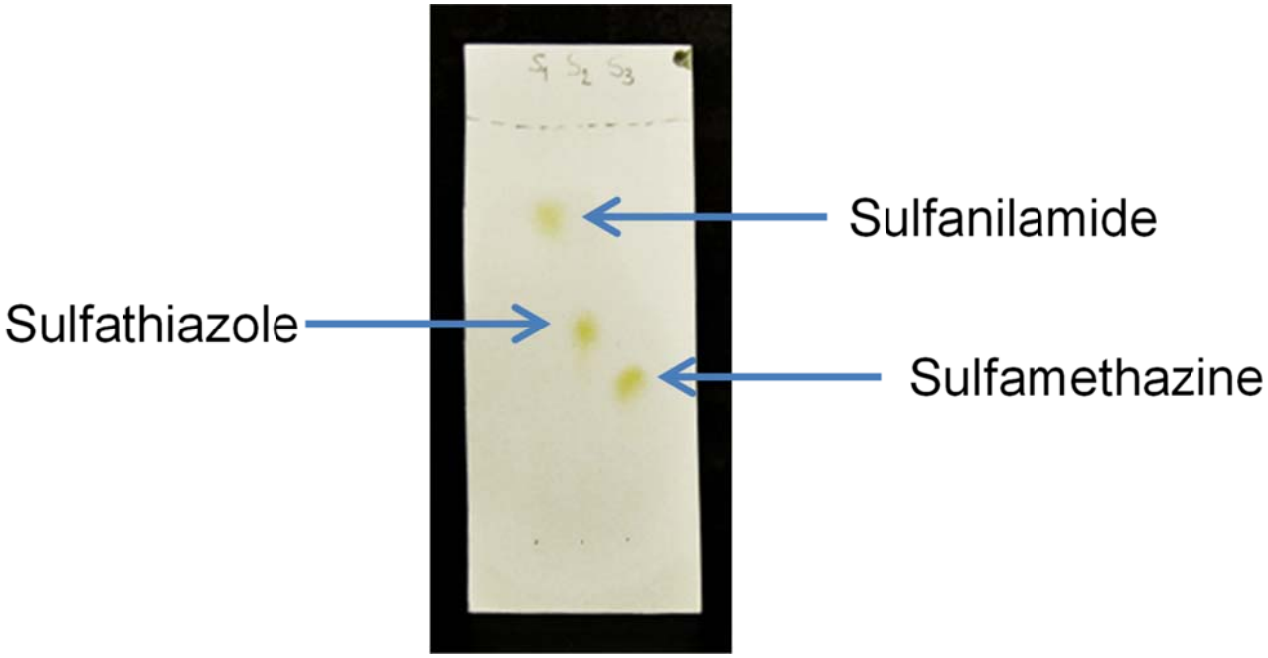


Figure SM 1.13.4 – TLC chromatogram of sulfanilamide **(S1)**, sulfathiazole **(S2)** and sulfamethazine **(S3)**. Silica gel HF_{254}; aqueous buffer at pH 7.4.

Tables

Table SM 1.13.1 – Log k and experimental log P[3] values for the seven sulfonamides.

Compound	t_R (min)	*k*	log *k*	experimental log *P*
Sulfaguanidine	2.58	0.66	-0.18	-1.07
Sulfanilamide	2.98	0.92	-0.03	-0.76
Sulfadiazine	5.32	2.43	0.39	-0.14
Sulfacetamide	5.73	2.70	0.43	-0.33
Sulfathiazole	7.83	4.05	0.61	-0.046
Sulfamerazine	9.77	5.30	0.72	0.10
Sulfamethazine	15.85	9.23	0.97	0.27

mean values with SD <10%

Table SM 1.13.2 – Rm, π, and log P values for sulfanilamide, sulfathiazole and sulfamethazine.

Compound	Rm	π	log P
Sulfanilamide	-0.06	---	-0.82 [4]
Sulfathiazole	0.42	1.02	0.20
Sulfamethazine	0.39	0.99	0.17

mean values with SD <15%

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[1] M. C. McMaster, *HPLC – A Practical User's Guide*, 2nd ed. Wiley-Interscience, New Jersey, 2007.

[2] *OCDE Guidelines for the Testing of Chemicals, Partition Coefficient (n-octanol/water), High Performance Liquid Chromatography (HPLC) Method*, 2004, **117**, 1-11 (see http://www.oecd-ilibrary.org/environment/test-no-117-partition-coefficient-n-octanol-water-hplc-method_9789264069824-en).

[3] S. Carda-Broch and A. Berthod, *Chromatographia*, 2004, **59**, 79.

[4] M. A. F. Prado. *J. Chem. Educ*., 2001, **78**, 533.