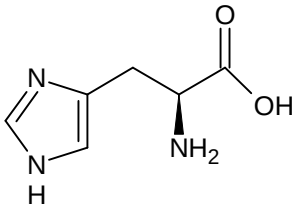
PRACTICAL PROBLEMS

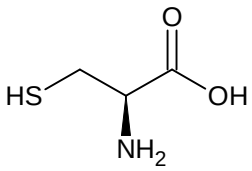
PROBLEM 1 (Practical)

Ion-exchange chromatography of amino acids

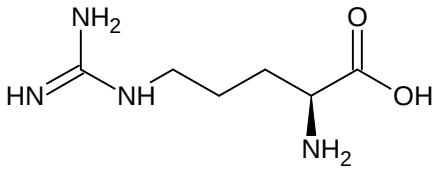
Ion-exchange chromatography is an important analytical and preparative method, which allows fractioning of charged substances. Interaction of ionic groups of the substances with counterions attached to the resin is behind the method. In this task you will have to carry out separation of a given mixture of three amino acids followed by quantitative assay of individual amino acids eluted from the column by using specific chromogenic reactions. Since queues of students are possible at spectrophotometers, we strongly suggest you starting the exam with Problem 1.



His



Cys



Arg

Three amino acids (see the structures above) are present in the mixture. These are histidine, cysteine, and arginine. Cross-linked sulfonated polystyrene is used as a cation-exchange resin (see the picture of the system below). At the beginning of the experiment the column is equilibrated with Eluent 1 (*pH* 4.9).

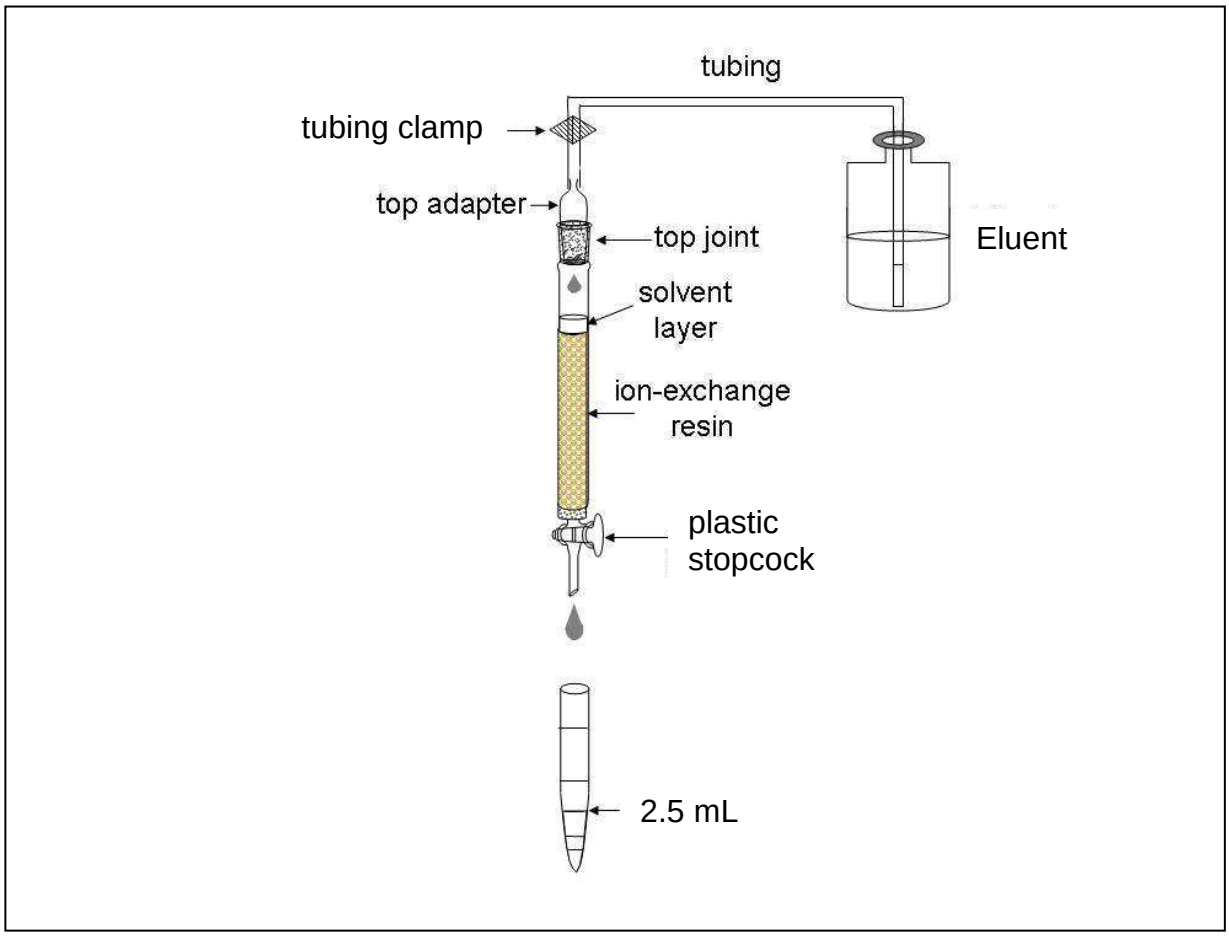
Procedure

Chromatography. Step 1

Apply the given solution of a mixture of amino acids to the column. First, open the stopcock to allow the solvent in the column draining into the Erlenmeyer flask labelled "Waste" so that the solvent is level with the top of packing material, still preventing the resin surface from drying off. Close the stopcock and carefully add the analyzed solution to the top of the column by using a syringe. Open the stopcock and let the sample soak inside the gel (drain the solvent into the "Waste" flask). Close the stopcock and add about 1 cm^3 of Eluent 1 (corresponds to ~ 1 cm of liquid in the column) by carefully releasing the

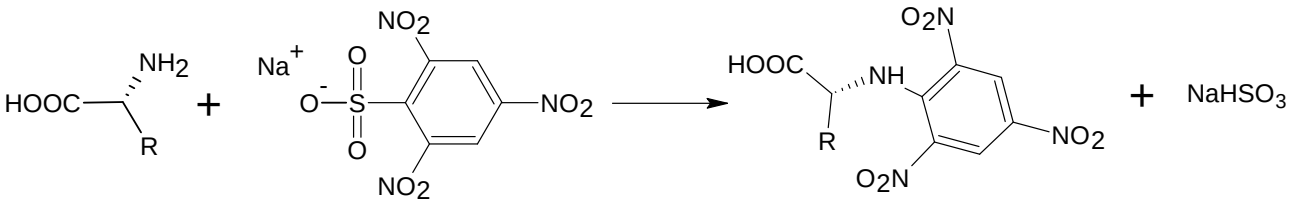
tubing clamp. Attach the top joint tightly, fixing the column with one hand and the adaptor with the other (be sure that the joint is fitted closely to the column). Replace the “Waste” flask at the stand with the test tubes in the rack. Release the tubing clamp and open the stopcock to let the eluent flow down through the column. Proceed with elution. (Always pen the stopcock to start elution and close the stopcock to stop it).

Collect the fractions in the test tubes up to the volume of 2.5 cm^3 (as shown in the Picture). If needed, label them with marker. After collecting each 4 to 8 fractions stop elution and carry out qualitative analysis of the collected samples.



Qualitative analysis of samples

Qualitative assay of amino acids is based on the reaction of their α-amino groups with sodium 2,4,6–trinitrobenzene sulfonate (TNBS):



The assay is carried out in the wells of a polystyrene plate, each well corresponding to a definite test tube. Before starting the assay, mix 1 cm^3 of TNBS solution with 10 cm^3 of carbonate buffer solution and place 0.1 cm^3 of the resulting mixture into half of the plate wells (from A1 to H5). Then add 0.1 cm^3 of the analyzed fraction into a well. Start with A1 well and continue with B1, C1, etc (move top to bottom and left to right). If an amino acid is present in the analyzed fraction, intense yellow coloration will develop in the corresponding well within 3 min. Use the coloration in the first well as the reference. To reliably estimate the coloration, place the plate on the white sheet of paper.

Note: all aliquots of 0.1 cm^3 should be added by using the pipettor. We expect you to use one tip for all fractions of a single peak.

1.1 a Draw the profile of coloration intensity (qualitatively) on the plate sketch in the Answer Sheet. Use the following symbols: (-) – no coloration, 1 – weak coloration, 2 – moderate coloration and 3 – intense coloration. Keep drawing the profile during the whole chromatography process.

Continue collecting fractions and analyzing them until you get at least two wells with coloration as in A1 well, which will indicate that the first amino acid has left the column completely (end of the first peak).

Chromatography. Step 2

As soon as you are finished with collecting the first peak, change to Eluent 2. To do so, close the stopcock, fix the tubing clamp (Important!), disconnect the tubing leading to the bottle with Eluent 1 and connect the tubing leading to the bottle with Eluent 2. Attach the top joint tightly.

1.1 b Indicate when the eluents have been changed by drawing lines between the corresponding wells on the plate sketch.

Continue elution, collecting fractions and carrying out qualitative analysis of samples as described above.

Chromatography. Step 3

As soon as you are finished with collecting the second peak, change to Eluent 3 as described in Step 2. Continue chromatography until the third amino acid leaves the column completely.

Stop chromatography by closing the stopcock and fixing the clamp.

Based on the results of qualitative analysis, choose the fractions which contain the amino acids.

1.1 Write down in the Answer Sheet the labels of wells corresponding to the chosen fractions.

1.2 Combine the fractions from each peak and measure the volumes of combined fractions using a measuring cylinder. Report the volumes of combined fractions excluding amounts used for the qualitative analysis. Write down the obtained results in the Answer Sheet.

Pour combined fractions in the amber glass vials labelled “Peak 1”, “Peak 2”, “Peak 3”. Prepare samples for quantitative spectrophotometric analysis as described below.

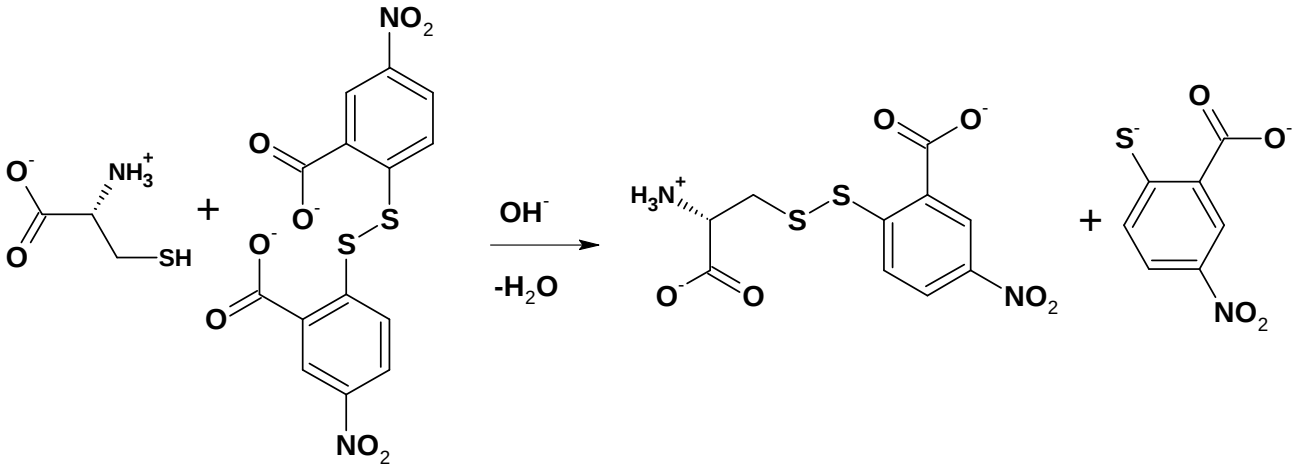
When finished with Practical exam, close the vials and leave them on the table. The combined fractions will be subsequently analyzed by lab staff.

Spectrophotometric analysis

For each probe, you should submit two cells to the operator. Prepare the probes as follows.

Important! When storing, always put cell in the cell holder! All cells have 2 ribbed and 2 working vertical surfaces. While operating with cells, do not touch working surfaces, otherwise you may get incorrect values of absorbance.

Assay 1 (peak 1). Cysteine concentration is determined by the Ellmann reaction:



Test tube A_1 (Reference).

Place 0.1 cm^3 of Eluent 1 from plastic microtube into a test tube and add 2.9 cm^3 of Ellmann reagent (DTNB).

Test tube B_1 (Sample).

Place 0.1 cm^3 of the analyzed solution into a test tube and add 2.9 cm^3 of Ellmann reagent (DTNB).

Mix the contents of the test tubes thoroughly and transfer each mixture to the corresponding cells labelled A_1 (for reference) and B_1 (for sample).

Assay 2 (peak 2). Determination of histidine concentration is based on the ability of imidazole moiety to react with diazonium compounds (Pauli reaction).

Test tube A_2 (Reference).

Place 2.8 cm^3 of Tris-HCl buffer solution into a test tube, add 0.1 cm^3 of Eluent 2 from plastic microtube and 0.1 cm^3 of Pauli reagent.

Test tube B_2 (Sample).

Place 2.8 cm^3 of Tris-HCl buffer solution into a test tube, add 0.1 cm^3 of the analyzed solution and 0.1 cm^3 of Pauli reagent.

Mix the contents of the test tubes thoroughly and transfer each mixture to the corresponding cells labelled A_2 (for reference) and B_2 (for sample).

Assay 3 (peak 3). Determination of arginine concentration is based on the ability of guanidinium moiety to react with some phenols under alkaline and oxidative conditions

(Sakaguchi reaction).

Test tube A_3 (Reference).

Place 0.1 cm^3 of Eluent 3 into a test tube and add 1.5 cm^3 of 10 % NaOH solution, 1 cm^3 of 8-hydroxyquinoline solution and 0.5 cm^3 of sodium hypobromite solution.

Test tube B_3 (Sample).

Place 0.1 cm^3 of the analyzed solution into a test tube and add 1.5 cm^3 of 10 % NaOH solution, 1 cm^3 8-hydroxyquinoline solution and 0.5 cm^3 of sodium hypobromite solution.

Shake the test tubes vigorously for 2 min (**Important!**) and observe formation of orange colour. Add 0.2 cm^3 of 8 M urea solution to each test tube, mix the contents and transfer about 3 cm^3 of each mixture to the corresponding cells labelled A_3 (for reference) and B_3 (for sample).

All mixtures should be analyzed by spectrophotometry not earlier than 10 min and not later than 2 h after preparation. Submit the set of 6 cells to the spectrophotometer operator. In case of a queue at the spectrophotometer, ask the operator to put your student code on the list at the signboard. You will be invited by the operator in due time. Meanwhile, you can answer the theoretical question and start fulfilling Problem No 2.

In case your sample(s) have not been subjected to studies within the proper time interval (which is quite improbable), prepare the sample(s) afresh.

Get the print-offs with the spectra of your samples and check it. Sign the print-offs and get the operator's signature.

1.3 Determine absorbance at the corresponding wavelengths and calculate the content (in mg) of each amino acid in the mixture you were given. The optical length is 1.0 cm. Complete the Answer Sheets taking into account that one mole of each amino acid gives one mole of the corresponding product.

Reference data:

The values of extinction coefficients:	Molar masses of the amino acids.
Product of Ellmann reaction: 13600 $M^{-1}cm^{-1}$ at 410 nm	Cysteine: 121 g mol^{-1}
Product of Pauli reaction: 6400 $M^{-1}cm^{-1}$ at 470 nm	Histidine: 155 g mol^{-1}
Product of Sakaguchi reaction: 7700 $M^{-1}cm^{-1}$ at 500 nm	Arginine: 174 g mol^{-1}

1.4 Draw three resonance structures of the species responsible for mixture coloration as a result of Ellmann reaction.

_____

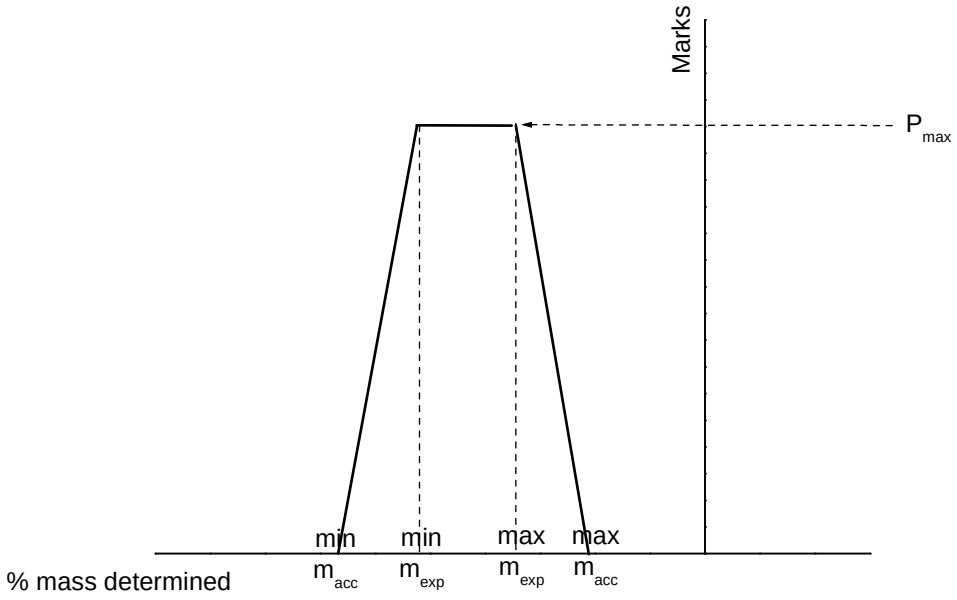
SOLUTION

1.2 – 1.3

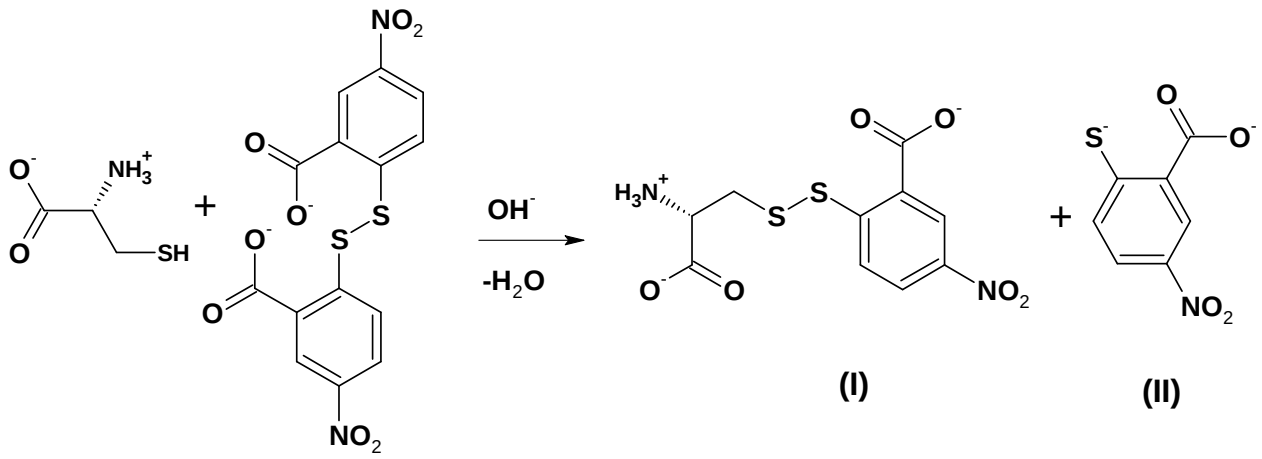
Content of an amino acid = $\frac{A_\lambda\, n\, V\, M}{\varepsilon\, l}$,

A_λ is the absorbance of the sample calculated from the spectra, l is the optical length (1.0 cm), n is the dilution factor determined as a ratio of the aliquot of analyzing solution (0.1 cm^3) and the final volume of the sample in the cells, V is the volume of the combined fraction from the corresponding peak, and M is the molar mass of the amino acid.

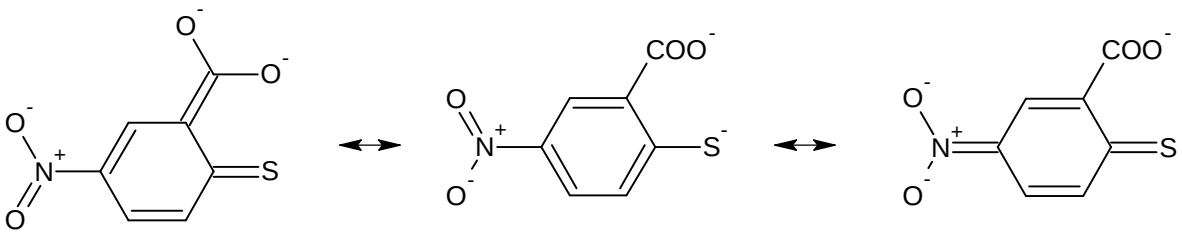
Content of each amino acid is determined by using the following plot (values recalculated from volumes reported by students and absorbance values recorded by spectrophotometer).



1.4 As it is given in the task text, mixed disulfide and 2-thio-5-nitrobenzoic acid are formed in the reaction.



Under slightly alkaline conditions, thiol group of (**II**) dissociates, and thiophenolate-anion is formed. Resonance structures can be realized for this compound:



The electronic structure of asymmetrical disulfide (**I**) does not differ considerably from that of the original Ellmann reagent. Therefore, it can be concluded that the compound responsible for coloration is thiophenolate-anion (a form containing C=S bond).

PROBLEM 2 (Practical)

Determination of Carbonate and Hydrogen Phosphate in an Abrasive Sample

Na_2CO_3, $CaCO_3$ and Na_2HPO_4 are the main constituents of abrasive powders. In this task you will have to determine carbonate and hydrogen phosphate ions in an abrasive sample by two acid-base titrations.

First, the exactly known amount of hydrochloric acid (taken in an excess) is added to the sample. As a result, hydrogen phosphates are transformed into H_3PO_4, whereas carbonates into CO_2 which is further removed by boiling. Calcium ions initially present in the sample pass into the solution. Because of possible interference in subsequent analysis, they are precipitated as CaC_2O_4 and filtered off prior to the titration.

Next, the phosphoric acid formed is subjected to two titrations with pre-standardized NaOH solution and two different indicators: Bromocresol Green (BCG) and Thymolphthalein (TP). First, H_3PO_4 (and excess of HCl) is titrated to $H_2PO_4^-$ ion, the endpoint lying in slightly acidic medium (*pH* of ~4.5). It corresponds to the colour change of BCG from yellow to blue. The second titration proceeds till HPO_4^{2-} is formed. The endpoint of the second titration corresponds to the color change of TP from colourless to blue (moderately alkaline medium, *pH* of ~10).

The content of CO_3^{2-} ions in the sample is calculated by finding the difference between:

a) the amount of the titrant equivalent to the initial amount of HCl (taken for the sample dissolution) and
b) the amount of the titrant corresponding to the second endpoint (TP).

The content of HPO_4^{2-} is calculated by finding the difference between the amounts of the titrant consumed to achieve two endpoints (TP and BCG).

Procedure

Step 1. <u>Dissolution of the sample and removal of CO_2</u>

To the sample of the abrasive powder in a beaker covered with watch glass add 10.00 cm^3 (exactly, with a pipette! Carefully, not removing the glass and avoiding losses because of splashing!) of ca. 1 mol dm^{-3} HCl (see the exact concentration of the acid on the label). After the most intensive stage of gas evolution is completed, heat

carefully the solution in the beaker (covered with watch glass) on a heating plate until the gas evolution stops. Then bring the solution to boiling and boil it carefully for 2 – 3 min.

Step 2. Precipitation of calcium

Remove the beaker from the plate; wash the steam condensate from the watch glass down to the beaker with distilled water. Add 1 – 2 cm^3 of 15 % $K_2C_2O_4$ solution with measuring cylinder. Put the beaker aside until the most part of the precipitate is formed (usually takes 10 to 20 min). Spend this time for standardization of the titrant solution of NaOH (see the procedure hereunder).

Step 3. Standardization of NaOH solution

Place with a pipette 10.00 cm^3 of HCl solution into a 100 cm^3 volumetric flask, make up to the mark with distilled water and mix. Fill the burette with NaOH solution. Transfer with a pipette 10.00 cm^3 of the diluted HCl solution from the volumetric flask to an Erlenmeyer flask. Add 1 – 2 drops of Thymolphthalein solution and titrate with NaOH solution until blue coloration stable on swirling for 5 – 10 s appears.

Here and after. Repeat the titrations as necessary. It is desirable that the highest and the lowest titrant volume values differ not more than by 0.10 cm^3. Report all the final volume values with 0.01 cm^3 accuracy.

2.1 a Complete the table in the Answer Sheet.

2.1 b Calculate the concentration of NaOH solution (in mol dm^{-3}).

Step 4. Filtering off calcium oxalate

After the most part of CaC_2O_4 precipitates filter the precipitate off collecting the filtrate into a 100 cm^3 volumetric flask. Slight turbidity in the filtrate is admissible, since small amounts of calcium oxalate do not interfere in the titration. Wash the filter with distilled water; make up the solution in the flask to the mark with distilled water and mix. Put the used filter into the waste basket.

Step 5. Sample titration against Bromocresol Green

Transfer with a pipette a 10.00 cm^3 aliquot of the sample solution coming from the

step 4 from the volumetric flask to an Erlenmeyer one, and add 3 drops of BCG solution. Prepare in another Erlenmeyer flask a reference solution by adding 3 drops of 15 % NaH_2PO_4 solution and 3 drops of BCG solution to 15 – 20 cm^3 of distilled water. Titrate the sample solution with NaOH solution until the colour coincides with that of the reference solution.

2.2 Complete the table in the Answer Sheet.

Step 6. Sample titration against thymolphthalein

Transfer with a pipette a 10.00 cm^3 aliquot of the sample solution coming from the step 4 from the volumetric flask to an Erlenmeyer one. Add 2 drops of TP solution and titrate with NaOH solution until blue coloration stable on mixing for 5 – 10 s appears.

2.3 Complete the table in the Answer Sheet.

Step 7. Calculations

2.4 Calculate the mass of CO_3^{2-} in the sample.

2.5 Calculate the mass of HPO_4^{2-} in the sample.

Step 8. Additional questions to the problem

Answer the additional questions in the Answer Sheets.

2.6a Indicate one reaction (write down the equation) for a process interfering in the sample analysis you have carried out in the presence of Ca^{2+}.

2.6b A list of mistakes possible at different steps is given in the table in the answer sheet. Indicate which of the mistakes can lead to errors in CO_3^{2-} and/or HPO_4^{2-} content determination. Use the following symbols: “0” if no error is expected, “+”or “–“ if the result is higher (positive error) or lower (negative error) than the true one.

SOLUTION

2.1 a, 2.2, 2.3

The values of the final volumes $V_{1,f}$, $V_{2,f}$, and $V_{3,f}$, (as reported in the Answer Sheet) were graded according to a scheme approved by the International Jury.

The values of $\Delta V_{expected}$ and $\Delta V_{acceptable}$ (in cm^3) are listed in the table below.

	ΔV, cm^3	
	expected	acceptable
$V_{1,f}$	**0.10**	**0.25**
$V_{2,f}$	**0.15**	**0.40**
$V_{3,f}$	**0.15**	**0.40**

2.1 b Calculation of NaOH concentration

$$c(\text{NaOH}) = \frac{c(\text{HCl}) \times V(\text{HCl}) \times V(\text{aliquot})}{V(\text{flask}) \times V(\text{NaOH})} = \frac{1.214\ \text{mol dm}^{-3} \times 10.00\ \text{cm}^3 \times 10.00\ \text{cm}^3}{100.0\ \text{cm}^3 \times V_{1,f}\text{cm}^3}$$

2.4 Calculation of the mass of CO_3^{2-}

$$m(CO_3^{2-}) = M(CO_3^{2-}) \times 1/2 \times \frac{c(\text{NaOH}) \times (V_{1,f} - V_{3,f}) \times V(\text{flask})}{V(\text{aliquot})} =$$

$$= 60.01\ \text{g mol}^{-1} \times 1/2 \times \frac{c(\text{NaOH})\ \text{mol dm}^{-3} \times (V_{1,f} - V_{3,f})\ \text{cm}^3 \times 100.0\ \text{cm}^3}{10.00\ \text{cm}^3} \times 0.001\ \text{dm}^3/\text{cm}^3 =$$

2.5 Calculation of the mass of HPO_4^{2-}

$$m(HPO_4^{2-}) = M(HPO_4^{2-}) \times \frac{c(\text{NaOH}) \times (V_{3,f} - V_{2,f}) \times V(\text{flask})}{V(\text{aliquot})} =$$

$$= 95.98\ \text{g mol}^{-1} \times \frac{c(\text{NaOH})\ \text{mol dm}^{-3} \times (V_{3,f} - V_{2,f})\ \text{cm}^3 \times 100.0\ \text{cm}^3}{10.00\ \text{cm}^3} \times 0.001\ \text{dm}^3/\text{cm}^3 =$$

2.6 a

$Ca^{2+} + H_2PO_4^- \rightarrow CaHPO_4 + H^+$

or

$3\ Ca^{2+} + 2\ HPO_4^{2-} \rightarrow Ca_3(PO_4)_2 + 2\ H^+$