Univerza v Ljubljani
Biotehniška fakulteta

DODATNO ŠTUDIJSKO GRADIVO ZA VAJE IZ ŽIVILSKE KEMIJE

ADDITIONAL STUDY MATERIALS FOR FOOD CHEMISTRY EXERCISES

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PREDGOVOR

Gradivo je namenjeno študentom pri laboratorijskih vajah pri predmetu Živilska kemija prvostopenjskega univerzitetnega študijskega programa Živilstvo in prehrana ter predstavlja dodatek k obstoječemu osnovnemu študijskemu gradivu “Abram in Zelenik-Blatnik, Vaje iz živilske kemije za študente živilske tehnologije, 2002”. Dodatno študijsko gradivo povzema in hkrati nadgrajuje teoretična izhodišča ter razširja raziskovalni pristop laboratorijskih vaj, ki so opisane v omenjenem osnovnem študijskemu gradivu. Avtorja meniva, da je za razumevanje relativno kompleksne problematike v veliko pomoč nazorna shematska ponazoritev teoretičnih osnov in praktične izvedbe ter izčrpna razlaga obdelave eksperimentalnih podatkov, kar sva vključila v dodatno študijsko gradivo.

V izvedbo predmeta Živilska kemija so hkrati z rednimi študenti vključeni študenti iz mednarodnega okolja (Erasmus izmenjava). Z namenom, da bi tudi tem študentom ponudili ustrezno, s slovenskim usklajeno gradivo, sva pripravila različico gradiva v angleškem jeziku.

S pričujočim gradivom želiva študentom omogočiti učinkovito pripravo in kvalitetnejšo izvedbo laboratorijskih vaj pri predmetu Živilska kemija, povečati varnost pri delu v laboratoriju, okrepiti razumevanje in samostojnost pri delu ter izboljšati ključne kompetence.

OSNOVE KEMIJSKEGA RAČUNANJA

Koncentracijske enote

Masni delež topljenca ($w_{topljenec}$) pove, kolikšen delež od mase raztopine ($m_{raztopina}$) predstavlja masa topljenca ($m_{topljenec}$). Enota: %.

$$w_{topljenec} = \frac{m_{topljenec}}{m_{raztopina}} \times 100\ \%$$

Za raztopino, ki je pripravljena kot zmes različnih tekočin, se vsebnost tekočega topljenca izrazi kot volumenski delež ($\varphi_{topljenec}$). Volumenski delež topljenca pove, kolikšen delež od celotne prostornine raztopine ($V_{raztopina}$) predstavlja volumen čistega tekočega topljenca ($V_{topljenec}$). Enota: %.

$$\varphi_{topljenec} = \frac{V_{topljenec}}{V_{raztopina}} \times 100\ \%$$

Masna koncentracija (γ) pove, kolikšna je masa topljenca v določenem volumnu raztopine. Enota: g/mL, mg/mL, g/L, ….

$$\gamma = \frac{m_{topljenec}}{V_{raztopina}}$$

Množinska (molarna) koncentracija (c) pove, kolikšna je množina topljenca v 1 L raztopine. Enota: mol/L.

$$c = \frac{n_{topljenec}}{V_{raztopina}}$$

V primeru razredčevanja bolj koncentrirane raztopine (izhodna raztopina) postopamo tako, da odmerimo določen volumen (najpogosteje s pipeto) izhodne raztopine ($V_{p\ izhodna}$) in dodamo toliko topila, da pripravimo ustrezni volumen razredčene raztopine ($V_{razredč\ raztopina}$). Koncentracijo topljenca v razredčeni raztopini izračunamo:

$$\text{koncentracija topljenca v razredčeni raztopini} = \frac{\text{koncentracija topljenca v izhodni raztopini} \times V_{p\ izhodna}}{V_{razredč\ raztopina}}$$

DOLOČITEV GLUKOZE IN SAHAROZE V SKUPNEM VZORCU

Glukoza

D(+)-glukoza (grozdni sladkor ali dekstroza) je aldoheksoza z molekulsko formulo $C_6H_{12}O_6$.

V večini živil je glukoza poleg fruktoze najbolj zastopan monosaharid. V razmeroma visokem deležu je prisotna v sadju, kjer vsebnost narašča s stopnjo zrelosti. Kot monomerna enota je gradnik številnih disaharidov (npr. saharoza, laktoza, maltoza), oligo- in polisaharidov (npr. β-glukani, amiloza in amilopektin (škrob), celuloza) ter spojin, ki nastanejo v reakciji med hemiacetalno –OH skupino glukoze in nesladkorno molekulo (ki ji rečemo aglikon), npr. z alkoholom, fenolno spojino, ….

V živilstvu predstavlja glukoza surovino za pridobivanje poliola sorbitola (redukcija karbonilne skupine), glukonske kisline in laktona (oksidacija karbonilne skupine) ter za proizvodnjo etanola (s fermentacijo).

V prisotnosti oksidantov, kot sta *Luffov* reagent in *Fehlingov* reagent (alkalna raztopina Cu^{2+} ionov), poteče oksidacija glukoze (karbonilna skupina) v karboksilno kislino. Pri tem se Cu^{2+} reducira v Cu^{+}. Zato pravimo, da je glukoza reducirajoči sladkor.

V alkalnih pogojih (ali encimsko katalizirana) poteče izomerizacija ketoze v aldozo, zato tudi fruktoza (ki kot ketoza ni reducirajoč sladkor) v takih pogojih deluje kot “reducirajoči sladkor”.

Saharoza

Saharoza (namizni sladkor, trsni sladkor, pesni sladkor) je α-D-glukopiranozil-β-D-fruktofuranozid z molekulsko formulo $C_{12}H_{22}O_{11}$. D-glukopiranoza in D-fruktofuranoza sta povezani z α,β-(1→2)-glikozidno vezjo. Reducirajoča konca obeh molekul sta vključena v glikozidno vez in zato saharoza ni reducirajoči sladkor (to pomeni, da je manj reaktivna).

Saharoza je najbolj pogost disaharid v rastlinah, kjer ima vlogo v transportu sladkorjev in kot energijska zaloga. V večjih količinah se kopiči v vegetativnih delih rastline, kot so listi in stebla (sladkorni trs do okoli 25 %), ter v sadežih (jabolka, pomaranče, marelice, breskve do okoli 8 %). V sadežih nekaterih vrst (npr. grozdje, fige) je saharoze malo, saj med dozorevanjem hidrolizira. V zelenjavi je znatna vsebnost saharoze v sladkorni pesi (do okoli 20 %) in čebuli (do okoli 10 %). Pri krompirju je pred polno zrelostjo v gomoljih vsebnost saharoze razmeroma visoka in med dozorevanjem pade.

Industrijski vir za pridobivanje saharoze sta sladkorna pesa in sladkorni trs. Na nekaterih geografskih območjih so v uporabi tudi drugi viri (npr. datelji, javorjev sok, ...).

V kislih pogojih ali po vplivom encima β-fruktofuranozidaza (invertaza) pride do cepitve (hidrolize) glikozidne vezi v molekuli saharoze. Pri cepitvi glikozidne vezi v molekuli saharoze nastane zmes glukoze in fruktoze. Pri cepitvi vezi v enem molu saharoze nastaneta dva mola monosaharidov; poleg tega je relativna sladkost glukozno-fruktoznega sirupa večja od sladkosti saharoze. Zato je raztopina nastale zmesi slajša od raztopine saharoze. Ime invertni sladkor za saharozo je zaradi inverzije: specifična sučnost linearno polarizirane svetlobe za saharozo je +66,5°, za zmes monosaharidov po hidrolizi saharoze pa –33,3°.

V živilstvu se saharoza uporablja kot sladilo, saj je po okusu sladka (tako kot večina mono- in disaharidov). Saharoza je zaradi veliko hidrofilnih –OH skupin v molekuli dobro topna v vodi (tako kot ostali mono- in disaharidi ter krajši oligosaharidi). To omogoča pripravo zelo koncentriranih raztopin (sirupi, med) z visoko osmolarnostjo, kar zavira rast mikroorganizmov. Saharoza v živilih deluje lahko tudi kot humektant.

V prehrani predstavlja saharoza vir energije. Encim β-fruktofuranozidaza v sluznici tankega črevesa katalizira cepitev glikozidne vezi. Nastala monosaharida se absorbirata ter preideta v kri.

Namen vaje

Določiti maso glukoze ($m_{glukoza}$) in maso saharoze ($m_{saharoza}$) v skupnem vzorcu z metodo po *Schoorl-Luffu*.

Princip vaje

Določitev glukoze temelji na oksidacijsko-redukcijski reakciji, ki poteče ob dodatku *Luffovega* reagenta (alkalna raztopina bakrovega citratnega kompleksa), ko glukoza (reducirajoči sladkor) v alkalnem mediju reducira Cu^{2+} do Cu_2O.

Določitev saharoze (ni reducirajoči sladkor) v skupnem vzorcu temelji na cepitvi glikozidne vezi (hidroliza) ter določitvi nastalih produktov hidrolize (glukoza in fruktoza) ter že sicer v vzorcu prisotne glukoze z dodatkom *Luffovega* reagenta. Pri tem v alkalnih pogojih poteče izomerizacija fruktoze v reducirajoči sladkor.

Ob dodatku raztopine reducirajočega sladkorja k *Luffovem* reagentu poteče oksidacijsko-redukcijska reakcija, v kateri se nastali Cu_2O izloči kot rdeče-rjava oborina.

Ob dodatku jodovice poteče naslednja oksidacijsko-redukcijska reakcija v prebitni množini joda:

$$Cu_2O + I_2 + 4\ HCl \rightarrow 2\ CuCl_2 + 2\ HI + H_2O$$

Izvedemo povratno titracijo, tako da določimo množino preostalega I_2 ($n_{I_2\ preostali}$; presežek glede na množino Cu_2O) s titracijo z raztopino $Na_2S_2O_3$; to je analiza vzorca.

$$2\ Na_2S_2O_3 + I_2 \rightarrow 2\ NaI + Na_2S_4O_6$$

Celotno množino dodanega I_2 ($n_{I_2\ celotni}$) določimo z analizo slepega vzorca, ki jo izvedemo tako kot analizo vzorca, le da namesto reducirajočega sladkorja k *Luffovem* reagentu dodamo vodo.

Množino reducirajočega sladkorja oz. množino Cu_2O, ki se sprosti v reakciji med reducirajočim sladkorjem in *Luffovim* reagentom, določa razlika:

$$n_{I_2\ zreagirani} = n_{I_2\ celotni} - n_{I_2\ preostali}$$

Eksperimentalni postopek

a) Priprava razredčenega vzorca

b) Določitev glukoze

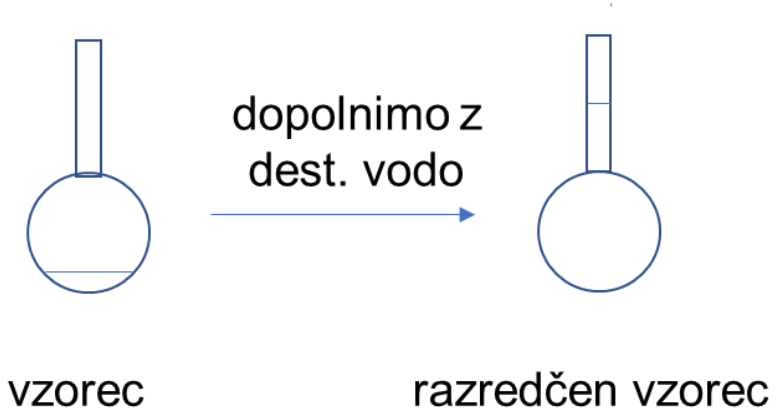
c) Hidroliza - po hidrolizi glikozidne vezi v saharozi so v hidrolizatu prisotni produkti hidrolize ter glukoza, ki je že sicer prisotna v vzorcu

d) Določitev vseh reducirajočih sladkorjev v vzorcu (glukoza, ki je že sicer prisotna v vzorcu + reducirajoči sladkorji nastali s hidrolizo)

e) Analiza slepega vzorca (voda namesto raztopine sladkorjev)

a) Priprava razredčenega vzorca

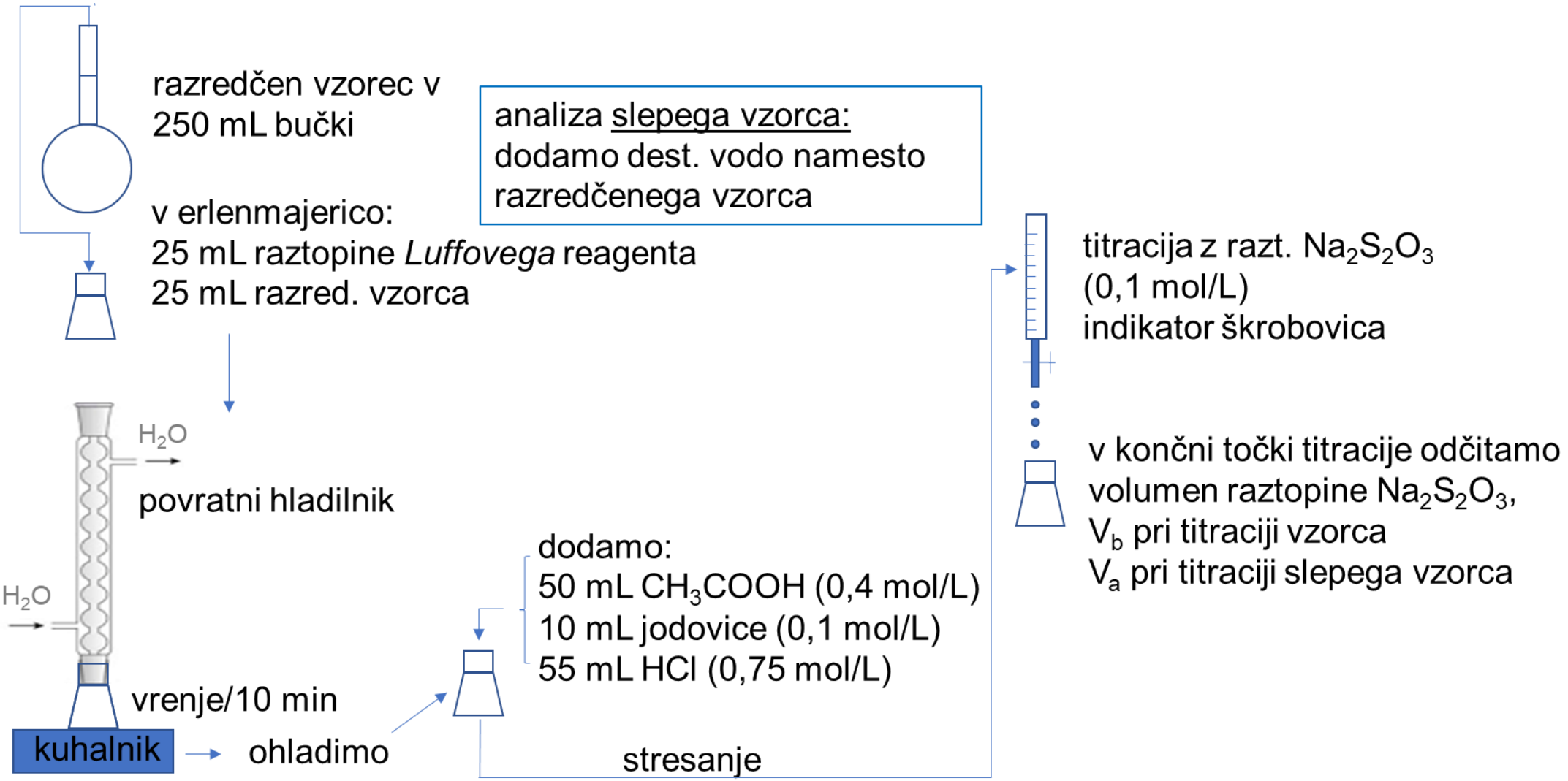
- Vzorec v 250 mL merilni bučki, ki vsebuje glukozo in saharozo, dopolnimo z destilirano vodo do oznake, zamašimo in premešamo - volumen razredčenega vzorca ($V_{razredčen\ vz}$) = 250 mL.



b) Določitev glukoze

- V erlenmajerico z brušenim zamaškom odmerimo 25 mL raztopine *Luffovega* reagenta in dodamo 25 mL razredčenega vzorca.
- Erlenmajerico postavimo na kuhalnik in priključimo na povratni hladilnik; segrevamo 10 min pri temperaturi vrenja; odstranimo s hladilnika, ohladimo na sobno temperaturo.
- Po ohladitvi dodamo: 50 mL raztopine CH_3COOH (0,4 mol/L; merilni valj), 10 mL jodovice (0,1 mol/L; polnilna pipeta) in 55 mL raztopine HCl (0,75 mol/L; merilni valj); vsebino premešamo (sprva počasi in previdno z odzračevanjem, zatem intenzivneje).
- Titriramo s standardno vodno raztopino $Na_2S_2O_3$ (0,1 mol/L) do preskoka barve iz temno modre v svetlo modro ob dodatku 1 mL raztopine škrobovice (1 %) kot indikatorja.
- V končni točki titracije odčitamo volumen raztopine $Na_2S_2O_3$, to je V_b.

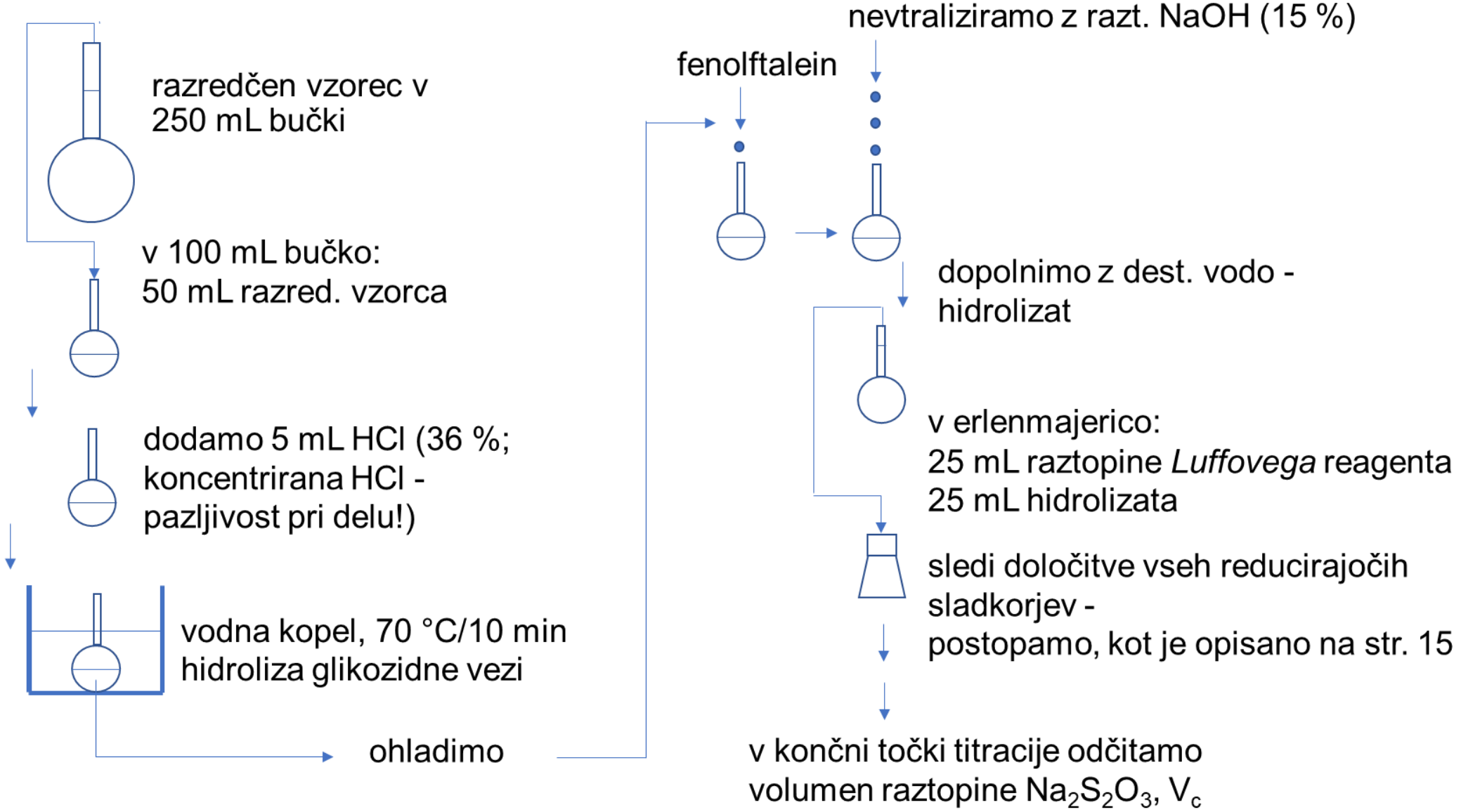
SHEMA POSTOPKA DOLOČITVE GLUKOZE



c) Hidroliza

- Iz 250 mL bučke prenesemo 50 mL raztopine razredčenega vzorca v 100 mL bučko.
- Dodamo 5 mL raztopine HCl (36 %; HCl je koncentrirana; potrebna je pazljivost pri delu).
- Bučko postavimo v vodno kopel pri 70 °C za 10 min (poteka kisla hidroliza glikozidne vezi v molekuli saharoze); vsebino v bučki nato ohladimo.
- Dodamo fenolftalein, nevtraliziramo z raztopino NaOH (15 %).
- Vsebino v 100 mL bučki (hidrolizat) dopolnimo z destilirano vodo do oznake.
- V 100 mL bučki (v hidrolizatu) so produkti cepitve glikozidne vezi v saharozi (glukoza in fruktoza) ter glukoza, ki je že sicer prisotna v vzorcu.

SHEMA POSTOPKA HIDROLIZE



d) Določitev vseh reducirajočih sladkorjev v hidrolizatu

- V erlenmajerico z brušenim zamaškom odmerimo 25 mL raztopine *Luffovega* reagenta in dodamo 25 mL hidrolizata iz 100 mL bučke.
- Naprej postopamo tako kot pri določanju glukoze (shema str.15).
- V končni točki titracije odčitamo volumen raztopine $Na_2S_2O_3$, to je V_c.

e) Analiza slepega vzorca

- V erlenmajerico z brušenim zamaškom odmerimo 25 mL raztopine *Luffovega* reagenta in dodamo 25 mL destilirane vode.
- Naprej postopamo tako kot pri določanju glukoze (shema str. 15).
- V končni točki titracije odčitamo volumen raztopine $Na_2S_2O_3$, to je V_a.

Izračuni

Iz podatkov titracije izračunamo:

- množino $Na_2S_2O_3$, ki je sorazmerna z $n_{I_2\ zreagirani}$ pri določitvi $m_{glukoza}$ v 25 mL razredčenega vzorca ($n_{Na_2S_2O_3\ glukoza}$):

$$n_{Na_2S_2O_3\ glukoza} = (V_a - V_b) \times c_{Na_2S_2O_3}$$

- množino $Na_2S_2O_3$, ki je sorazmerna z $n_{I_2\ zreagirani}$ pri določitvi $m_{vsi\ reduc\ sladk}$ v 25 mL hidrolizata ($n_{Na_2S_2O_3\ vsi\ reduc\ sladk}$):

$$n_{Na_2S_2O_3\ vsi\ reduc\ sladk} = (V_a - V_c) \times c_{Na_2S_2O_3}$$

Za izračun mase sladkorja ne uporabimo stehiometrijskega razmerja med množino sladkorja in množino $Na_2S_2O_3$, pač pa eksperimentalno določeno razmerje (preglednica 1 na str. 20).

Iz podatkov v preglednici 1 na str. 20 narišemo graf in iz grafa odčitamo:

- $m_{glukoza}$ v 25 mL razredčenega vzorca pri izračunanem $n_{Na_2S_2O_3\ glukoza}$ in
- $m_{vsi\ reduc\ sladk}$ v 25 mL hidrolizata pri izračunanem $n_{Na_2S_2O_3\ vsi\ reduc\ sladk}$.

Preglednica 1: Pretvorba množine $Na_2S_2O_3$ v maso reducirajočega sladkorja v 25 mL razredčenega vzorca oz. hidrolizata za opisani postopek določitve.

$n_{Na_2S_2O_3}$ (mmol)	$m_{reduc\ sladk}$ (mg)
0,1	2,4
0,2	4,8
0,3	7,2
0,4	9,7
0,5	12,2
0,6	14,7
0,7	17,2
0,8	19,8

Izračun mase glukoze v vzorcu

Glede na opis eksp. postopka: iz 250 mL bučke odmerimo v erlenmajerico z brušenim zamaškom 25 mL razredčenega vzorca, torej:

$m_{glukoza}$ v 250 mL razredčenega vzorca = $m_{glukoza}$ v 25 mL razredčenega vzorca $\times$ 10

$m_{glukoza}$ v vzorcu = $m_{glukoza}$ v 250 mL razredčenega vzorca

Izračun mase saharoze v vzorcu

Glede na opis eksp. postopka: iz 100 mL bučke s hidrolizatom odmerimo 25 mL hidrolizata, torej:

$m_{vsi\ reduc\ sladk}$ v 100 mL hidrolizata = $m_{vsi\ reduc\ sladk}$ v 25 mL hidrolizata $\times$ 4 =

= $m_{vsi\ reduc\ sladk}$ v 50 mL razredčenega vzorca odmerjenega v 100 mL bučko

Glede na opis eksp. postopka: 50 mL razredčenega vzorca odmerimo iz 250 mL bučke, torej:

$m_{vsi\ reduc\ sladk}$ v 250 mL razredčenega vzorca = $m_{vsi\ reduc\ sladk}$ v 50 mL razredčenega vzorca $\times$ 5

$m_{vsi\ reduc\ sladk}$ v vzorcu = $m_{vsi\ reduc\ sladk}$ v 250 mL razredčenega vzorca

Upoštevamo dejstvo, da se pri povezavi dveh monosaharidov v disaharid odcepi voda, torej:

$M_{saharoza}$ = 2 $\times$ 180 g/mol – 18 g/mol

masa disaharida = masa dveh monosaharidov $\times$ 0,95

$m_{saharoza}$ v vzorcu = ($m_{vsi\ reduc\ sladk}$ v vzorcu – $m_{glukoza}$ v vzorcu) $\times$ 0,95

Poročilo

Eksperimentalni podatki

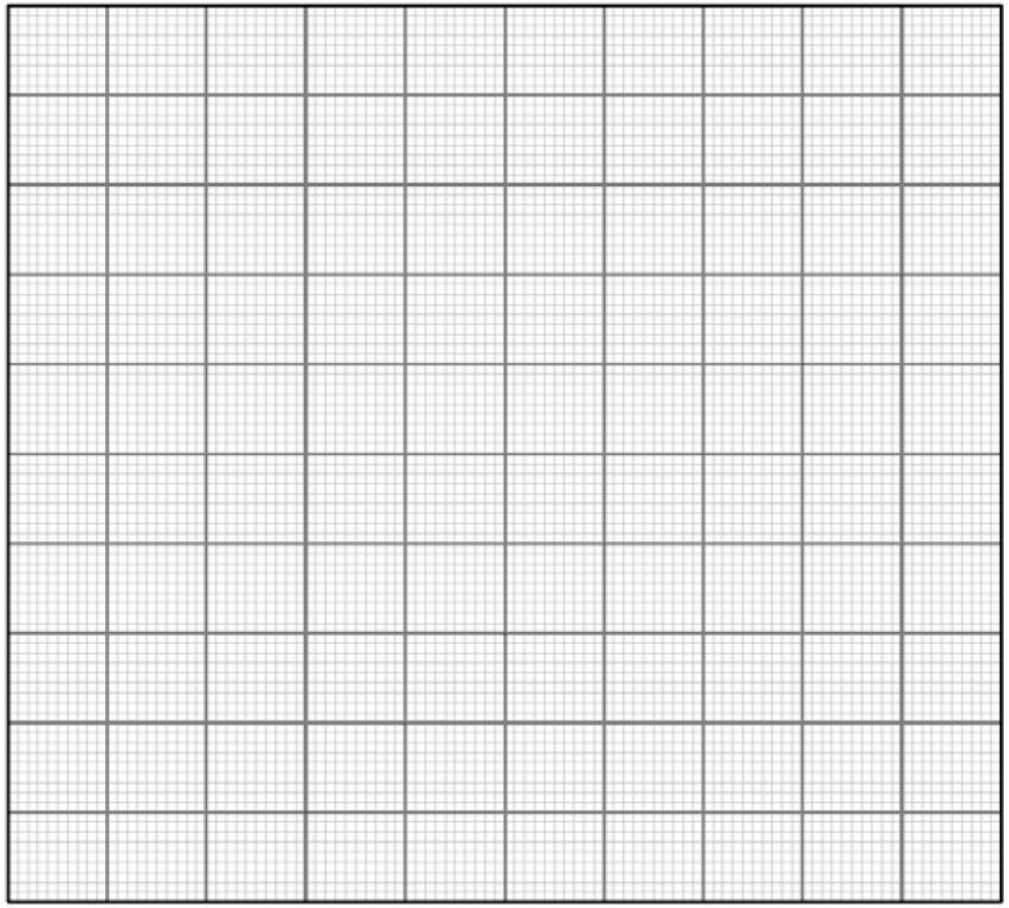
V_a = ……..…. V_b = ……..…… V_c = ……..…. $c_{Na_2S_2O_3}$ = ……

Ostali podatki so navedeni v opisu eksperimentalnega postopka.

Izračuni in graf

$n_{Na_2S_2O_3\ glukoza}$ =

$n_{Na_2S_2O_3\ vsi\ reduc\ sladk}$ =



Odvisnost mase reducirajočega sladkorja od množine $Na_2S_2O_3$

Iz grafa odčitana masa glukoze v 25 mL razredčenega vzorca pri izračunani vrednosti $n_{Na_2S_2O_3\ glukoza}$ = …….

$m_{glukoza}$ v 250 mL razredčenega vzorca =

$m_{glukoza}$ v vzorcu =

Iz grafa odčitana masa vseh reducirajočih sladkorjev v 25 mL hidrolizata pri izračunani vrednosti $n_{Na_2S_2O_3\ vsi\ reduc\ sladk}$ = ……..

$m_{vsi\ reduc\ sladk}$ v 100 mL hidrolizata =

$m_{vsi\ reduc\ sladk}$ v 250 mL razredčenega vzorca =

$m_{vsi\ reduc\ sladk}$ v vzorcu =

$m_{saharoza}$ v vzorcu =

Rezultat

številka vzorca ……

$m_{glukoza}$ v vzorcu =

$m_{saharoza}$ v vzorcu =

DOLOČITEV AKTIVNOSTI TRIPSINSKEGA INHIBITORJA V SOJI

Inhibitorji tripsina

Inhibitorji encimov so različne snovi, ki zmanjšajo encimsko aktivnost, in so v živilih naravno prisotne ali dodane. S prehranskega vidika so še posebno pomembni inhibitorji prebavnih proteolitičnih encimov, proteaz.

Inhibitorje proteaz najdemo v rastlinah (predvsem v stročnicah, tudi v žitaricah, krompirju, paradižniku), kjer naj bi imeli različne vloge - zaščita rastline pred endogenimi proteazami, ki se sprostijo ob poškodovanju rastlinskega tkiva; zaščita pred proteazami mikroorganizmov, insektov, …. Največjo vsebnost omenjenih inhibitorjev so določili v semenih, pa tudi v listih in v gomoljih. Poznani so tudi inhibitorji proteaz, ki so mikrobnega ali živalskega izvora.

Inhibitorji proteaz so pogostokrat proteini oz. polipeptidi. Glede na katalitični tip proteaze, ki jo inhibirajo, razvrstimo inhibitorje proteaz v štiri skupine.

Med inhibitorje serinskih proteaz v soji uvrščamo:

- inhibitorje Kunitzovega tipa, ki vsebujejo malo cisteinskih ostankov in tvorijo dva disulfidna mostička. So termično manj stabilni.
- inhibitorje Bowman-Birkovega tipa, ki vsebujejo več cisteinskih ostankov in tvorijo več disulfidnih mostičkov. So termično stabilnejši. V primerjavi z inhibitorji Kunitzovega tipa imajo manjšo molsko maso.

Oba tipa inhibitorjev serinskih proteaz sta učinkovita inhibitorja tripsina.

Tripsin je endoproteaza in katalizira cepitev peptidne vezi v proteinskih in peptidnih substratih na karboksilni strani aminokislin lizin ali arginin. Inhibicija tripsina je posledica tvorbe zelo stabilnega kompleksa med inhibitorjem in encimom.

Inhibicija tripsina pri človeku vodi v motnje v prebavi proteinov, kar zmanjša biološko razpoložljivost aminokislin iz proteinov. Inhibicija tripsina povzroči prekomerno produkcijo in izločanje tripsina, kar v skrajnih primerih lahko vodi v hipertrofijo (povečanje) trebušne slinavke. Poleg tega povečana sinteza pankreatičnih encimov oslabi sintezo drugih proteinov v telesu, kar ima za posledico počasnejšo rast.

Aktivnost tripsinskih inhibitorjev se zmanjša s termično obdelavo, saj pride pri povišani temperaturi do denaturacije zaradi rušenje nativne strukture. V prisotnosti reducentov, kjer pride do redukcije disulfidnih vezi v inhibitorjih, rezultira termična obdelava v še učinkovitejši denaturaciji.

Ker je uporaba stročnic, ki vsebujejo še posebno veliko inhibitorjev tripsina (predvsem soja), v prehrani precej razširjena, je potrebna pozornost pri njihovi zadostni toplotni obdelavi. Način, čas in temperatura toplotne obdelave, velikost delcev in vsebnost vlage v semenih vplivajo na to, v kolikšni meri pride do zmanjšanja aktivnosti tripsinskih inhibitorjev. Kot učinkovito se je izkazalo kuhanje v vreli vodi ter uporaba mikrovalov. Pri tem je pomembno zadostno predhodno namakanje semen (~24 ur), saj je učinek večji pri semenih z večjo vsebnostjo vlage.

Namen vaje

Določiti aktivnost tripsinskega inhibitorja v rumeni in rdeči soji.

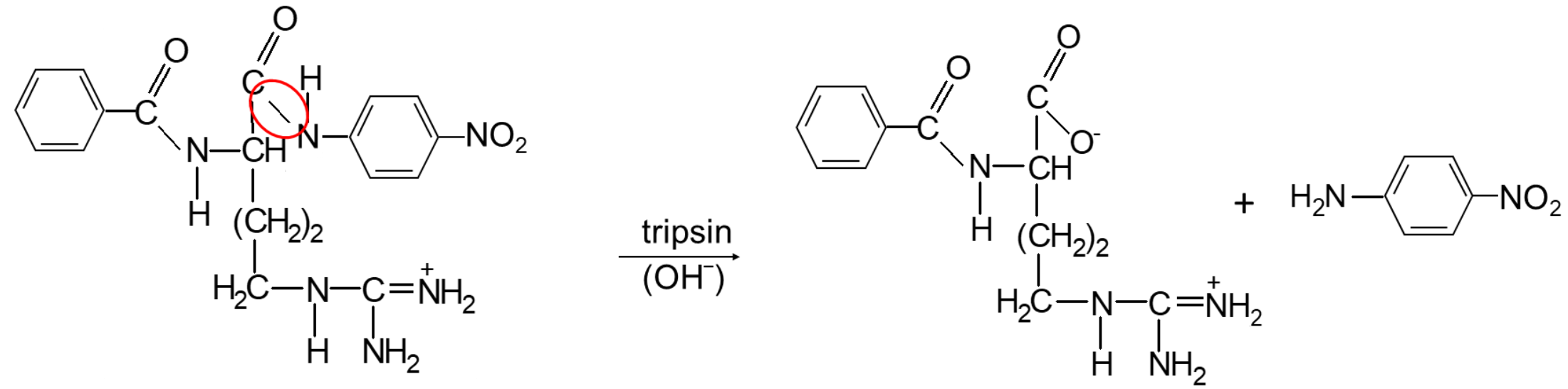
Določiti vpliv toplotne obdelave soje (3 ure kuhanje v vreli vodi (A); 3 ure v ventilatorskem sušilniku pri 104 °C (B); 6 ur v ventilatorskem sušilniku pri 104 °C (C)) na aktivnost tripsinskega inhibitorja.

Princip vaje

Določitev aktivnosti tripsinskega inhibitorja temelji na tem, da tripsin katalizira odcepitev *p*-nitroanilina iz sintetičnega substrata N-benzoil-D,L-arginin-*p*-nitroanilid (BAPNA).

Tripsinski inhibitor v ekstraktu iz soje zavira delovanje tripsina in zato nastane manj produkta *p*-nitroanilina.

To, koliko manj produkta nastane v določenem času, je sorazmerno z aktivnostjo tripsinskega inhibitorja oziroma z vsebnostjo tripsinskega inhibitorja v reakcijski zmesi.



N-benzoil-D,L-arginin-*p*-nitroanilid (BAPNA)

N-benzoil-D,L-arginin

p-nitroanilin

Vsebnost reakcijskega produkta *p*-nitroanilina določimo spektrofotometrično.

Eksperimentalni postopek

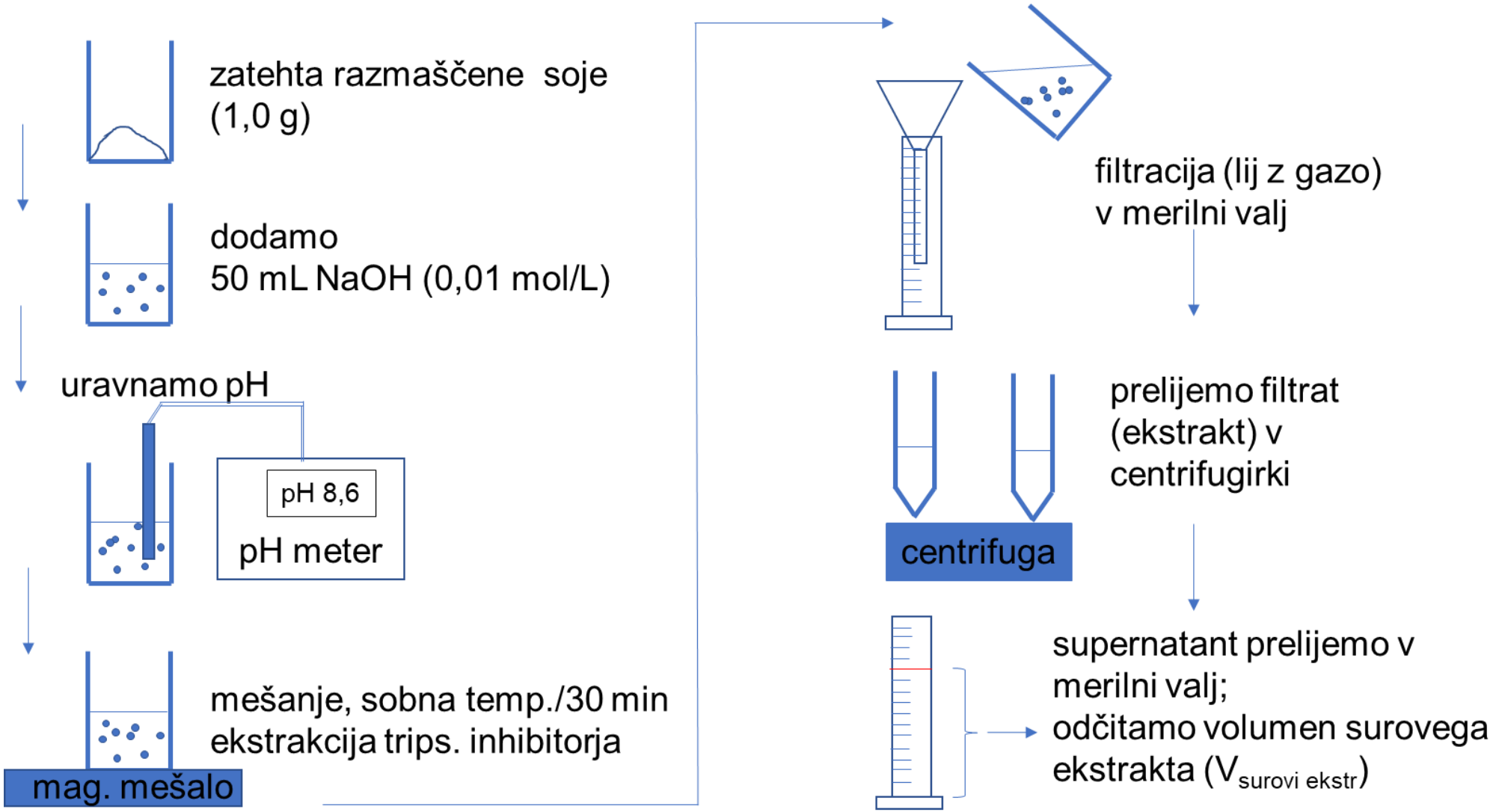
a) Ekstrakcija tripsinskega inhibitorja iz soje (izvedemo postopek določitve aktivnosti tripsinskega inhibitorja v rumeni in rdeči soji ter v rumeni soji, ki je toplotno obdelana na tri različne načine)

b) Spektrofotometrična določitev aktivnosti tripsinskega inhibitorja

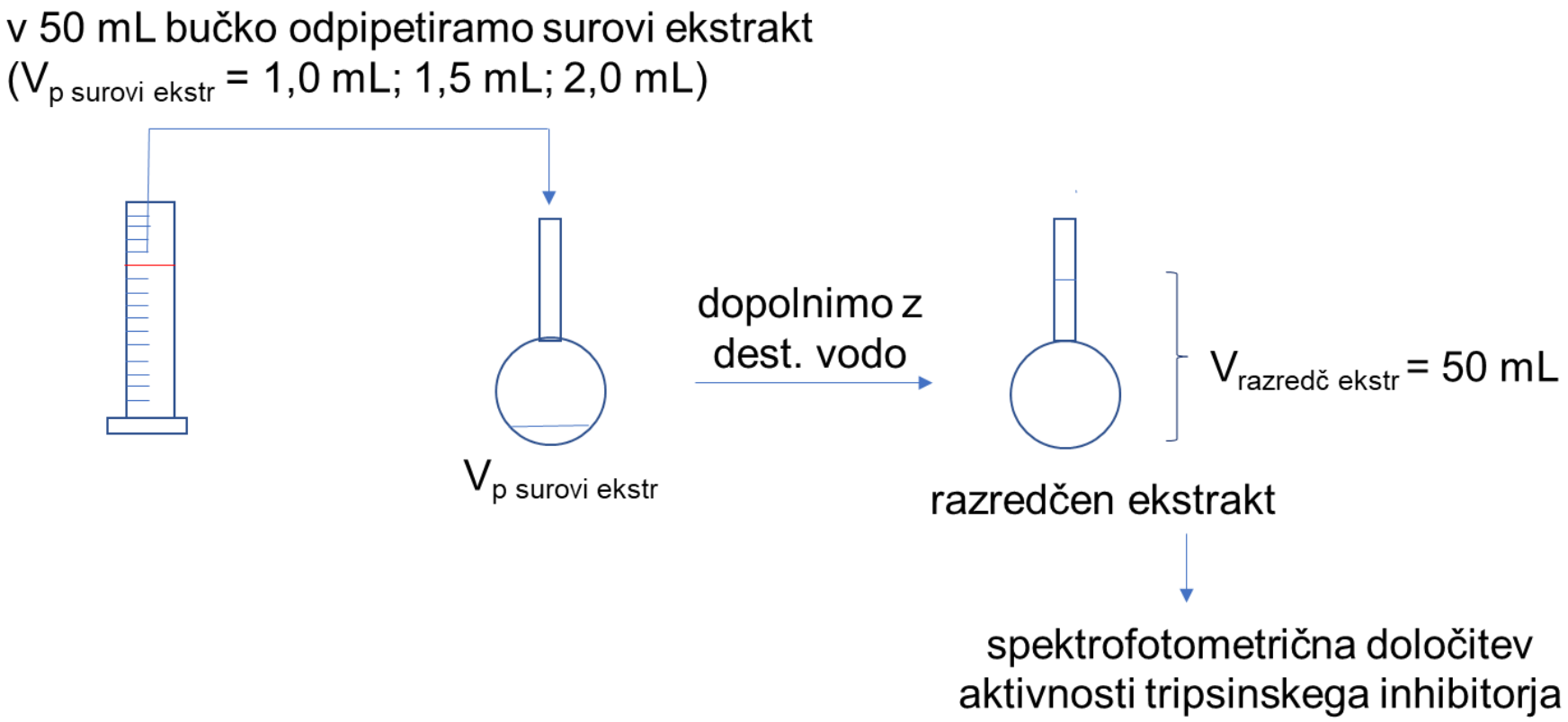
a) Ekstrakcija tripsinskega inhibitorja iz soje (priprava ekstrakta)

- Zatehtamo 1,0 g zmlete in razmaščene soje ($m_{razmašč\ soja}$) v 100 mL čašo; soja je razmaščena z dietil etrom.
- Dodamo 50 mL ekstrakcijskega topila (raztopina NaOH (0,01 mol/L)); uravnamo pH (raztopina HCl oz. NaOH) na 8,6 (pH meter).
- Mešanje na magnetnem mešalu 30 min pri sobni temperaturi; poteka ekstrakcija tripsinskega inhibitorja iz soje.
- Prefiltriramo v merilni valj skozi lij z gazo; filtrat (ekstrakt tripsinskega inhibitorja) je moten; potrebujemo bister ekstrakt, saj je določitev aktivnosti spektrofotometrična; zato prelijemo filtrat (ekstrakt) v dve centrifugirki; pozorni smo na to, da sta centrifugirki z vsebino vred po masi izenačeni; sledi centrifugiranje.
- Supernatant (bistri del nad usedlino po centrifugiranju v centrifugirki) previdno prelijemo v merilni valj in v merilnem valju odčitamo volumen surovega ekstrakta ($V_{surovi\ ekstr}$).
- Pripravimo razredčen ekstrakt: v vsako posamezno 50 mL bučko odmerimo določen volumen surovega ekstrakta ($V_{p\ surovi\ ekstr}$ = 1,0 mL; 1,5 mL; 2,0 mL), dopolnimo z destilirano vodo do oznake, bučke zapremo in vsebino premešamo; volumen razredčenega ekstrakta ($V_{razredč\ ekstr}$) je 50 mL.

SHEMA POSTOPKA EKSTRAKCIJE TRIPSINSKEGA INHIBITORJA



SHEMA POSTOPKA PRIPRAVE RAZREDČENEGA EKSTRAKTA



Različni $V_{p\ surovi\ ekstr}$ pomenijo različne količine tripsinskega inhibitorja v reakcijski zmesi pri spektrofotometrični določitvi.

b) Spektrofotometrična določitev aktivnosti tripsinskega inhibitorja

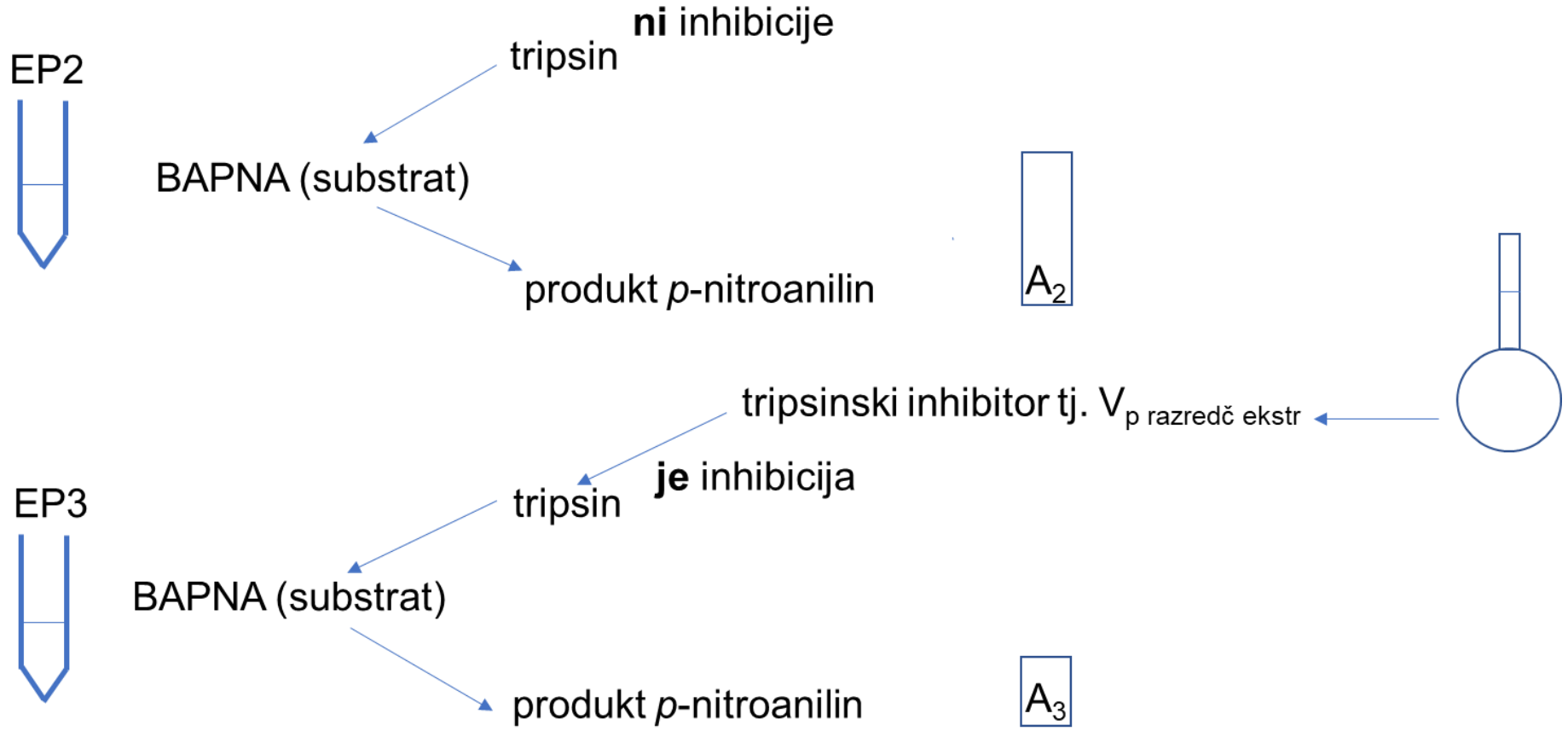
- Za vsak posamezni razredčen ekstrakt pripravimo tri epruvete: EP1 (slepi vzorec), EP2 (ni trips. inh.), EP3 (vsebuje trips. inh.).
- V epruveto EP1 (slepi vzorec) odmerimo: 2,0 mL razredčenega ekstrakta, 1,0 mL raztopine ocetne kisline (30 %), 5,0 mL raztopine BAPNA in 2,0 mL raztopine tripsina (zakaj tak vrstni red?); premešamo na vrtinčniku.
- V epruveti EP2 ter EP3 odmerimo določen volumen razredčenega ekstrakta ($V_{p\ razredč\ ekstr}$), vodo in tripsin (preglednica 1 na str. 36); premešamo na vrtinčniku.

Preglednica 1: Volumen razredčenega ekstrakta ($V_{p\ razredč\ ekstr}$), vode ($V_{p\ voda}$) in tripsina ($V_{p\ tripsin}$) za odmerjanje v EP2 in EP3.

epruveta	$V_{p\ razredč\ ekstr}$ (mL)	$V_{p\ voda}$ (mL)	$V_{p\ tripsin}$ (mL)
EP2	-	2,0	2,0
EP3	2,0	-	2,0

- Raztopino BAPNA ter EP1, EP2 in EP3 z opisano vsebino postavimo v vodno kopel pri 37 °C za približno 5 min.
- Zatem v EP2 in EP3 odmerimo 5,0 mL raztopine BAPNA (substrat).
- Reakcijo v EP2 in v EP3 pustimo teči točno 10 min (vodna kopel pri 37 °C).
- Reakcijo v EP2 in EP3 prekinemo z dodatkom 1,0 mL raztopine ocetne kisline (30 %) (zakaj z raztopino ocetne kisline?); premešamo na vrtinčniku.
- Vsebino iz epruvet prelijemo v kivete in izmerimo absorbanco raztopine v EP2 (A_2) in EP3 (A_3) proti slepemu vzorcu (EP1) pri 410 nm.

SHEMA POSTOPKA SPEKTROFOTOMETRIČNE DOLOČITVE



Koncentracija produkta *p*-nitroanilina v EP2 > koncentracija produkta *p*-nitroanilina v EP3;
torej: $A_2 > A_3$

Izračuni

Aktivnost tripsinskega inhibitorja je sorazmerna z ΔA ($\Delta A = A_2 - A_3$).

Večja kot je inhibicija tripsina, manj nastane produkta in manjša je A_3, torej je večja ΔA.

Aktivnost tripsinskega inhibitorja izrazimo kot število tripsinskih enot, ki so se inhibirale (TIU).

Po definiciji: 1 enota tripsinske inhibicije (1TIU) ustreza $\Delta A = 0{,}010$, določeno pri opisanih pogojih.

$$\text{aktivnost trips. inh. v reakcijski zmesi} = \frac{\Delta A}{0{,}010}$$

Glede na opis eksp. postopka (preglednica 1 na str. 36): v reakcijsko zmes (v EP3) smo odmerili določen $V_{\text{p razredč ekstr}}$, torej:

$$\text{aktivnost trips. inh. v } V_{\text{p razredč ekstr}} = \text{aktivnost trips. inh. v reakcijski zmesi}$$

$$\text{aktivnost trips. inh. / mL razredč. ekstr.} = \frac{\text{aktivnost trips. inh. v } V_{\text{p razredč ekstr}}}{V_{\text{p razredč ekstr}}}$$

aktivnost trips. inh. v celotnem $V_{razredč\ ekstr}$ = $V_{razredč\ ekstr}$ × aktivnost trips. inh. / mL razredč. ekstr.

Glede na opis eksp. postopka (str. 33, 35): $V_{razredč\ ekstr}$ = 50 mL; razredčen ekstrakt pripravimo tako, da odmerimo določen $V_{p\ surovi\ ekstr}$ in dodamo vodo, torej:

aktivnost trips. inh. v $V_{p\ surovi\ ekstr}$ = aktivnost trips. inh. v celotnem $V_{razredč\ ekstr}$

$$\text{aktivnost trips. inh. / mL surovega ekstr.} = \frac{\text{aktivnost trips. inh. v } V_{p\ surovi\ ekstr}}{V_{p\ surovi\ ekstr}} \qquad \text{(enota: TIU/mL)}$$

Glede na opis eksp. postopka (str. 33, 34): tripsinski inhibitor smo ekstrahirali iz soje in po filtraciji in centrifugiranju odčitali $V_{surovi\ ekstr}$, torej:

aktivnost trips. inh. v celotnem $V_{surovi\ ekstr}$ = $V_{surovi\ ekstr}$ × aktivnost trips. inh. / mL surovega ekstr.

aktivnost trips. inh. v razmašč. soji = aktivnost trips. inh. v celotnem $V_{surovi\ ekstr}$

$$\text{aktivnost trips. inh. / mg razmašč. soje} = \frac{\text{aktivnost trips. inh. v razmašč. soji}}{m_{razmašč\ soja}} \qquad \text{(enota: TIU/mg)}$$

Primer: iz razmaščene soje ($m_{razmašč\ soja}$ = 1000 mg) pripravimo ekstrakt ($V_{surovi\ ekstr}$ = 42 mL) in po opisanem eksperimentalnem postopku ($V_{p\ surovi\ ekstr}$ = 1,5 mL, $V_{razredč\ ekstr}$ = 50 mL, $V_{p\ razredč\ ekstr}$ = 2,0 mL) določimo ΔA = 0,050.

$$\text{aktivnost trips. inh. v reakcijski zmesi} = \frac{\Delta A}{0{,}010} = \frac{0{,}050}{0{,}010} = 5{,}0\ \text{TIU}$$

$$\text{aktivnost trips. inh. v } V_{p\ razredč\ ekstr} = \text{aktivnost trips. inh. v reakcijski zmesi} = 5{,}0\ \text{TIU}$$

$$\text{aktivnost trips. inh. / mL razredč. ekstr.} = \frac{\text{aktivnost trips. inh. v } V_{p\ razredč\ ekstr}}{V_{p\ razredč\ ekstr}} = \frac{5{,}0\ \text{TIU}}{2{,}0\ \text{mL}} = 2{,}5\ \text{TIU/mL}$$

$$\text{aktivnost trips. inh. v celotnem } V_{razredč\ ekstr} = V_{razredč\ ekstr} \times \text{aktivnost trips. inh. / mL razredč. ekstr.} =$$

$$= 50\ \text{mL} \times 2{,}5\ \text{TIU/mL} = 125\ \text{TIU}$$

$$\text{aktivnost trips. inh. v } V_{p\ surovi\ ekstr} = \text{aktivnost trips. inh. v celotnem } V_{razredč\ ekstr} = 125\ \text{TIU}$$

$$\text{aktivnost trips. inh. / mL surovega ekstr.} = \frac{\text{aktivnost trips. inh. v } V_{\text{p surovi ekstr}}}{V_{\text{p surovi ekstr}}} = \frac{125\ \text{TIU}}{1{,}5\ \text{mL}} =$$

$$= 83{,}3\ \text{TIU/mL}$$

$$\text{aktivnost trips. inh. v celotnem } V_{\text{surovi ekstr}} = V_{\text{surovi ekstr}} \times \text{aktivnost trips. inh. / mL surovega ekstr.} =$$

$$= 42\ \text{mL} \times 83{,}3\ \text{TIU/mL} = 3500\ \text{TIU}$$

$$\text{aktivnost trips. inh. v razmašč. soji} = \text{aktivnost trips. inh. v celotnem } V_{\text{surovi ekstr}} = 3500\ \text{TIU}$$

$$\text{aktivnost trips. inh. / mg razmašč. soje} = \frac{\text{aktivnost trips. inh. v razmašč. soji}}{m_{\text{razmašč soja}}} =$$

$$= \frac{3500\ \text{TIU}}{1000\ \text{mg}} = 3{,}5\ \text{TIU/mg}$$

Poročilo

Eksperimentalni podatki

preiskovani vzorec soje: ……..

$m_{razmašč\ soja}$ = …… $V_{surovi\ ekstr}$ = …… $V_{p\ surovi\ ekstr}$ = …… A_2 = …… A_3 = ……

Ostali podatki sledijo vrednostim, ki so navedene v opisu eksperimentalnega postopka.

Izračuni

ΔA =

aktivnost trips. inh. v reakcijski zmesi =

aktivnost trips. inh. v $V_{p\ razredč\ ekstr}$ =

aktivnost trips. inh. / mL razredč. ekstr. =

aktivnost trips. inh. v celotnem $V_{razredč\ ekstr}$ =

aktivnost trips. inh. v $V_{p\ surovi\ ekstr}$ =

aktivnost trips. inh. / mL surovega ekstr. =

aktivnost trips. inh. v celotnem $V_{surovi\ ekstr}$ =

aktivnost trips. inh. v razmašč. soji =

aktivnost trips. inh. / mg razmašč. soje =

Izpolnite preglednico in komentirajte aktivnost tripsinskega inhibitorja v soji glede na sorto in vpliv toplotne obdelave.

Preglednica 2: Aktivnost tripsinskega inhibitorja v soji.

	rdeča soja			rumena soja			topl. obd. rumena soja (A)			topl. obd. rumena soja (B)			topl. obd. rumena soja (C)		
$V_{p\ surovi\ ekstr}$ (mL)	1,0	1,5	2,0	1,0	1,5	2,0	1,0	1,5	2,0	1,0	1,5	2,0	1,0	1,5	2,0
ΔA															
aktivnost trips. inh. v reakcijski zmesi (TIU)															
aktivnost trips. inh. v $V_{p\ razredč\ ekstr}$ (TIU)															
aktivnost trips. inh. / mL razredč. ekstr. (TIU/mL)															
aktivnost trips. inh. v celotnem $V_{razredč\ ekstr}$ (TIU)															
aktivnost trips. inh. v $V_{p\ surovi\ ekstr}$ (TIU)															
aktivnost trips. inh. / mL surovega ekstr. (TIU/mL)															
aktivnost trips. inh. v celotnem $V_{surovi\ ekstr}$ (TIU)															
aktivnost trips. inh. v razmašč. soji (TIU)															
aktivnost trips. inh. / mg razmašč. soje (TIU/mg)															

Komentar

KVANTITATIVNA DOLOČITEV ŠKROBA V MOKI

Škrob

Škrob je zmes dveh različnih polimerov: amiloza in amilopektin.

Amiloza sestoji iz nekaj 100 do nekaj 1000 glukoznih enot, ki so med seboj povezane z α-(1→4)-glikozidno vezjo. Molekula amiloze ima en reducirajoči konec. Polimer oblikuje vijačnico. Med –OH skupinami glukoznih enot v polimeru so vzpostavljene vodikove vezi, kar stabilizira strukturo vijačnice. Molska masa amiloze je odvisna od botanične pripadnosti (vrste, sorte). V pšeničnem škrobu je v povprečju 1000-2000 glukoznih enot, v krompirjevem tudi do 4500.

Polimer amilopektina je večji od amiloze in sestoji iz 60000 do 3000000 glukoznih enot. Polimer je razvejan. Razvejanje je na 10 do 100 glukoznih enot v verigi. V polimeru so glukozne enote povezane z α-(1→4)-glikozidno vezjo. Na mestu razvejanja je molekula glukoze iz C1 povezana na osnovno verigo z α-(1→6)-glikozidno vezjo. Stranske verige amilopektina oblikujejo dvojne vijačnice. Molekula amilopektina ima en reducirajoči konec v osnovni verigi.

Običajno vsebujejo rastline več amilopektina. Masno razmerje med amilopektinom in amilozo je odvisno od botanične pripadnosti in v splošnem znaša 3:1.

Sinteza škroba poteka v organelih rastlinskih celic, v kloroplastih v listih in v amiloplastih, kjer se skladišči v obliki škrobnih granul. Ta t.i. rezervni škrob, ki se nahaja predvsem v endospermu semen ter v koreninah in gomoljih, predstavlja energijsko zalogo. Velikost in oblika škrobnih granul je odvisna od botanične pripadnosti (vrste, sorte). Premer škrobnih granul pri koruzi je 15 µm, pri pšenici 25 µm in pri krompirju 40 µm.

Zaradi tesne ureditve škrobnih polimerov v granulah je hidratacija v hladni vodi precej omejena, kar ima za posledico slabšo topnost. Struktura granul se znatno ne spremeni. V hladni vodi škrob tvori suspenzijo z majhno viskoznostjo. Do opaznih sprememb pride pri zvišani temperaturi (55-70 ºC), ki povzroči oslabitev jakosti vodikovih vezi med segmenti polimerov. To omogoči razmikanje polimerov. Struktura postane bolj amorfna. Posledično vse več vode vstopi v granule, kar vodi do povečane hidratacije in nabrekanja. Molekule amiloze in manjši del molekul amilopektina se sprosti iz granul in granule razpadejo.

Glavni vir škroba v humani prehrani so gomoljnice, žitarice, stročnice, korenovke, kostanj, oreški in tudi sadje. Z dozorevanjem sadja vsebnost škroba pade. Zrelo sadje (z nekaj izjemami, kot je npr. banana) ne vsebuje škroba.

Škrob je ključno hranilo v humani prehrani. Prebava škroba se začne v ustih pod vplivom encima α-amilaze v slini, nadaljuje se v tankem črevesu pod vplivom α-amilaze iz trebušne slinavke in zatem pod vplivom maltaze in izomaltaze, ki nastale oligosaharide in maltozo razgradijo do glukoze.

Škrob je pomembna industrijska surovina. Z namenom izboljšanja funkcionalnosti (ustreznejših tehnoloških in senzoričnih lastnosti) se iz nativnega škroba s kemijsko, fizikalno in/ali encimsko modifikacijo pridobiva modificiran škrob. Nativni in modificirani škrobi se v živilstvu uporabljajo kot gostila, stabilizatorji pen, emulzij in suspenzij, kot emulgatorji, želirna sredstva, humektanti, polnila, kriostabilizatorji, sredstva za enkapsulacijo, nosilci arom, nadomestki maščob.

Škrobni hidrolizati so produkti modifikacije škroba, kjer cepimo glikozidne vezi med glukoznimi enotami s pomočjo kisline (HCl) in ustreznih encimov. Produkti hidrolize so različni dekstrini, oligosaharidi, maltoza in glukoza. Kislinska hidroliza je v primerjavi z encimsko manj specifična. Na industrijskem nivoju se za encimsko hidrolizo škroba uporabljajo endoamilaze (npr. α-amilaza) in eksoamilaze (npr. β-amilaza ter amiloglukozidaza).

Namen vaje

Določiti masni delež škroba ($w_{škrob}$) v pšenični moki.

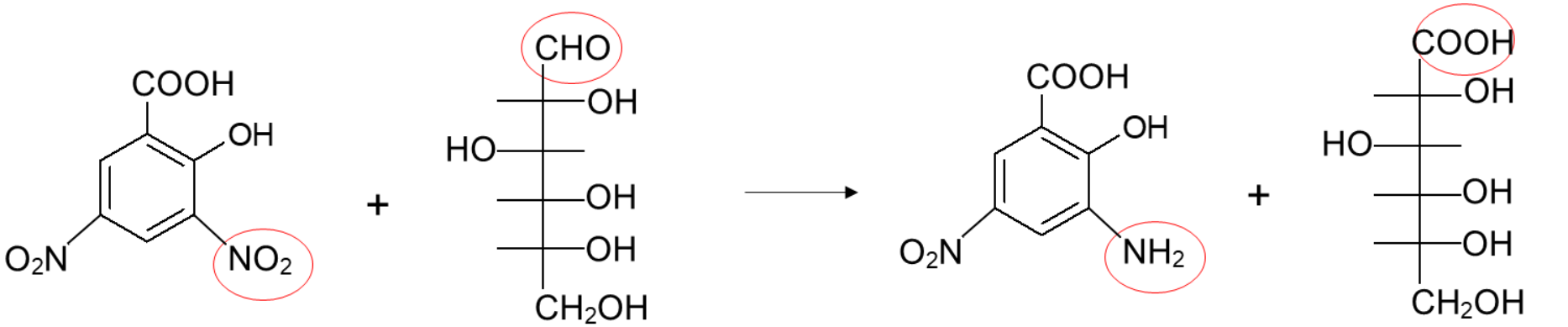
Določiti vpliv postopka razgradnje škroba na vsebnost glukoze v hidrolizatu škroba.

Princip vaje

Namakanje škroba v vreli vodi omogoči boljšo hidratacijo in nabrekanje granul, zaradi česar je bolj sprejemljiv za kisline in encime, kar vodi v učinkovitejšo razgradnjo škroba.

Pri kislinski hidrolizi (je sicer manj specifična) in hidrolizi z encimoma α-amilaza (cepi α-(1→4)-glikozidne vezi v notranjosti verige) in amiloglukozidaza (cepi α-(1→4)-glikozidne vezi z ne-reducirajočega konca oligosaharidov ter α-(1→6)-glikozidne vezi (počasneje)) je končni produkt razgradnje glukoza.

Določitev glukoze v hidrolizatu temelji na tem, da je glukoza močan reducent in reducira nitro skupino v 3,5-dinitrosalicilni kislini do aminoskupine.



3,5-dinitrosalicilna kislina (rumena)

glukoza

3-amino-5-nitrosalicilna kislina (oranžno-rdeča)

glukonska kislina

Vsebnost reakcijskega produkta 3-amino-5-nitrosalicilno kislino določimo spektrofotometrično. Absorbanca je sorazmerna s koncentracijo reakcijskega produkta. Koncentracija reakcijskega produkta je sorazmerna z vsebnostjo glukoze v hidrolizatu škroba.

Z namenom, da bi ovrednotili, kako postopek razgradnje škroba (izpustimo določene korake) vpliva na vsebnost glukoze v hidrolizatu, izvedemo eksperimentalni postopek na 8 različnih načinov (Preglednica 1 na str. 53).

Preglednica 1: Postopek razgradnje škroba.

	dodatek kisline	segrevanje	dodatek encimov
1A	✓	✓	✓
1B	✓	✓	x
2A	✓	x	✓
2B	✓	x	x
3A	x	✓	✓
3B	x	✓	x
4A	x	x	✓
4B	x	x	x

Eksperimentalni postopek

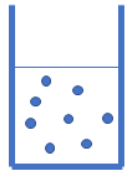
a) Kislinska hidroliza
b) Encimska hidroliza
c) Spektrofotometrična določitev glukoze v hidrolizatu škroba
d) Umeritvena krivulja za določitev glukoze v hidrolizatu škroba

a) Kislinska hidroliza

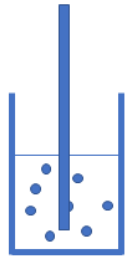
- Zatehtamo 0,80 g moke (m_{moka}) v 100 ml čašo.
- Dodamo 25 mL raztopine HCl (2 mol/L) in 25 mL destilirane vode.
- Mešanje s stekleno plačko pri sobni temperaturi 20 min.
- Čašo postavimo na ogreto ploščo in počakamo, da raztopina zavre; vrenje 20 min.
- Ohladimo na sobno temperaturo.
- Uravnamo pH raztopine na vrednost 6,9 z raztopino NaOH (3 mol/L).
- Raztopino iz čaše kvantitativno prenesemo v 100 mL merilno bučko, dopolnimo z destilirano vodo do oznake, zamašimo in vsebino premešamo.

SHEMA POSTOPKA

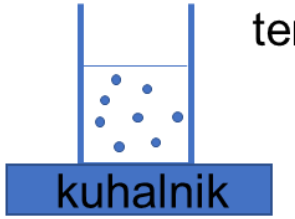
zatehta moke
(0,80 g)



dodamo:
25 mL HCl (2 mol/L)
25 mL vode



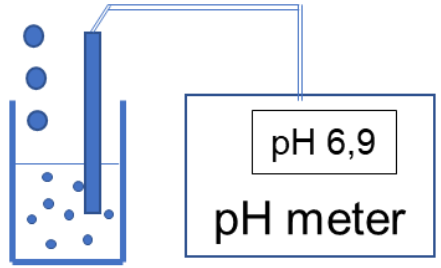
mešanje
sobna temp./20 min

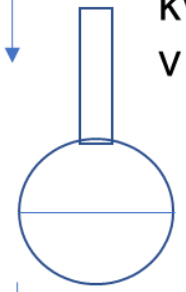


temp. vrenja/20 min

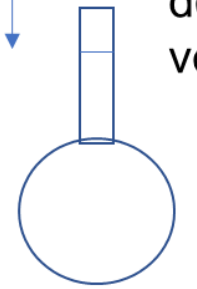
ohladimo

uravnamo pH z
NaOH (3 mol/L)





kvantitativno prenesemo
v 100 mL bučko



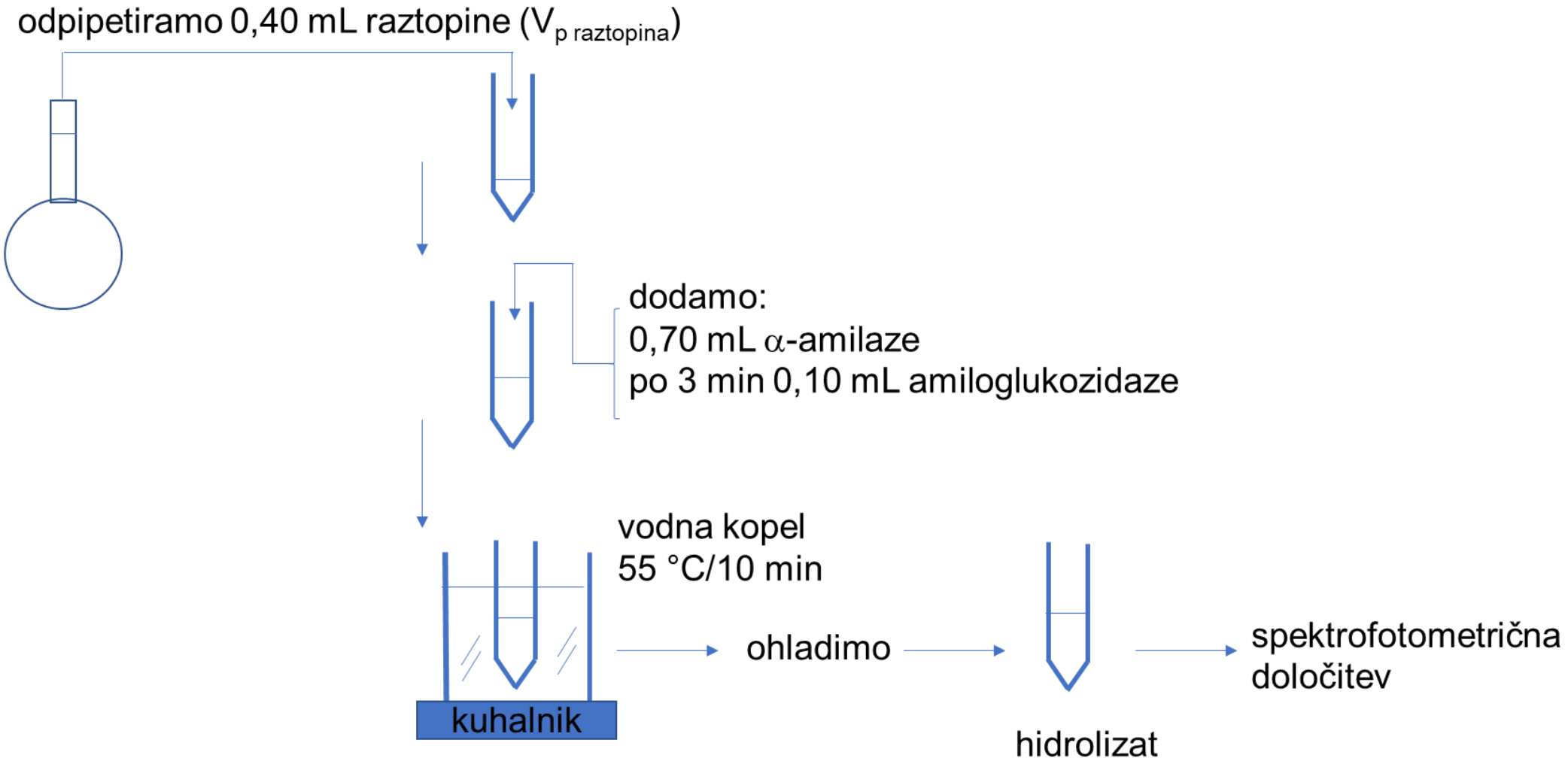
dopolnimo z destilirano
vodo

encimska hidroliza

b) Encimska hidroliza

- Iz 100 mL merilne bučke odmerimo 0,40 mL raztopine ($V_{p\ raztopina}$) v epruveto.
- Dodamo 0,70 mL raztopine α-amilaze.
- Pustimo učinkovati točno 3 min.
- Dodamo 0,10 mL raztopine amiloglukozidaze.
- Inkubacija 10 min v vodni kopeli pri 55 °C.
- Ohladitev na sobno temperaturo.

SHEMA POSTOPKA ENCIMSKE HIDROLIZE



Vpliv postopka razgradnje škroba na vsebnost glukoze v hidrolizatu:

1A in 1B: zatehta, dodatek 25 mL kisline, 25 mL vode, sobna temp./20 min, vrenje/20 min, ohladitev, nevtralizacija, kvantitativno v 100 mL merilno bučko, zatem:

1A: 0,40 mL v epruveto, dodatek 0,70 mL α-amilaze, 0,10 mL amiloglukozidaze, vodna kopel
1B: 0,40 mL v epruveto, dodatek 0,80 mL vode, vodna kopel.

2A in 2B: zatehta, dodatek 25 mL kisline, 25 mL vode, sobna temp./40 min, ni ohlajanja, nevtralizacija, kvantitativno v 100 mL merilno bučko, zatem:

2A: 0,40 mL v epruveto, dodatek 0,70 mL α-amilaze, 0,10 mL amiloglukozidaze, vodna kopel
2B: 0,40 mL v epruveto, dodatek 0,80 mL vode, vodna kopel.

3A in 3B: zatehta, dodatek 50 mL vode, sobna temp./20 min, vrenje/20 min, ohladitev, ni nevtralizacije, kvantitativno v 100 mL merilno bučko, zatem:

3A: 0,40 mL v epruveto, dodatek 0,70 mL α-amilaze, 0,10 mL amiloglukozidaze, vodna kopel
3B: 0,40 mL v epruveto, dodatek 0,80 mL vode, vodna kopel.

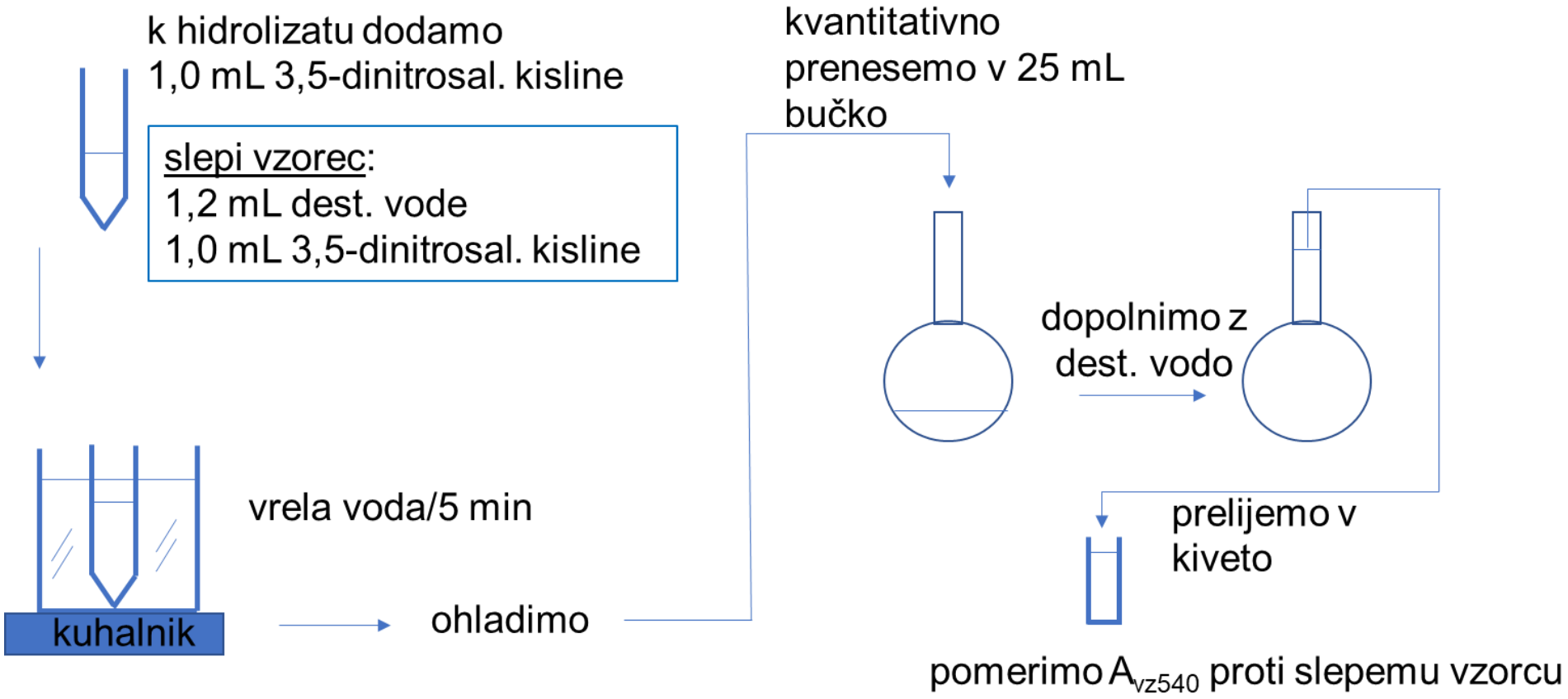
4A in 4B: zatehta, dodatek 50 mL vode, sobna temp./40 min, ni ohlajanja, ni nevtralizacije, kvantitativno v 100 mL merilno bučko, zatem:

4A: 0,40 mL v epruveto, dodatek 0,70 mL α-amilaze, 0,10 mL amiloglukozidaze, vodna kopel
4B: 0,40 mL v epruveto, dodatek 0,80 mL vode, vodna kopel.

c) Spektrofotometrična določitev glukoze v hidrolizatu škroba

- Po končani hidrolizi v epruveto k hidrolizatu škroba dodamo 1,0 mL raztopine 3,5-dinitrosalicilne kisline (1 g/100 mL), premešamo.
- V drugi epruveti pripravimo slepi vzorec: v epruveto odmerimo 1,2 mL destilirane vode in 1,0 mL raztopine 3,5-dinitrosalicilne kisline, premešamo.
- Obe epruveti postavimo v kopel z vrelo vodo za 5 min, zatem ohladimo na sobno temperaturo.
- Vsebino iz posamezne epruvete kvantitativno prenesemo v 25 mL merilno bučko, dopolnimo z destilirano vodo do oznake, zamašimo in vsebino premešamo.
- Pripravimo dve kiveti: kiveto za vzorec (KV_{vz}) in kiveto za slepi vzorec ($KV_{sl\ vz}$).
- Vsebino iz bučk prelijemo v ustrezno kiveto.
- Pomerimo absorbanco vzorca proti slepemu vzorcu pri 540 nm (A_{vz540}).

SHEMA SPEKTROFOTOMETRIČNE DOLOČITVE GLUKOZE V HIDROLIZATU ŠKROBA



d) Umeritvena krivulja za določitev glukoze v hidrolizatu škroba

- Uporabimo izhodno raztopino glukoze z masno koncentracijo 2,0 mg/mL ($\gamma_{glu\ izh}$).
- Pripravimo 6 epruvet; v vsako odmerimo ustrezen volumen izhodne raztopine glukoze ($V_{p\ izh\ razt\ glu}$) in destilirane vode ($V_{p\ voda}$), kot je navedeno v preglednici 2 na str. 68.
- V vsako epruveto dodamo po 1,0 mL raztopine 3,5-dinitrosalicilne kisline, premešamo, epruvete postavimo v kopel z vrelo vodo za 5 min; zatem ohladimo na sobno temperaturo.
- Pripravimo šest 25 mL merilnih bučk; vsebino iz posamezne epruvete kvantitativno prenesemo v merilno bučko, dopolnimo z destilirano vodo do oznake, zamašimo, vsebino premešamo.
- Pripravimo 6 kivet; vsebino iz posamezne bučke prelijemo v kiveto in pomerimo A_{540} vseh raztopin proti slepemu vzorcu; vrednosti vnesemo v preglednico 2 na str. 68 ($m_{glukoza}$ v 25 mL bučki izračunamo iz $\gamma_{glu\ izh}$).

SHEMA PRIPRAVE UMERITVENE KRIVULJE

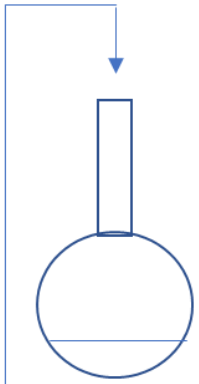
pripravimo 6 epruvet
v vsako:
$V_{p\ izh\ razt\ glu}$ ($\gamma_{glu\ izh}$ = 2,0 mg/mL)
$V_{p\ voda}$
1,0 mL 3,5-dinitrosal. kisline

slepi vzorec:
1,2 mL vode
1,0 mL 3,5-dinitrosal. kisline

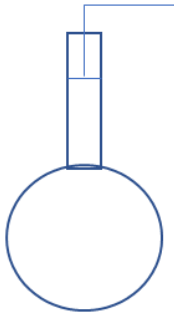
vrela voda/5 min

ohladimo

kvantitativno prenesemo v 25 mL bučko



dopolnimo z dest. vodo



prelijemo v kiveto

pomerimo A_{540} proti slepemu vzorcu

Izračuni

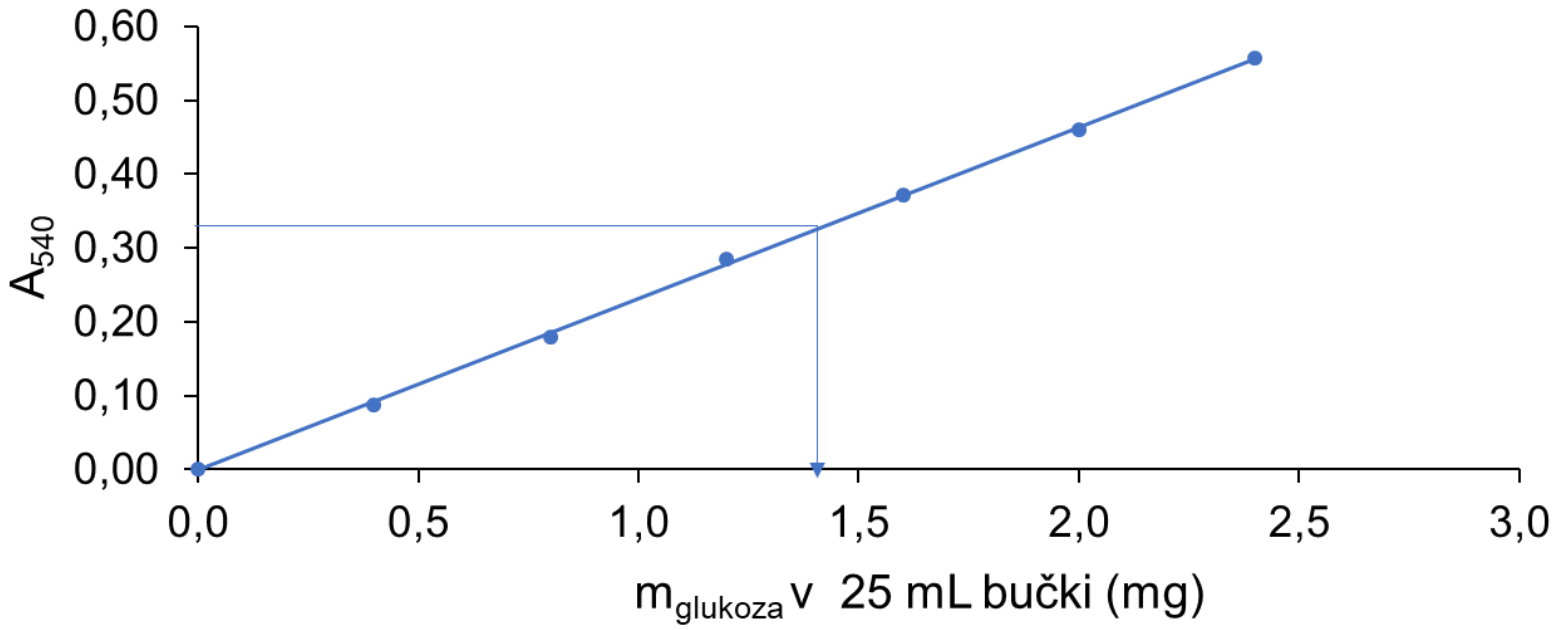
Upoštevajoč vrednosti v preglednici 2 na str. 68 narišemo graf odvisnosti A_{540} od $m_{glukoza}$ v 25 mL bučki. Iz grafa odčitamo ustrezno vrednost $m_{glukoza}$ v 25 mL bučki pri pomerjeni A_{vz540} za preiskovani hidrolizat škroba.

Na primer, da smo za hidrolizat škroba določili vrednost A_{vz540} = 0,316, odčitamo $m_{glukoza}$ v 25 mL bučki, kot je prikazano na str. 65.

Odvisnost A_{540} od $m_{glukoza}$ v 25 mL bučki opišemo lahko tudi z enačbo premice: $A_{540} = k \times m_{glukoza}$ v 25 mL bučki.

Smerni koeficient premice (k) določimo z linearno regresijsko analizo vrednosti A_{540} in $m_{glukoza}$ v 25 mL bučki (preglednica 2 na str. 68). Za preiskovani hidrolizat škroba izračunamo pri pomerjeni A_{vz540} ustrezno vrednost $m_{glukoza}$ v 25 mL bučki. Linearno regresijsko analizo opravimo z uporabo ustreznih računalniških programov (MS Excel ali Origin).

Kako iz $m_{glukoza}$ v hidrolizatu škroba v 25 mL bučki izračunamo maso škroba v zatehti moke ($m_{škrob}$), je razloženo na str. 66, 67.



Odvisnost A_{540} od $m_{glukoza}$ v 25 mL bučki

$m_{glukoza}$ v hidrolizatu škroba v 25 mL bučki = $m_{glukoza}$ v hidrolizatu škroba v epruveti

$m_{glukoza}$ v hidrolizatu škroba v epruveti = $m_{glukoza}$ v $V_{p\ raztopina}$

Glede na opis eksp. postopka: v epruveto odmerimo 0,40 mL raztopine iz 100 mL bučke ($V_{p\ raztopina}$ = 0,40 mL), torej:

$$m_{glukoza}\ v\ 100\ mL\ bučki = \frac{m_{glukoza}\ v\ V_{p\ raztopina}}{V_{p\ raztopina}} \times 100\ mL$$

Upoštevamo dejstvo, da se pri tvorbi glikozidne vezi med glukoznimi enotami v polisaharidu odcepi voda ($M_{glukoza}$ = 180 g/mol, M_{voda} = 18 g/mol), torej:

$M_{glukozna\ enota}$ v polisaharidu = $M_{glukoza} \times 0{,}9$ oziroma: $m_{glukoznih\ enot}$ v polimeru škroba = $m_{glukoza} \times 0{,}9$

$m_{glukoznih\ enot}$ v 100 mL bučki = $m_{glukoza}$ v 100 mL bučki $\times$ 0,9

Glede na opis eksp. postopka: pri pripravi hidrolizata vsebino iz čaše kvantitativno prenesemo v 100 mL merilno bučko, torej:

$$m_{\text{glukoznih enot}} \text{ v 100 mL bučki} = m_{\text{glukoznih enot}} \text{ v polimeru škroba v moki} =$$

$$= m_{\text{škrob}} \text{ v moki}$$

$$w_{\text{škrob}} \text{ v moki} = \frac{m_{\text{škrob}} \text{ v moki}}{m_{\text{moka}}} \times 100\ \%$$

Poročilo

Analiza hidrolizata škroba

postopek razgradnje škroba: …….

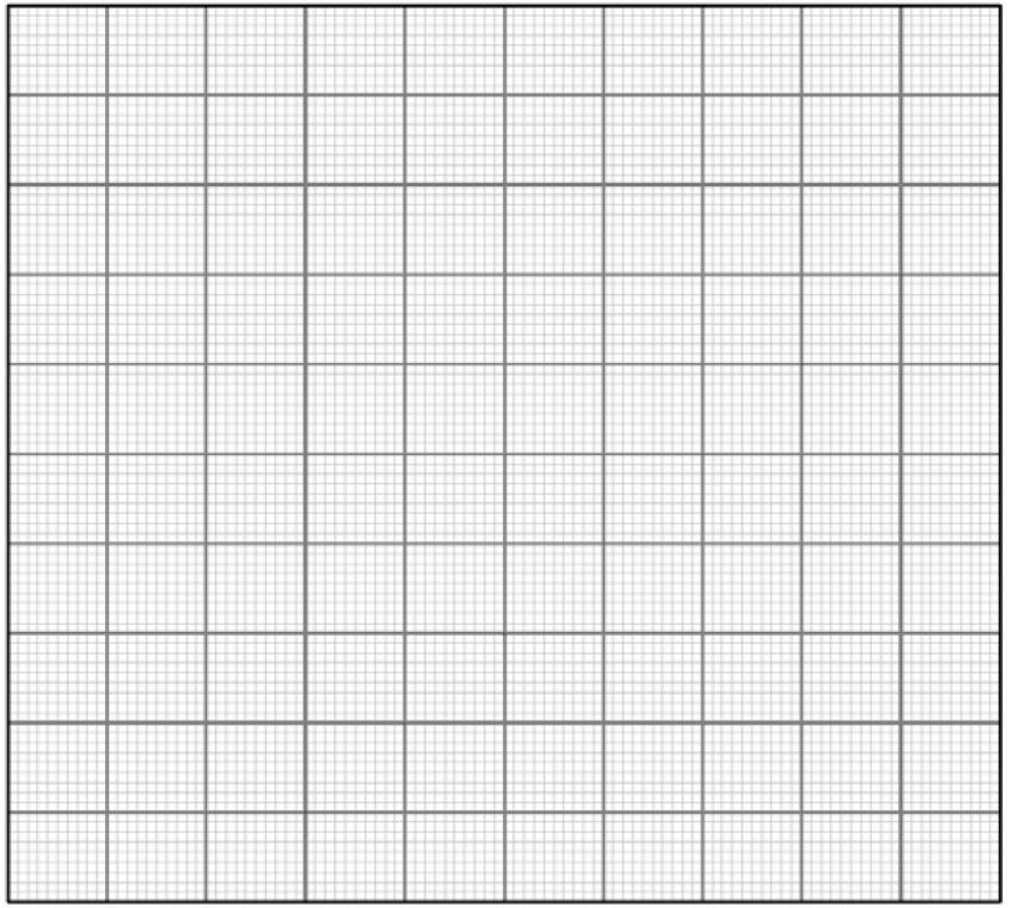
m_{moka} = …….. A_{vz540} = ……..

Ostali podatki so navedeni v opisu eksperimentalnega postopka.

Umeritvena krivulja

Preglednica 2: Odvisnost A_{540} od $m_{glukoza}$ v 25 mL bučki.

epruveta	$V_{p\ izh\ razt\ glu}$ (mL)	$m_{glukoza}$ v 25 mL bučki (mg)	$V_{p\ voda}$ (mL)	A_{540}
1	0,20	0,40	1,00	
2	0,40	0,80	0,80	
3	0,60	1,20	0,60	
4	0,80	1,60	0,40	
5	1,00	2,00	0,20	
6	1,20	2,40	0,00	



Odvisnost A_{540} od $m_{glukoza}$ v 25 mL bučki

Izračun $m_{glukoza}$ v hidrolizatu škroba in masnega deleža škroba v moki ($w_{škrob}$)

Iz umeritvene krivulje odčitana ali s pomočjo k (k = …….) izračunana vrednost $m_{glukoza}$ v hidrolizatu škroba v 25 mL bučki pri pomerjeni A_{vz540}: ……..

Izračunajte $w_{škrob}$; napišite celoten izračun s številkami in enotami.

$m_{glukoza}$ v hidrolizatu škroba v epruveti =

$m_{glukoza}$ v 100 mL bučki =

$m_{glukoznih\ enot}$ v polimeru škroba =

$m_{škrob}$ v moki =

$w_{škrob}$ v moki =

Izpolnite preglednico in komentirajte vpliv postopka razgradnje škroba na vsebnost glukoze v hidrolizatu škroba in na izračunan masni delež škroba.

Preglednica 3: Vsebnost glukoze v pripravljenih hidrolizatih škroba in izračunan masni delež škroba ($w_{škrob}$).

postopek razgradnje škroba	A_{vz540}	$m_{glukoza}$ v hidrolizatu škroba v 25 mL bučki (mg)	$w_{škrob}$ (%)
1A			
1B			
2A			
2B			
3A			
3B			
4A			
4B			

Komentar

KVANTITATIVNA DOLOČITEV HOLESTEROLA V MLEKU

Holesterol

Holesterol sodi v skupino lipidov, poimenovano steroidi. Steroli so spojine, kjer je na steroidni skelet vezana −OH skupina.

Holesterol je sestavni del celičnih membran in vpliva na fluidnost oz. togost membrane. V veliki količini je prisoten v živčnem tkivu, predvsem v možganih. Je tudi prekurzor za sintezo žolčnih kislin, vitamina D_3 (holekalciferola) in steroidnih hormonov. Lahko ga sami sintetiziramo (predvsem v jetrih) ali zaužijemo s hrano. Zaradi slabe topnosti v vodi se po organizmu v krvi in ostalih zunajceličnih tekočinah transportira kot del lipidnih kompleksov s proteini, tj. lipoproteinov.

Holesterol sodi med t.i. živalske sterole in je prisoten v živilih, kot so meso, mast, jajčni rumenjak, mleko in mlečni izdelki. V mleku se holesterol nahaja v lipoproteinskih kompleksih in sicer pretežno v fosfolipidnem monosloju, ki obdaja maščobne kroglice, manjši del (kot ester z maščobnimi kislinami) pa v njihovi notranjosti. Vsebnost holesterola v mleku je povezana z vsebnostjo mlečne maščobe. Če je prehranski vnos holesterola previsok ali se pojavljajo metabolne motnje, se koncentracija holesterola v krvi poveča, kar predstavlja povečano tveganje za srčno-žilne bolezni. To poudarja pomen določitve holesterola v živilih.

Rastline sintetizirajo različne t.i. rastlinske sterole, fitosterole (*β*-sitosterol, stigmasterol, …). Najdemo jih predvsem v rastlinskih oljih. Ker naj bi fitosteroli ob zaužitju v prebavnem traktu zmanjšali absorpcijo holesterola in s tem zmanjšali koncentracijo holesterola krvi, se dodajajo v nekatera živila.

Namen vaje

Določiti masno koncentracijo holesterola v vzorcih mleka (γ_{holM}) z 0,5 %, 1,5 % oz. 3,5 % mlečne maščobe.

Ugotoviti, kako je koncentracija holesterola v mleku povezana z odstotkom mlečne maščobe.

Princip vaje

V vzorcu mleka s saponifikacijo razgradimo triacilglicerole in sprostimo holesterol iz holesterolnih estrov in lipoproteinskega kompleksa.

Po ekstrakciji prostega holesterola v organsko fazo sledi spektrofotometrična določitev produkta, ki nastane v naslednji reakciji v prisotnosti močne kisline:

holesterol + *o*-ftalaldehid ⟶ reakcijski produkt (vijoličasta)

Absorbanca je sorazmerna s koncentracijo reakcijskega produkta in koncentracija reakcijskega produkta je sorazmerna z vsebnostjo holesterola.

Eksperimentalni postopek

a) Saponifikacija s KOH v etanolu
b) Ekstrakcija holesterola v heksan in odparitev heksana
c) Spektrofotometrična določitev holesterola
d) Umeritvena krivulja za določitev holesterola

a) Saponifikacija s KOH v etanolu

- V epruveto z brušenim zamaškom odmerimo 2 mL mleka ($V_{p\ mleko}$), dodamo 3 mL etanola (95 %) in 2 mL raztopine KOH (50 %); epruveto zapremo in vsebino premešamo.
- Segrevamo 15 min pri 60 °C v vodni kopeli; pri tem se s saponifikacijo razgradijo triacilgliceroli in sprosti holesterol iz lipoproteinskega kompleksa.
- Ohladimo na sobno temperaturo.

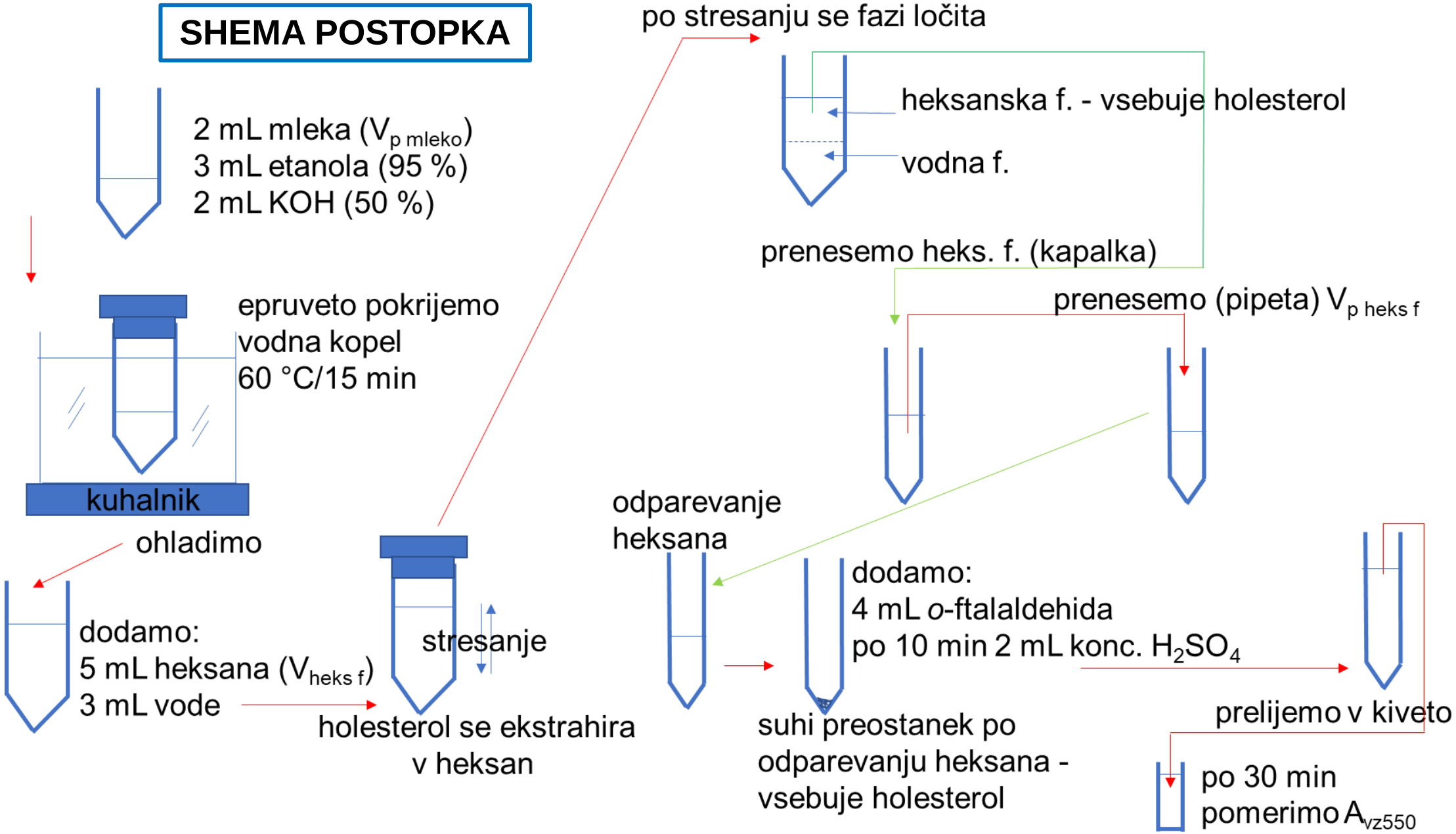
b) Ekstrakcija holesterola v heksan in odparitev heksana

- Dodamo 5 mL heksana ($V_{heks\ f}$) in 3 mL vode.
- Epruveto pokrijemo in stresamo; pri tem poteka ekstrakcija holesterola iz vodne faze v heksan.
- Nato epruveto odložimo v stojalo in počakamo, da se vodna in heksanska faza ločita; vodna faza je v spodnjem delu epruvete, heksanska faza je nad njo; meja med obema fazama je vidna.
- S kapalko prenesemo heksansko fazo v drugo čisto epruveto; pri tem pazimo, da s kapalko ne zajamemo vodne faze.
- Z merilno pipeto odmerimo določen volumen heksanske faze ($V_{p\ heks\ f}$) v naslednjo čisto epruveto; pri mleku z 0,5 % maščobe, $V_{p\ heks\ f}$ = 3 mL; pri mleku z 1,5 % oz. 3,5 % maščobe, $V_{p\ heks\ f}$ = 2 mL.
- Odstranimo heksan z odparevanjem v vakuumskem sušilniku (30 min, 40 °C, 30 mbar).

c) Spektrofotometrična določitev holesterola

- Suhemu preostanku po odparevanju heksana (vsebuje holesterol) dodamo 4 mL raztopine *o*-ftalaldehida v koncentrirani ocetni kislini, previdno premešamo; po 10 min dodamo 2 mL koncentrirane raztopine H_2SO_4 (potrebna je pazljivost pri delu!).
- Po 30 min pomerimo absorbanco pri 550 nm (A_{vz550}) proti slepemu vzorcu.
- Slepi vzorec sestoji iz 4 mL raztopine *o*-ftalaldehida in 2 mL koncentrirane raztopine H_2SO_4.

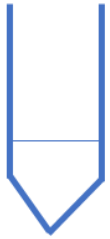
SHEMA POSTOPKA



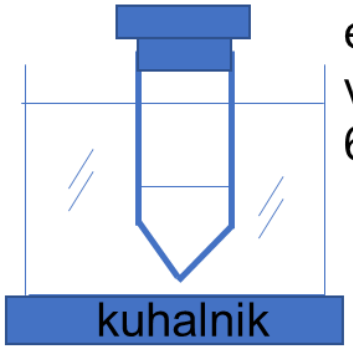
d) Umeritvena krivulja za določitev holesterola

- Uporabimo izhodno raztopino holesterola v 95 % etanolu z masno koncentracijo 0,10 mg/mL ($\gamma_{hol\ izh}$).
- Pripravimo 5 epruvet z brušenim zamaškom; v vsako odmerimo ustrezen volumen izhodne raztopine holesterola ($V_{p\ izh\ razt\ hol}$) in 95 % etanola ($V_{95\%\ etanol}$), kot je navedeno v preglednici 1 na str. 88.
- Nato dodamo v vsako epruveto po 2 mL raztopine KOH (50 %), segrevamo 15 min pri 60 °C v vodni kopeli in postopamo podobno kot pri določitvi holesterola v mleku.
- Razlika je le, da pred korakom odparevanja heksana odmerimo 4 mL heksanske faze ($V_{p\ heks\ f}$ = 4 mL) in ne 2 mL oz. 3 mL kot pri določitvi holesterola v mleku.
- Pomerimo A_{550} vseh raztopin in vrednosti vnesemo v preglednico 1 na str. 88; kako izračunamo koncentracijo holesterola v reakcijski zmesi ($\gamma_{hol\ reakc\ zm}$) za umeritveno krivuljo, je razloženo na str. 85.

SHEMA PRIPRAVE UMERITVENE KRIVULJE

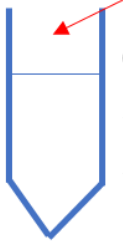


pripravimo 5 epruvet
v vsako:
$V_{p\ izh\ razt\ hol}$ ($\gamma_{hol\ izh}$ = 0,10 mg/mL)
$V_{95\%\ etanol}$
2 mL KOH (50 %)



epruveto pokrijemo
vodna kopel
60 °C/15 min

ohladimo

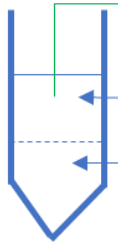


dodamo
5 mL heksana ($V_{heks\ f}$)
3 mL vode

stresanje

holesterol se ekstrahira
v heksan

po stresanju se fazi ločita

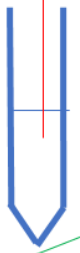


heksanska f. - vsebuje holesterol

vodna f.

prenesemo heks. f. (kapalka)

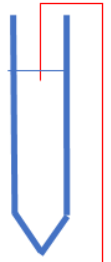
prenesemo (pipeta) $V_{p\ heks\ f}$ = 4 mL



odparevanje
heksana

dodamo:
4 mL *o*-ftalaldehida
po 10 min 2 mL konc. H_2SO_4

suhi preostanek po
odparevanju heksana -
vsebuje holesterol



prelijemo v kiveto

po 30 min
pomerimo A_{550}

Izračuni

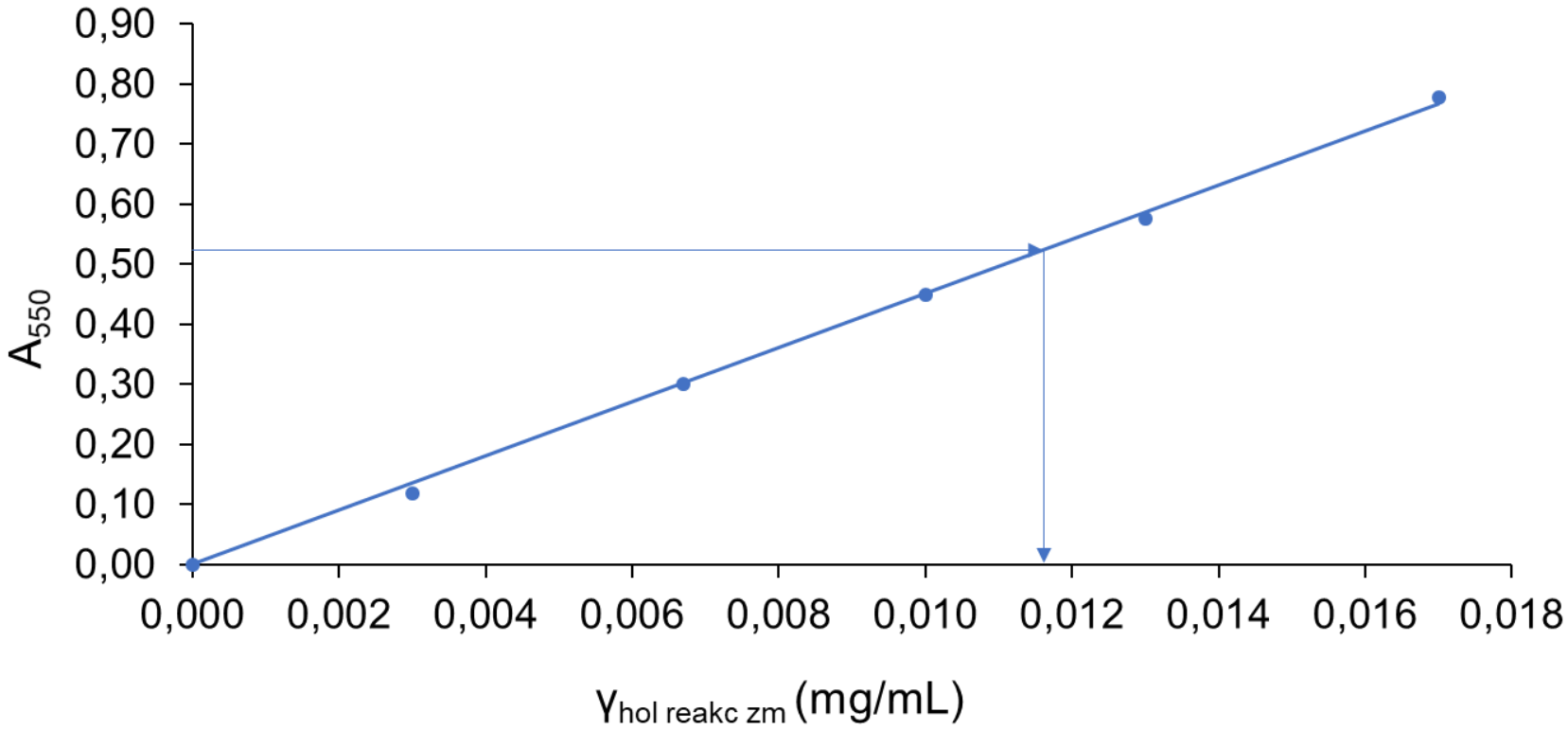
Upoštevajoč vrednosti v preglednici 1 na str. 88 narišemo graf odvisnosti A_{550} od $\gamma_{hol\ reakc\ zm}$. Pri pomerjeni A_{vz550} odčitamo iz grafa ustrezno vrednost $\gamma_{hol\ reakc\ zm}$ za vzorec mleka.

Na primer, da smo določili vrednost $A_{vz550} = 0{,}525$, odčitamo $\gamma_{hol\ reakc\ zm}$, kot je prikazano na str. 84.

Odvisnost A_{550} od $\gamma_{hol\ reakc\ zm}$ opišemo lahko tudi z enačbo premice: $A_{550} = k \times \gamma_{hol\ reakc\ zm}$.

Smerni koeficient premice (k) določimo z linearno regresijsko analizo vrednosti A_{550} in $\gamma_{hol\ reakc\ zm}$ (preglednica 1 na str. 88). Pri pomerjeni A_{vz550} izračunamo ustrezno vrednost $\gamma_{hol\ reakc\ zm}$ za vzorec mleka. Linearno regresijsko analizo opravimo z uporabo ustreznih računalniških programov (MS Excel ali Origin).

Kako za preiskovani vzorec mleka iz $\gamma_{hol\ reakc\ zm}$ izračunamo γ_{holM}, je razloženo na str. 86, 87.



Odvisnost A_{550} od $\gamma_{hol\ reakc\ zm}$

Izračun koncentracije holesterola v reakcijski zmesi ($\gamma_{hol\ reakc\ zm}$) za umeritveno krivuljo

$$m_{hol}\ v\ V_{p\ izh\ razt\ hol} = \gamma_{hol\ izh} \times V_{p\ izh\ razt\ hol}$$

Vrednosti za $V_{p\ izh\ razt\ hol}$ so navedene v preglednici 1 na str. 88; $\gamma_{hol\ izh} = 0{,}10$ mg/mL.

Holesterol se iz izhodne raztopine ekstrahira v heksan, torej:

$$m_{hol}\ v\ celotnem\ volumnu\ heksanske\ faze = m_{hol}\ v\ V_{p\ izh\ razt\ hol}$$

$$\gamma_{hol\ heks\ f} = \frac{m_{hol}\ v\ celotnem\ volumnu\ heksanske\ faze}{V_{heks\ f}}$$

Glede na opis eksp. postopka: $V_{heks\ f} = 5$ mL; pri pripravi umeritvene k. prenesemo v novo epruveto $V_{p\ heks\ f} = 4$ mL.

$$m_{hol}\ v\ V_{p\ heks\ f} = \gamma_{hol\ heks\ f} \times V_{p\ heks\ f}$$

$m_{hol}\ v\ V_{p\ heks\ f} = m_{hol}$ pred odparitvijo heksana = m_{hol} po odparitvi heksana = m_{hol} v reakcijski zmesi, torej:

$$\gamma_{hol\ reakc\ zm} = \frac{m_{hol}\ v\ reakcijski\ zmesi}{V_{reakc\ zm}}$$

Glede na opis eksp. postopka: $V_{rekc\ zm} = 6$ mL.

Izračun koncentracije holesterola v mleku (γ_{holM})

Iz umeritvene krivulje odčitamo ali s pomočjo k izračunamo za preiskovani vzorec mleka ustrezno vrednost $\gamma_{hol\ reakc\ zm}$ pri pomerjeni A_{vz550}.

$$m_{hol}\ v\ reakcijski\ zmesi = \gamma_{hol\ reakc\ zm} \times V_{rekc\ zm}$$

Glede na opis eksp. postopka: $V_{rekc\ zm} = 6$ mL.

m_{hol} v reakcijski zmesi = m_{hol} po odparitvi heksana = m_{hol} pred odparitvijo heksana = m_{hol} v $V_{p\ heks\ f}$, torej:

$$\gamma_{hol\ heks\ f} = \frac{m_{hol}\ v\ V_{p\ heks\ f}}{V_{p\ heks\ f}}$$

Glede na opis eksp. postopka: pri mleku z 0,5 % maščobe, $V_{p\ heks\ f} = 3$ mL; pri mleku z 1,5 % oz. 3,5 % maščobe, $V_{p\ heks\ f} = 2$ mL.

$$m_{hol}\ v\ celotnem\ volumnu\ heksanske\ faze = \gamma_{hol\ heks\ f} \times V_{heks\ f}$$

Glede na opis eksp. postopka: $V_{heks\ f} = 5$ mL.

Holesterol se iz mleka ekstrahira v heksan, torej:

$$m_{hol} \text{ v } V_{p\ mleko} = m_{hol} \text{ v celotnem volumnu heksanske faze}$$

$$\gamma_{holM} = \frac{m_{hol \text{ v } V_{p\ mleko}}}{V_{p\ mleko}}$$

Poročilo

Analiza vzorca mleka

vsebnost mlečne maščobe = …….

$V_{p\ heks\ f}$ = ……. A_{vz550} = ……..

Ostali podatki so navedeni v opisu eksperimentalnega postopka.

Umeritvena krivulja

Preglednica 1: Odvisnost A_{550} od $\gamma_{hol\ reakc\ zm}$.

epruveta	$V_{p\ izh\ razt\ hol}$ (mL)	$V_{95\%\ etanol}$ (mL)	$\gamma_{hol\ reakc\ zm}$ (mg/mL)	A_{550}
1	0,25	2,75	0,0033	
2	0,50	2,50	0,0067	
3	0,75	2,25	0,0100	
4	1,00	2,00	0,0133	
5	1,25	1,75	0,0167	

Izračun koncentracije holesterola v reakcijski zmesi ($\gamma_{hol\ reakc\ zm}$) za umeritveno krivuljo

Za vsako od epruvet izračunajte $\gamma_{hol\ reakc\ zm}$ in preverite, ali se vaša izračunana vrednost sklada z vrednostjo v preglednici 1 na str. 88.

m_{hol} v $V_{p\ izh\ razt\ hol}$ =

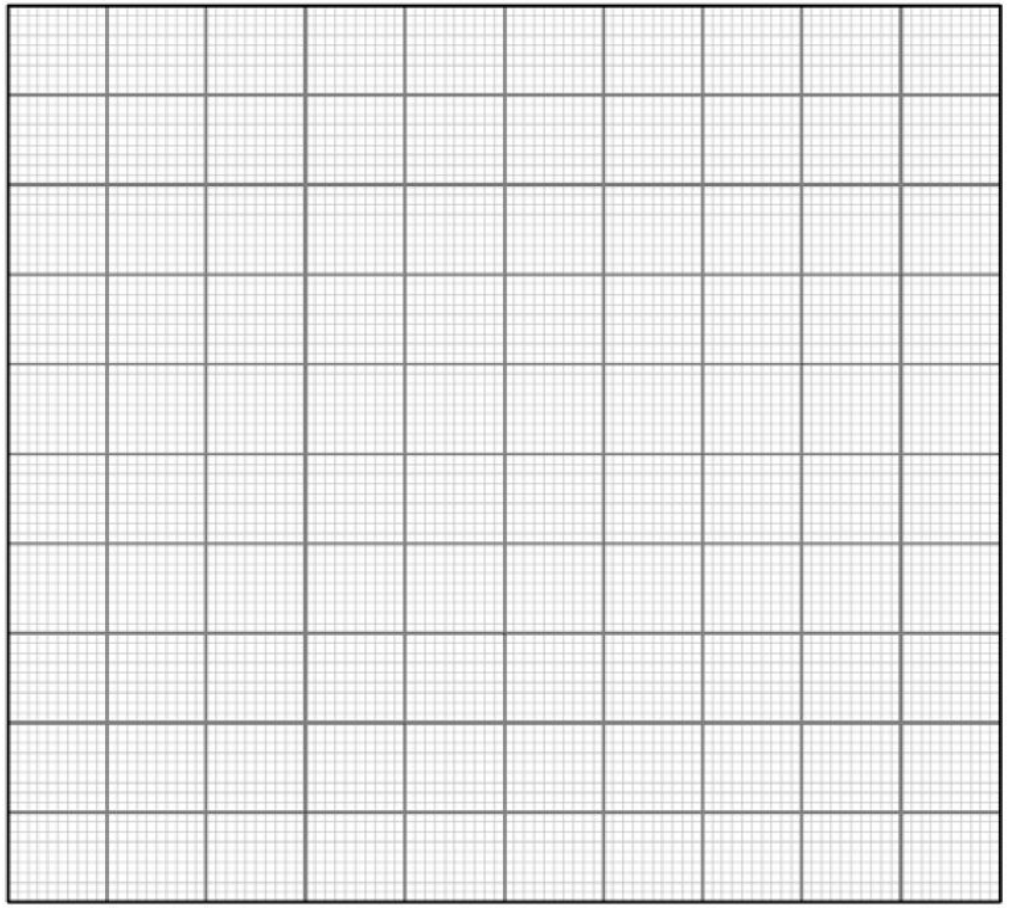
m_{hol} v celotnem volumnu heksanske faze =

$\gamma_{hol\ heks\ f}$ =

m_{hol} v $V_{p\ heks\ f}$ =

m_{hol} v reakcijski zmesi =

$\gamma_{hol\ reakc\ zm}$ =



Odvisnost A_{550} od $\gamma_{hol\ reakc\ zm}$

Izračun koncentracije holesterola v mleku (γ_{holM})

Iz umeritvene krivulje odčitana ali s pomočjo k (k = ……) izračunana vrednost $\gamma_{hol\ reakc\ zm}$ za preiskovani vzorec mleka pri vrednosti A_{vz550}: ……..

Izračunajte γ_{holM}; napišite celoten izračun s številkami in enotami.

m_{hol} v reakcijski zmesi =

m_{hol} v $V_{p\ heks\ f}$ =

$\gamma_{hol\ heks\ f}$ =

m_{hol} v celotnem volumnu heksanske faze =

m_{hol} v $V_{p\ mleko}$ =

γ_{holM} =

Izpolnite preglednico in komentirajte določeno vsebnost holesterola glede na vsebnost maščobe v mleku.

Preglednica 2: Odvisnost koncentracije holesterola od vsebnosti maščobe v mleku.

vsebnost mlečne maščobe (%)	0,5	1,5	3,5
$\gamma_{hol\ reakc\ zm}$ (mg/mL)			
m_{hol} v reakcijski zmesi (mg)			
m_{hol} v $V_{p\ heks\ f}$ (mg)			
$\gamma_{hol\ heks\ f}$ (mg/mL)			
m_{hol} v celotnem volumnu heksanske faze (mg)			
m_{hol} v $V_{p\ mleko}$ (mg)			
γ_{holM} (mg/mL)			

Komentar

DOLOČITEV AKTIVNOSTI POLIFENOL OKSIDAZE V KROMPIRJU

Fenol oksidaze

Fenol oksidaze so encimi, ki katalizirajo aerobno oksidacijo fenolnih spojin, kar v nadaljevanju vodi v tvorbo različnih rjavo obarvanih visokomolekularnih produktov.

Pri tem gre za reakcijo hidroksilacije monofenolov v *o*-difenole:

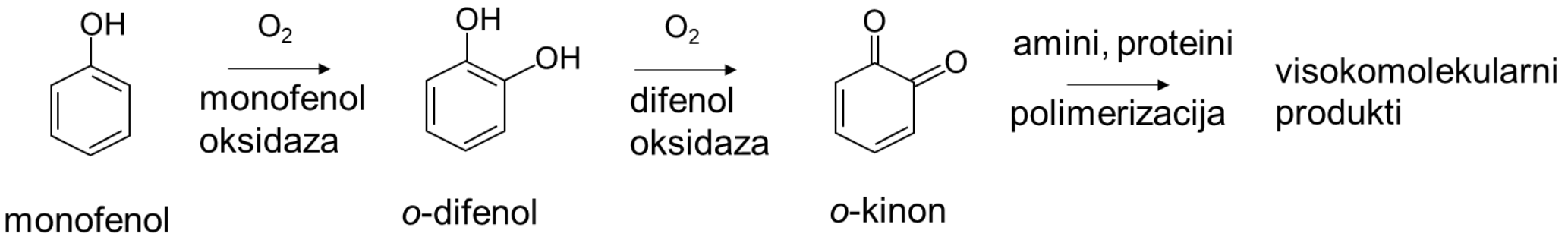
monofenol + O_2 + $2H^+$ → *o*-difenol + H_2O

in za reakcijo oksidacije *o*-difenolov v *o*-kinone:

o-difenol + ½ O_2 → *o*-kinon + H_2O

Monofenoli so spojine, kjer je na benzenov obroč vezana ena –OH skupina. *o*-difenoli (katehol) so spojine, kjer sta na benzenov obroč vezani medsebojno v *orto* položaju dve –OH skupini.

Encimi, ki katalizirajo hidroksilacije monofenolov v *o*-difenole, sodijo med monofenol oksidaze. Encimi, ki katalizirajo oksidacijo *o*-difenolov v *o*-kinone, sodijo med difenol (katehol) oksidaze. Encimi, ki katalizirajo hidroksilacijo monofenolov v *o*-difenole in oksidacijo le teh v *o*-kinone, sodijo med tirozinaze.



V katalitično aktivnost encima so vključeni bakrovi ioni.

Nastali *o*-kinoni so reaktivne molekule. Rjavo obarvani visokomolekularni produkti se tvorijo s polimerizacijo *o*-kinonov. V reakcijo polimerizacije, ki ni encimsko katalizirana, so vključeni amini in proteini.

Fenol oksidaze so prisotne v mikroorganizmih ter v rastlinskih in živalskih celicah, kjer njihova aktivnost vodi v nastanek pigmentov melaninov. V rastlinah naj bi prisotnost omenjenih encimov predstavljala zaščito pred mrčesom in patogeni. V rastlinski celici se nahajajo v kloroplastih in kromoplastih. Ob poškodbi tkiva se izlijejo iz omenjenih organelov, pridejo v stik s substratom (fenolne spojine) in se zaradi različnih dejavnikov aktivirajo. Nastali *o*-kinoni lahko pri škodljivcu povzročijo deaktivacijo encimov. Polimerni produkti *o*-kinonov pa naj bi predstavljali tudi fizično zaščito.

V živilstvu posvečamo fenol oksidazam pozornost, ker med obdelavo in skladiščenjem povzročijo encimsko porjavitev sadja in zelenjave. Do porjavitve pride na površini tkiva, ki je izpostavljeno mehanski obdelavi oz. poškodbi (lupljenje, rezanje, mletje, …). Do porjavitve pride lahko tudi v sadnem oz. zelenjavnem soku, pireju, …. Zaradi poslabšanja senzoričnih lastnosti (videz), so takšna živila s strani potrošnika manj sprejemljiva.

Nastali zavržki povzročajo ekonomske izgube in predstavljajo obremenitev za okolje. V nekaterih primerih, kot so npr. rozine, suhe slive, kakav, črni čaj, kava, pa je pojav encimske porjavitve zaželen.

Optimalni pogoji za encimsko aktivnost so v pH območju med 4,0 in 7,0 ter temperatura od 30 do 50 °C. Encim je relativno stabilen tudi v temperaturnem območju od 55 do 80 °C. Zato obstaja možnost, da se katalitična aktivnost fenol oksidaz pokaže tudi med kratkotrajnejšo toplotno obdelavo, saj pride pri ~60 °C do poškodovanja celičnih membran, kar omogoči stik encima s substratom.

Pojav encimske porjavitve se ublaži/prepreči z izvedbo različnih fizikalnih in kemijskih postopkov.

Fizikalni postopki: dehidracija, zamrzovanje, toplotna obdelava (blanširanje) z namenom deaktivacije encima; obdelava v modificirani atmosferi, oblivanje s sladkornim sirupom, uporaba zaščitnih filmov z namenom zmanjšanja dostopnosti O_2.

Izvedba kemijskih postopkov za inhibicijo encimov vključuje uporabo kislin (citronska, jabolčna, fosforna(V) kislina) z namenom znižanja pH, saj so fenol oksidaze zelo slabo aktivne pri pH < 3; dodatek kelatorjev (EDTA, oksalna kislina, citronska kislina), ki vežejo bakrove ione; dodatek reducentov (sulfit, askorbinska kislina, cistein), ki reducirajo *o*-kinone v difenole.

Fenolne spojine

To je številčna skupina strukturno raznolikih spojin. Med fenolne spojine uvrščamo: enostavne fenole ali benzokinone, fenolne kisline, naftokinone, ksantone, stilbene, flavonoide, lignane, biflavonoide, lignine, kumarine in kondenzirane tanine. Prehransko so najpomembnejše fenolne kisline, flavonoidi in tanini. Flavonoidi so med fenolnimi spojinami največja skupina.

Fenolne spojine so prisotne v naravi kot sekundarni metaboliti rastlinske celice. Razporeditev teh spojin v celici/rastlini ni enakomerna. Nahajajo se v vakuoli (proste, konjugati) in vezane na celično steno. Akumulirajo se v zunanjih slojih in epidermalnem tkivu rastline. Rastlini nudijo zaščito pred zunanjimi stresnimi dejavniki, delujejo kot vizualni markerji in imajo pomen pri rasti in reprodukciji.

Vplivajo na senzorične lastnosti (barva, okus, aroma) živil. Še posebno veliko fenolnih spojin je v začimbah, jagodičevju, čaju in oljčnem olju.

Namen vaje

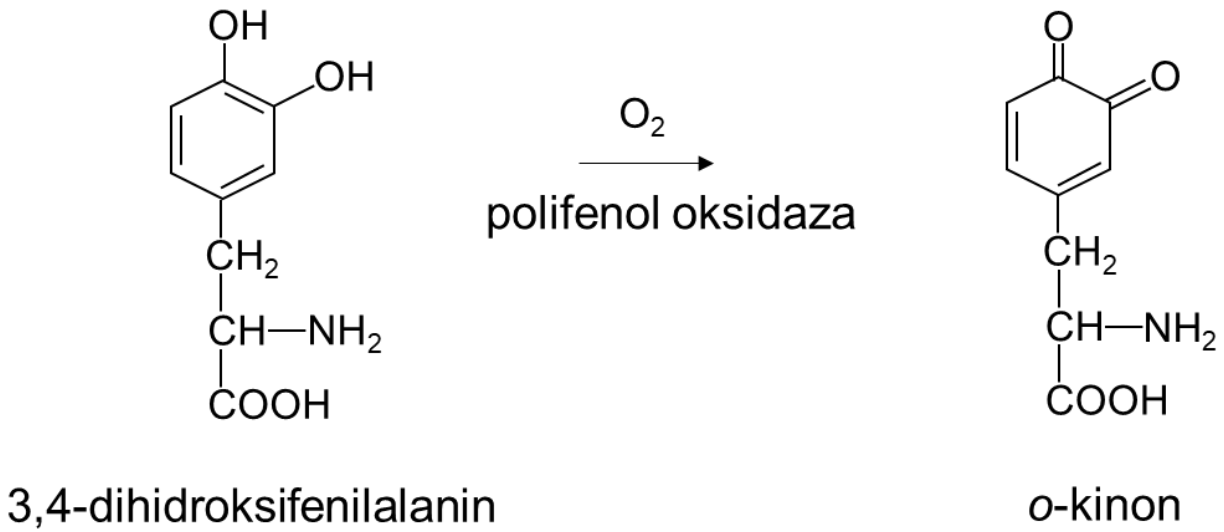
Določiti aktivnost polifenol oksidaze v starem in v mladem krompirju.

Določiti vpliv toplotne obdelave krompirja (45 min kuhanje v vreli vodi) na aktivnost polifenol oksidaze.

Princip vaje

Polifenol oksidaza (PFO) v ekstraktu iz krompirja katalizira aerobno oksidacijo fenolne spojine 3,4-dihidroksifenilalanin (DOPA).

Hitrost nastajanja reakcijskega produkta (*o*-kinona) je sorazmerna z aktivnostjo polifenol oksidaze in z vsebnostjo polifenol oksidaze v reakcijski zmesi.



3,4-dihidroksifenilalanin

o-kinon

Koncentracijo reakcijskega produkta (*o*-kinona) oziroma hitrost njegovega nastajanja spremljamo spektrofotometrično.

Eksperimentalni postopek

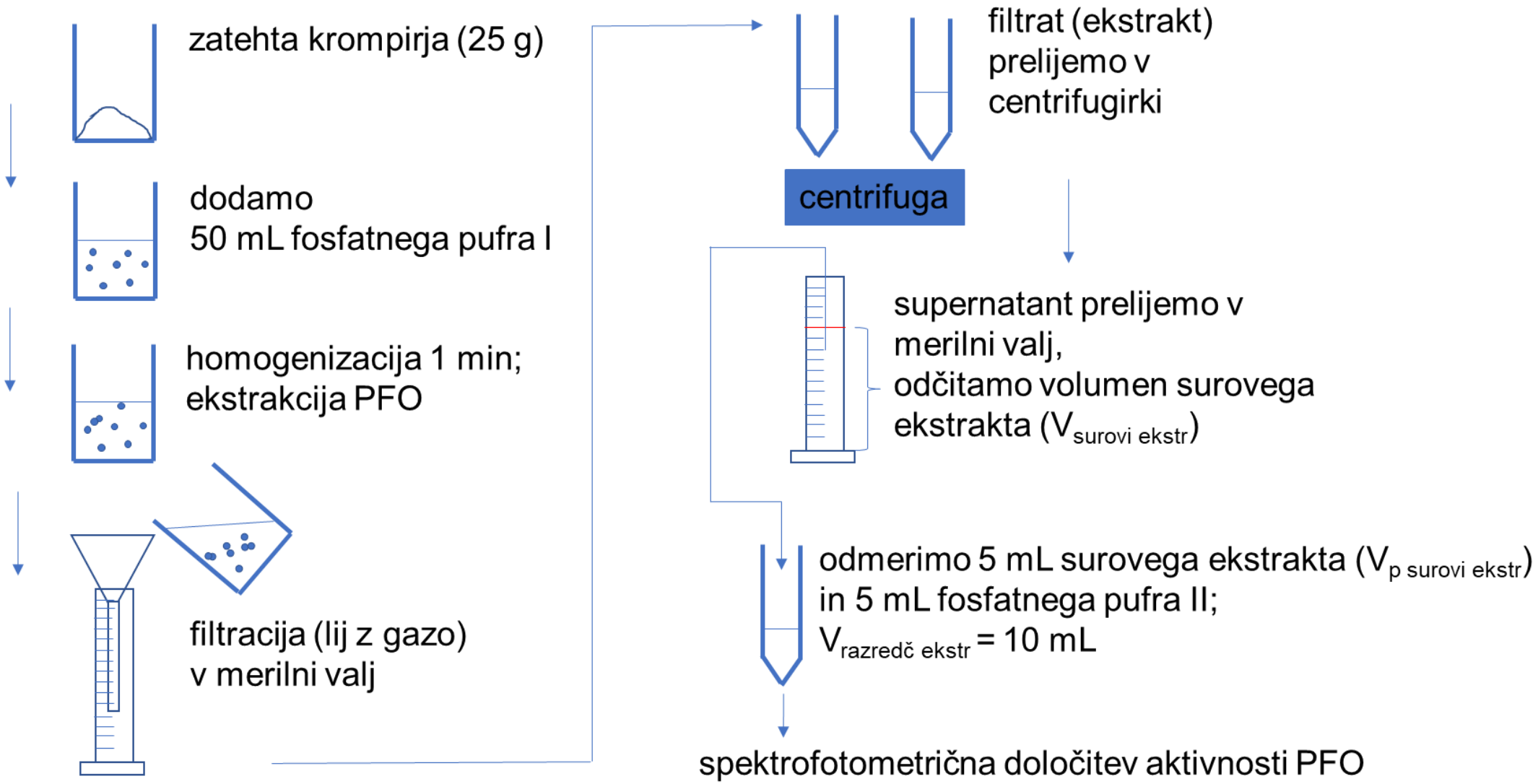
a) Ekstrakcija polifenol oksidaze iz krompirja (izvedemo postopek določitve aktivnosti polifenol oksidaze v starem in v mladem krompirju ter v toplotno obdelanem mladem krompirju)

b) Spektrofotometrična določitev aktivnosti polifenol oksidaze

a) Ekstrakcija polifenol oksidaze iz krompirja (priprava ekstrakta)

- Krompir olupimo, narežemo in zatehtamo 25 g ($m_{krompir}$) v sekljalnik.
- Dodamo 50 mL ledenomrzlega fosfatnega pufra I (0,1 mol/L, pH 6,8; vsebuje NaF).
- Sledi homogenizacija 1 min.
- Prefiltriramo v merilni valj skozi lij z gazo; filtrat (ekstrakt PFO) je moten; potrebujemo bister ekstrakt, saj je določitev aktivnosti spektrofotometrična; zato prelijemo filtrat (ekstrakt) v dve centrifugirki; pozorni smo na to, da sta centrifugirki z vsebino vred po masi izenačeni; sledi centrifugiranje.
- Supernatant (to je bistri del nad usedlino po centrifugiranju v centrifugirki) previdno prelijemo v merilni valj in v merilnem valju odčitamo volumen surovega ekstrakta ($V_{surovi\ ekstr}$).
- Pripravimo razredčen ekstrakt: v epruveto odmerimo 5 mL surovega ekstrakta (to je $V_{p\ surovi\ ekstr}$), dodamo 5 mL fosfatnega pufra II (0,1 mol/L, pH 6,8) in premešamo na vrtinčniku; volumen razredčenega ekstrakta ($V_{razredč\ ekstr}$) je 10 mL.

SHEMA POSTOPKA EKSTRAKCIJE POLIFENOL OKSIDAZE



b) Spektrofotometrična določitev aktivnosti polifenol oksidaze

- Za vsak posamezni razredčen ekstrakt pripravimo: kivete za vzorec (KV_{vz}), kiveto za kontrolni vzorec (KV_{kontr}) in kiveto za slepi vzorec ($KV_{sl\ vz}$).
- V kiveti $KV_{sl\ vz}$ je voda.
- V kiveto KV_{kontr} odmerimo pufer II in raztopino DOPA (preglednica 1 na str. 106), premešamo in pomerimo absorbanco pri 475 nm (A_{kontr}) proti slepemu vzorcu.
- V kivete KV_{vz} odmerimo pufer II, raztopino DOPA in določen volumen razredčenega ekstrakta ($V_{p\ razredč\ ekstr}$) (preglednica 1 na str. 106); ekstrakt odmerimo pri spektrofotometru tik pred začetkom meritev.
- Ko odmerimo ekstrakt, sprožimo štoparico, vsebino v kiveti dobro premešamo, kiveto postavimo v spektrofotometer in začnemo z meritvami absorbance pri 475 nm proti slepemu vzorcu (A_{izm}).
- A_{izm} pomerimo vsakih 15 s do t = 90 s, zatem vsakih 30 s do t = 300 s.

Preglednica 1: Volumen pufra II ($V_{p\ pufer\ II}$), raztopine DOPA ($V_{p\ DOPA}$) in razredčenega ekstrakta ($V_{p\ razredč\ ekstr}$) za odmerjanje v kivete.

kiveta	$V_{p\ pufer\ II}$ (mL)	$V_{p\ DOPA}$ (mL)	$V_{p\ razredč\ ekstr}$ (mL)
KV_{kontr}	2,00	1,00	-
KV_{vz} 1	1,95	1,00	0,05
KV_{vz} 2	1,90	1,00	0,10
KV_{vz} 3	1,80	1,00	0,20
KV_{vz} 4	1,70	1,00	0,30

Različni $V_{p\ razredč\ ekstr}$ pomenijo različne količine encima v reakcijski zmesi.

Izračuni

Absorbanco reakcijskega produkta (A_{vz}) izračunamo tako, da od vrednosti A_{izm} odštejemo vrednost A_{kontr} ($A_{vz} = A_{izm} - A_{kontr}$). Vrednosti zapišemo v preglednico 2 na str. 113 in narišemo graf odvisnosti A_{vz} od časa.

Da lahko določimo začetno hitrost, potegnemo tangento na krivuljo pri času t = 0, kot je prikazano na str. 108, in odčitamo A_{vz} pri 60 sek (1 min); to je ΔA_{vz} / min.

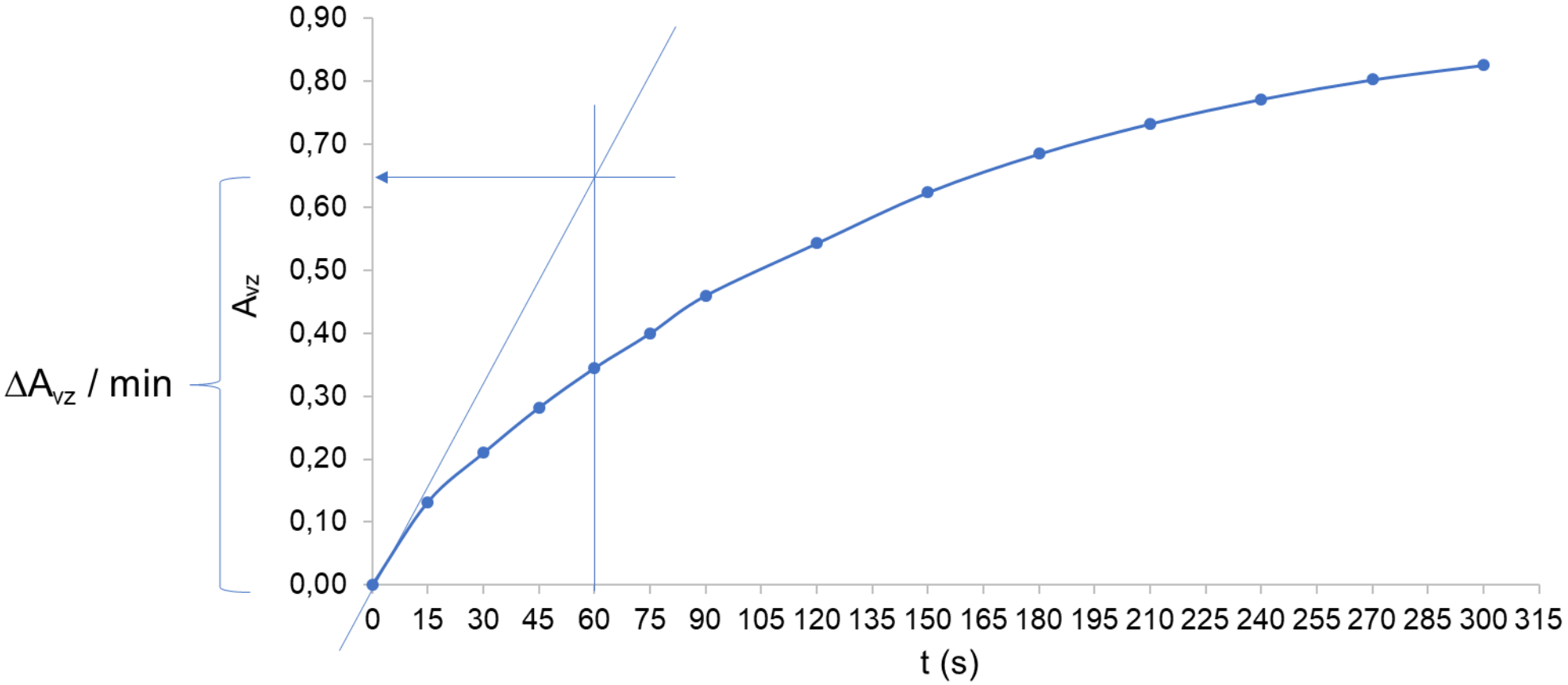
Aktivnost PFO je sorazmerna z množino produkta v danem volumnu reakcijske zmesi ($V_{reakc\ zm}$), ki nastane v eni minuti ($n_{produkt}$ / min), in $n_{produkt}$ / min je sorazmerno z ΔA_{vz} / min.

$n_{produkt}$ / min izračunamo upoštevajoč Beer-Lambertov zakon ($A = \varepsilon \times l \times c$).

Koncentracija produkta v reakcijski zmesi ($c_{produkt}$) je podana kot:

$$c_{produkt} = \frac{n_{produkt}}{V_{reakc\ zm}} = \frac{A_{vz}}{\varepsilon \times l} \qquad \text{oziroma:} \qquad \frac{n_{produkt}\ /\ \text{min}}{V_{reakc\ zm}} = \frac{\Delta A_{vz}\ /\ \text{min}}{\varepsilon \times l}$$

ε je molski absorpcijski koeficient (5012 $L \times mol^{-1} \times cm^{-1}$); l je dolžina optične poti (dimenzija kivete 1 cm).



Odvisnost A_{vz} od časa inkubacije

$$n_{produkt} / \min = \frac{\Delta A_{vz} / \min}{\varepsilon \times l} \times V_{reakc\ zm}$$

Volumen reakcijske zmesi ($V_{reakc\ zm}$) je seštevek volumnov, ki jih odmerimo v kiveto KV_{vz}; glede na opis eksp. postopka: $V_{reakc\ zm} = 3$ mL.

$n_{produkt}$ izrazimo v enoti µmol (1 µmol = 1×10^{-6} mol)

Aktivnost polifenol oksidaze, ki je sorazmerna z $n_{produkt} / \min$, izrazimo kot število encimskih enot (U).

Po definiciji: 1 enota encimske aktivnosti (1 U) katalizira nastanek 1 µmol produkta v 1 min, določeno pri opisanih pogojih, torej:

$$\text{aktivnost PFO v reakcijski zmesi} = \frac{1\ \text{U} \times n_{produkt} / \min}{1\ \mu\text{mol/min}}$$

Na primer, če izračunamo, da je v 1 min nastalo 0,02 µmol produkta ($n_{produkt} / \min = 0{,}02$ µmol/min), je aktivnost PFO v reakcijski zmesi = 0,02 U.

Glede na opis eksp. postopka (preglednica 1 na str. 106): v kiveto KV_{vz} (reakcijska zmes) smo odmerili določen $V_{p\ razredč\ ekstr}$, torej:

aktivnost PFO v $V_{p\ razredč\ ekstr}$ = aktivnost PFO v reakcijski zmesi

$$\text{aktivnost PFO / mL razredč. ekstr.} = \frac{\text{aktivnost PFO v } V_{p\ razredč\ ekstr}}{V_{p\ razredč\ ekstr}}$$

aktivnost PFO v celotnem $V_{razredč\ ekstr}$ = $V_{razredč\ ekstr}$ × aktivnost PFO / mL razredč. ekstr.

Glede na opis eksp. postopka (str. 103, 104): $V_{razredč\ ekstr}$ = 10 mL; razredčen ekstrakt pripravimo tako, da odmerimo 5 mL surovega ekstrakta (to je $V_{p\ surovi\ ekstr}$) + 5 mL pufra II, torej:

aktivnost PFO v $V_{p\ surovi\ ekstr}$ = aktivnost PFO v celotnem $V_{razredč\ ekstr}$

$$\text{aktivnost PFO / mL surovega ekstr.} = \frac{\text{aktivnost PFO v } V_{p\ surovi\ ekstr}}{V_{p\ surovi\ ekstr}} \qquad \text{(enota: U/mL)}$$

Glede na opis eksp. postopka (str. 103, 104): iz krompirja smo polifenol oksidazo ekstrahirali in po filtraciji in centrifugiranju odčitali $V_{surovi\ ekstr}$, torej:

aktivnost PFO v celotnem $V_{surovi\ ekstr}$ = $V_{surovi\ ekstr}$ × aktivnost PFO / mL surovega ekstr.

aktivnost PFO v krompirju = aktivnost PFO v celotnem $V_{surovi\ ekstr}$

$$\text{aktivnost PFO / mg krompirja} = \frac{\text{aktivnost PFO v krompirju}}{m_{krompir}} \qquad \text{(enota: U/mg)}$$

Poročilo

Eksperimentalni podatki

preiskovani vzorec krompirja: ……..

$m_{krompir}$ = …….

$V_{surovi\ ekstr}$ = …….

$V_{p\ razredč\ ekstr}$ = …….

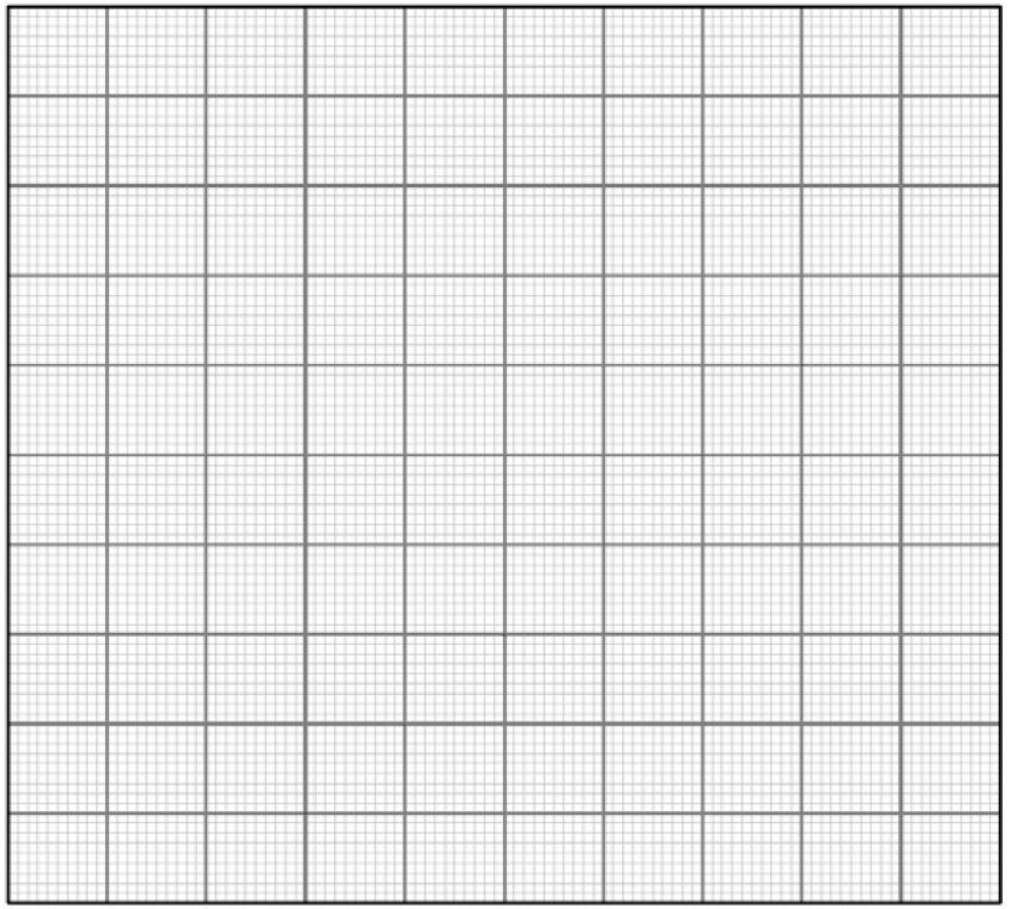
A_{kontr} = …….

Ostali podatki sledijo vrednostim, ki so navedene v opisu eksperimentalnega postopka.

Preglednica 2: Odvisnost A_{vz} od časa inkubacije.

$V_{p\ razredč\ ekstr}$ (mL)	stari krompir				mladi krompir				topl. obdelan mladi krompir			
	0,05	0,10	0,20	0,30	0,05	0,10	0,20	0,30	0,05	0,10	0,20	0,30
t(s)	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}	A_{vz}
0												
15												
30												
45												
60												
75												
90												
120												
150												
180												
210												
240												
270												
300												

Izračuni in graf



Odvisnost A_{vz} od časa

ΔA_{vz} / min =

$n_{produkt}$ / min =

aktivnost PFO v reakcijski zmesi =

aktivnost PFO v $V_{p\ razredč\ ekstr}$ =

aktivnost PFO / mL razredč. ekstr.

aktivnost PFO v celotnem $V_{razredč\ ekstr}$ =

aktivnost PFO v $V_{p\ surovi\ ekstr}$ =

aktivnost PFO / mL surovega ekstr. =

aktivnost PFO v celotnem $V_{surovi\ ekstr}$ =

aktivnost PFO v krompirju =

aktivnost PFO / mg krompirja =

Izpolnite preglednico in komentirajte aktivnost polifenol oksidaze glede na izbrani krompir in vpliv toplotne obdelave.

Preglednica 3: Aktivnost polifenol oksidaze v krompirju.

	stari krompir				mladi krompir				topl. obdelan mladi krompir			
$V_{p\ razredč\ ekstr}$ (mL)	0,05	0,10	0,20	0,30	0,05	0,10	0,20	0,30	0,05	0,10	0,20	0,30
ΔA_{vz} / min												
$n_{produkt}$ / min (µmol/min)												
aktivnost PFO v reakc. zmesi (U)												
aktivnost PFO v $V_{p\ razredč\ ekstr}$ (U)												
aktivnost PFO / mL razredč. ekstr. (U/mL)												
aktivnost PFO v celotnem $V_{razredč\ ekstr}$ (U)												
aktivnost PFO v $V_{p\ surovi\ ekstr}$ (U)												
aktivnost PFO / mL surovega ekstr. (U/mL)												
aktivnost PFO v celotnem $V_{surovi\ ekstr}$ (U)												
aktivnost PFO v krompirju (U)												
aktivnost PFO / mg krompirja (U/mg)												

Komentar

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FOREWORD

The material is intended for students in laboratory exercises in the subject Food Chemistry of the academic study programme Food Science and Nutrition and is a supplement to the existing study material "Abram and Zelenik-Blatnik, Exercises in food chemistry for students of food technology, 2002". The additional study material summarizes and at the same time builds on the theoretical starting points and extends the research approach of the laboratory exercises described in the aforementioned basic study material. The authors believe that a clear schematic presentation of the theoretical basis and practical implementations, as well as a comprehensive explanation of the processing of experimental data, which we have included in the additional study material, are a great help in understanding a relatively complex issue.

Students from an international environment (Erasmus exchange) participate in the Food Chemistry course at the same time as regular students. We have prepared an English version of the material to offer those students suitable material that is harmonized with the Slovenian one.

With this material, we would like to encourage students to effectively prepare and carry out laboratory exercises in the subject Food Chemistry, increase their safety when working in the laboratory, strengthen their understanding and independence in their work, and improve their key skills.

BASICS OF CHEMICAL CALCULATIONS

Concentration units

Mass fraction of the solute (w_{solute}) indicates the ratio between the mass of the solute (m_{solute}) and the total mas of the solution ($m_{solution}$). Unit: %.

$$w_{solute} = \frac{m_{solute}}{m_{solution}} \times 100\ \%$$

For a solution prepared as a mixture of different liquids, the liquid solute content is expressed as a volume fraction (φ_{solute}). The volume fraction of the solute indicates the ratio between the volume of the pure liquid solute (V_{solute}) and the total volume of the solution ($V_{solution}$). Unit: %.

$$\varphi_{solute} = \frac{V_{solute}}{V_{solution}} \times 100\ \%$$

Mass concentration (γ) indicates the mass of solute in a certain volume of solution. Unit: g/mL, mg/mL, g/L, ….

$$\gamma = \frac{m_{solute}}{V_{solution}}$$

Molar concentration (c) indicates the number of moles of solute in 1 L of solution. Unit: mol/L.

$$c = \frac{n_{solute}}{V_{solution}}$$

When we dilute a more concentrated solution (stock solution), we measure a certain volume (usually with a pipette) of the stock solution ($V_{p\ stock}$) and add sufficient solvent to prepare the corresponding volume of the diluted solution ($V_{diluted\ slution}$). The solute concentration in a diluted solution is calculated:

$$\text{solute concentration in diluted solution} = \frac{\text{solute concentration in stock solution} \times V_{p\ stock}}{V_{diluted\ slution}}$$

DETERMINATION OF GLUCOSE AND SUCROSE IN THE MIXTURE

Glucose

D(+)-glucose (grape sugar or dextrose) is an aldohexose with the molecular formula $C_6H_{12}O_6$.

Next to fructose, glucose is the most common monosaccharide in foods. It is present in a relatively high proportion in fruit, where its content increases with the degree of ripeness. As a monomeric unit, it is a building block of many disaccharides (e.g. sucrose, lactose, maltose), oligo- and polysaccharides (e.g. β-glucans, amylose and amylopectin (starch), cellulose), and compounds that are formed in the reaction between the hemiacetal –OH group of glucose and a non-sugar molecule (called aglycone), e.g. with alcohol, a phenolic compound, ….

In the food industry, glucose is a raw material for the production of the polyol sorbitol (reduction of the carbonyl group), gluconic acid and lactone (oxidation of the carbonyl group), and for the production of ethanol (by fermentation).

In the presence of oxidizing agents such as the *Luff* reagent and the *Fehling* reagent (alkaline solution of Cu^{2+} ions), glucose (carbonyl group) is oxidized to carboxylic acid. Here, Cu^{2+} is reduced to Cu^{+}. This is why we say that glucose is a reducing sugar.

In an alkaline environment (or enzyme-catalysed), the isomerization of ketose to aldose takes place. Under these conditions, even fructose (which, like ketose, is not a reducing sugar) acts as a "reducing sugar".

Sucrose

Sucrose (table sugar, cane sugar, beet sugar) is α-D-glucopyranosyl-β-D-fructofuranoside with the molecular formula $C_{12}H_{22}O_{11}$. D-glucopyranose and D-fructofuranose are linked by an α,β-(1→2)-glycosidic bond. The reducing ends of both molecules are involved in a glycosidic bond; therefore, sucrose is not a reducing sugar (meaning it is less reactive).

Sucrose is the most abundant disaccharide in plants, where it plays a role in the transport of sugars and as an energy store. It accumulates in larger amounts in the vegetative parts of the plant, such as leaves and stems (sugar cane up to about 25%), and in fruit (apples, oranges, apricots, peaches up to about 8%). Some fruits (e.g. grapes, figs) contain small amounts of sucrose, as this is hydrolyzed during ripening. In vegetables, the sucrose content is considerable in sugar beet (up to about 20%) and onions (up to 10%). In potatoes, the sucrose content in the tubers is relatively high before full ripening and decreases during ripening.

The industrial source of sucrose is sugar beet and sugar cane. In some geographical areas, other sources are also used (e.g. dates, maple sap, ...).

Under acidic conditions or in the presence of the enzyme β-fructofuranosidase (invertase), the glycosidic bond in the sucrose molecule is cleaved (hydrolysed). When the glycosidic bond in the sucrose molecule is cleaved, a mixture of glucose and fructose is formed. The cleavage of the bonds in one mole of sucrose produces two moles of monosaccharides; in addition, the relative sweetness of glucose-fructose syrup is higher than that of sucrose. Therefore, the solution of the resulting mixture is sweeter than the sucrose solution. The name invert sugar for sucrose is due to the inversion: the specific rotation of linearly polarised light for sucrose is +66.5°, and for the mixture of monosaccharides after the hydrolysis of sucrose it is –33.3°.

Sucrose is used as a sweetener in foods because it has a sweet taste (like most mono- and disaccharides). Due to the high number of hydrophilic –OH groups in the molecule, sucrose (like other mono- and disaccharides and shorter oligosaccharides) is very soluble in water. This enables the preparation of very concentrated solutions (syrup, honey) with high osmolarity, which inhibits the growth of microorganisms. Sucrose in food can also act as a humectant.

In the diet, sucrose is a source of energy. The enzyme β-fructofuranosidase in the mucosa of the small intestine catalyses the cleavage of the glycosidic bond. The resulting monosaccharides are absorbed and transported into the blood.

Objective

Determine the mass of glucose ($m_{glucose}$) and the mass of sucrose ($m_{sucrose}$) in the mixture by the *Schoorl-Luff* method.

Principle of method

The determination of glucose is based on an oxidation-reduction reaction that takes place after adding *Luff* reagent (alkaline solution of copper citrate complex) when glucose (reducing sugar) reduces Cu^{2+} to Cu_2O in an alkaline medium.

The determination of sucrose (not a reducing sugar) in the mixture is based on the cleavage of the glycosidic bond (hydrolysis) and the determination of the resulting hydrolysis products (glucose and fructose) and the glucose already present in the sample by adding *Luff* reagent. Here, the isomerization of fructose to a reducing sugar takes place in an alkaline environment.

When a reducing sugar solution is added to *Luff* reagent, an oxidation-reduction reaction takes place, in which the Cu_2O is formed as a reddish-brown precipitate.

When iodine solution is added, the following oxidation-reduction reaction takes place in the excess amount of iodine:

$$Cu_2O + I_2 + 4\ HCl \rightarrow 2\ CuCl_2 + 2\ HI + H_2O$$

We perform a back titration by determining the amount of remaining I_2 ($n_{I_2\ remaining}$; excess with respect to the amount of Cu_2O) by titration with $Na_2S_2O_3$ solution; this is the sample analysis.

$$2\ Na_2S_2O_3 + I_2 \rightarrow 2\ NaI + Na_2S_4O_6$$

The total amount of added I_2 ($n_{I_2\ total}$) is determined by the blank sample analysis, which is performed in the same way as the sample analysis, except that water is added to *Luff* reagent instead of reducing sugar.

The amount of reducing sugar or the amount of Cu_2O formed in the reaction between the reducing sugar and *Luff* reagent is determined as:

$$n_{I_2\ reacted} = n_{I_2\ total} - n_{I_2\ remaining}$$

Experimental procedure

a) Diluted sample preparation

b) Glucose determination

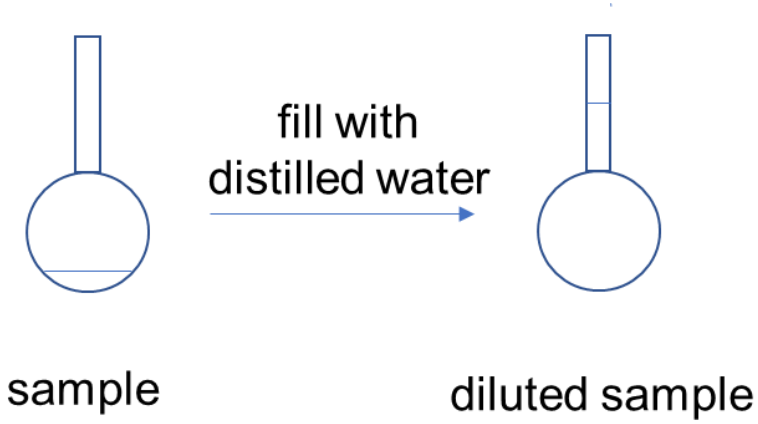
c) Hydrolysis - after hydrolysis of the glycosidic bond in sucrose, the hydrolysate contains the hydrolysis products and the glucose already present in the sample

d) Determination of all reducing sugars in hydrolysate (glucose already present in the sample + reducing sugars formed by hydrolysis)

e) Blank sample analysis (water instead of the sugar solution)

a) Diluted sample preparation

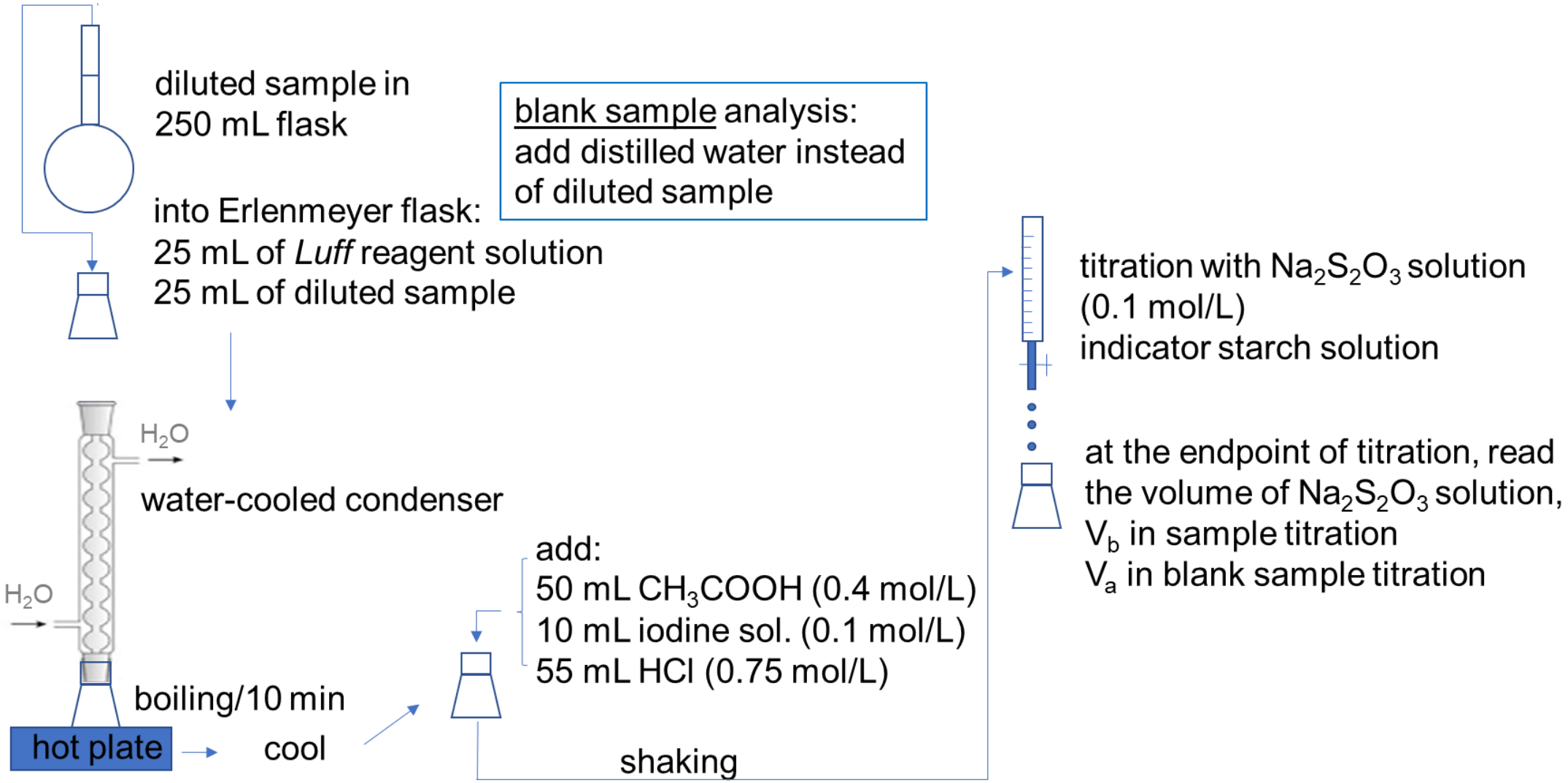
- The sample in a 250 mL volumetric flask containing glucose and sucrose is filled to the mark with distilled water, the flask is closed and the contents are mixed - the volume of the diluted sample ($V_{diluted\ sample}$) = 250 mL.



b) Glucose determination

- Measure 25 mL of *Luff* reagent solution into a glass-stoppered Erlenmeyer flask and add 25 mL of the diluted sample.
- Place the flask on a hot plate and connect it to a water-cooled condenser; heat at boiling temperature for 10 min; remove from the condenser; cool to room temperature.
- After cooling, add: 50 mL of CH_3COOH solution (0.4 mol/L; measuring cylinder), 10 mL of iodine solution (0.1 mol/L; volumetric pipette) and 55 mL of HCl solution (0.75 mol/L; measuring cylinder); shake the contents (first slowly and carefully, then more intensively).
- Titrate with a standard aqueous solution of $Na_2S_2O_3$ (0.1 mol/L) to the color change from dark blue to light blue using 1 mL of starch solution (1%) as an indicator.
- At the endpoint of the titration, read the volume of the $Na_2S_2O_3$ solution, i.e. V_b.

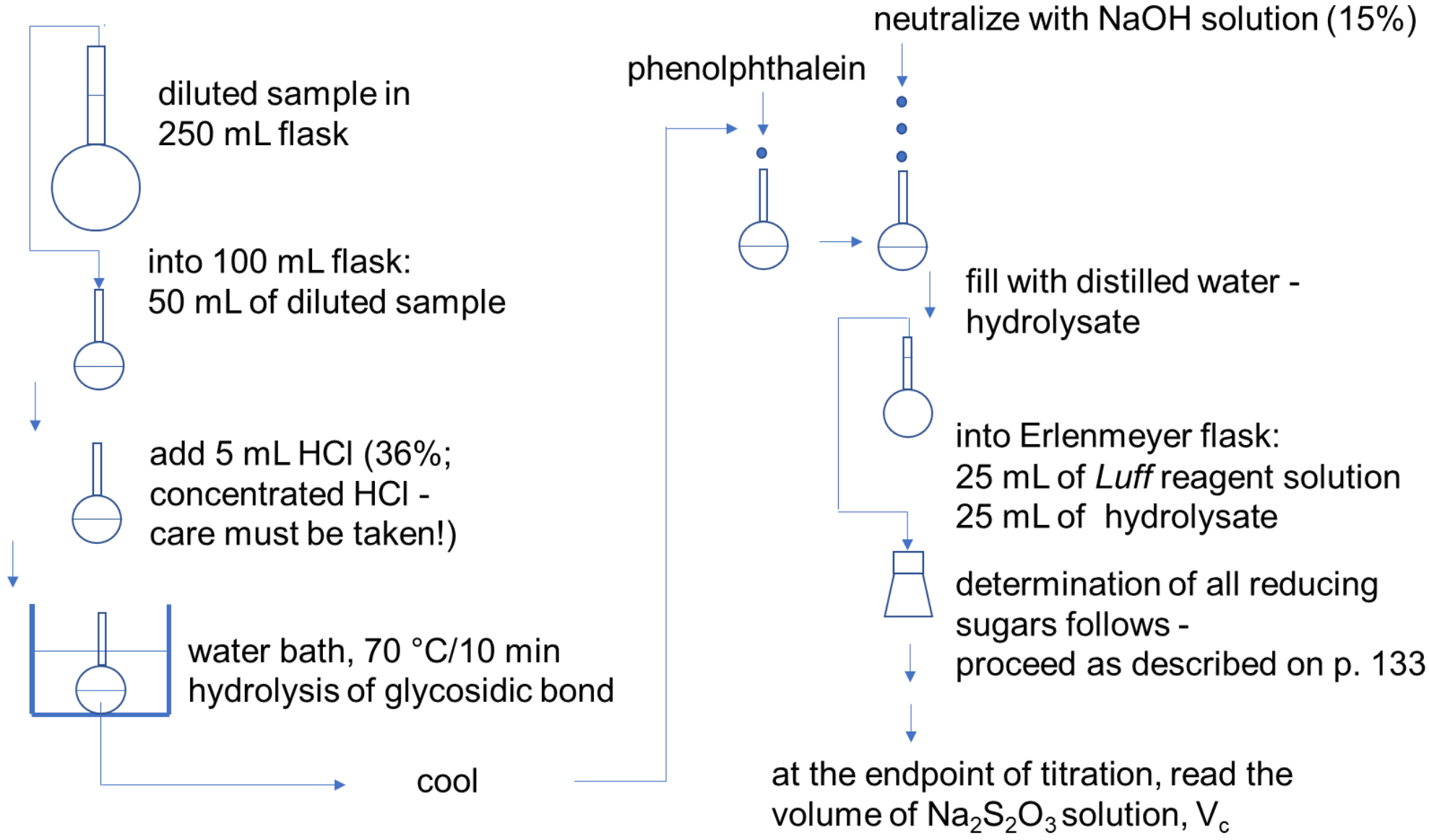
SCHEME OF GLUCOSE DETERMINATION PROCEDURE



c) Hydrolysis

- Measure 50 mL of the diluted sample from the 250 mL flask into a 100 mL flask.
- Add 5 mL of HCl solution (36%; HCl is concentrated; care must be taken).
- Place the flask in a water bath at 70 °C for 10 min (acid hydrolysis of the glycosidic bond in the sucrose molecule is taking place); then cool the contents of the flask.
- Add phenolphthalein and neutralize with NaOH solution (15%).
- Fill the contents in the 100 mL flask (hydrolysate) to the mark with distilled water.
- The products of the cleavage of the glycosidic bond in sucrose (glucose and fructose) and glucose already present in the sample are in a 100 mL flask (in the hydrolysate).

SCHEME OF HYDROLYSIS PROCEDURE



d) Determination of all reducing sugars in hydrolysate

- Measure 25 mL of *Luff* reagent solution into a glass-stoppered Erlenmeyer flask and add 25 mL of the hydrolysate from a 100 mL flask.
- Proceed in the same way as for the determination of glucose (scheme p. 133).
- At the endpoint of titration, read the volume of the $Na_2S_2O_3$ solution, i.e. V_c.

e) Blank sample analysis

- Measure 25 mL of *Luff* reagent solution into a glass-stoppered Erlenmeyer flask and add 25 mL of distilled water.
- Proceed in the same way as for the determination of glucose (scheme p. 133).
- At the endpoint of titration, read the volume of the $Na_2S_2O_3$ solution, i.e. V_a.

Calculations

From titration data we calculate:

- the amount of $Na_2S_2O_3$ which is proportional to $n_{I_2\ reacted}$ in the determination of $m_{glucose}$ in 25 mL of diluted sample ($n_{Na_2S_2O_3\ glucose}$):

$$n_{Na_2S_2O_3\ glucose} = (V_a - V_b) \times c_{Na_2S_2O_3}$$

- the amount of $Na_2S_2O_3$ which is proportional to $n_{I_2\ reacted}$ in the determination of $m_{all\ reduc\ sugars}$ in 25 mL of hydrolysate ($n_{Na_2S_2O_3\ all\ reduc\ sugars}$):

$$n_{Na_2S_2O_3\ all\ reduc\ sugars} = (V_a - V_c) \times c_{Na_2S_2O_3}$$

To calculate the mass of the sugar, we do not use the stoichiometric ratio between the amount of sugar and the amount of $n_{Na_2S_2O_3}$, but the experimentally determined ratio (Table 1 on p. 138).

Using the data in Table 1 on p. 138, we draw a graph and read:

- $m_{glucose}$ in 25 mL of diluted sample at calculated $n_{Na_2S_2O_3\ glucose}$ and
- $m_{all\ reduc\ sugars}$ in 25 mL of hydrolysate at calculated $n_{Na_2S_2O_3\ all\ reduc\ sugars}$.

Table 1: Conversion of the $n_{Na_2S_2O_3}$ amount to the mass of reducing sugar in 25 mL of diluted sample or hydrolysate for the determination method described.

$n_{Na_2S_2O_3}$ (mmol)	$m_{reduc\ sugar}$ (mg)
0.1	2.4
0.2	4.8
0.3	7.2
0.4	9.7
0.5	12.2
0.6	14.7
0.7	17.2
0.8	19.8

Calculation of the mass of glucose in the sample

According to the experiment description: 25 mL of the diluted sample is measured from a 250 mL flask into a glass-stoppered Erlenmeyer flask, therefore:

$m_{glucose}$ in 250 mL of diluted sample = $m_{glucose}$ in 25 mL of diluted sample $\times$ 10

$m_{glucose}$ in the sample = $m_{glucose}$ in 250 mL of diluted sample

Calculation of the mass of sucrose in the sample

According to the experiment description: 25 mL of hydrolysate is measured from a 100 mL flask containing hydrolysate, therefore:

$m_{\text{all reduc sugars}}$ in 100 mL of hydrolysate = $m_{\text{all reduc sugars}}$ in 25 mL of hydrolysate $\times$ 4 =

= $m_{\text{all reduc sugars}}$ in 50 mL of diluted sample measured into 100 mL flask

According to the experiment description: 50 mL of diluted sample is measured from a 250 mL flask, therefore:

$m_{\text{all reduc sugars}}$ in 250 mL of diluted sample = $m_{\text{all reduc sugars}}$ in 50 mL of diluted sample $\times$ 5

$m_{\text{all reduc sugars}}$ in the sample = $m_{\text{all reduc sugars}}$ in 250 mL of diluted sample

We take into account the fact that water is released when two monosaccharides are joined to form a disaccharide, therefore: $M_{\text{sucrose}} = 2 \times 180$ g/mol – 18 g/mol

mass of disaccharide = mass of two monosaccharides $\times$ 0.95

m_{sucrose} in the sample = ($m_{\text{all reduc sugars}}$ in the sample – m_{glucose} in the sample) $\times$ 0.95

Report

Experimental data

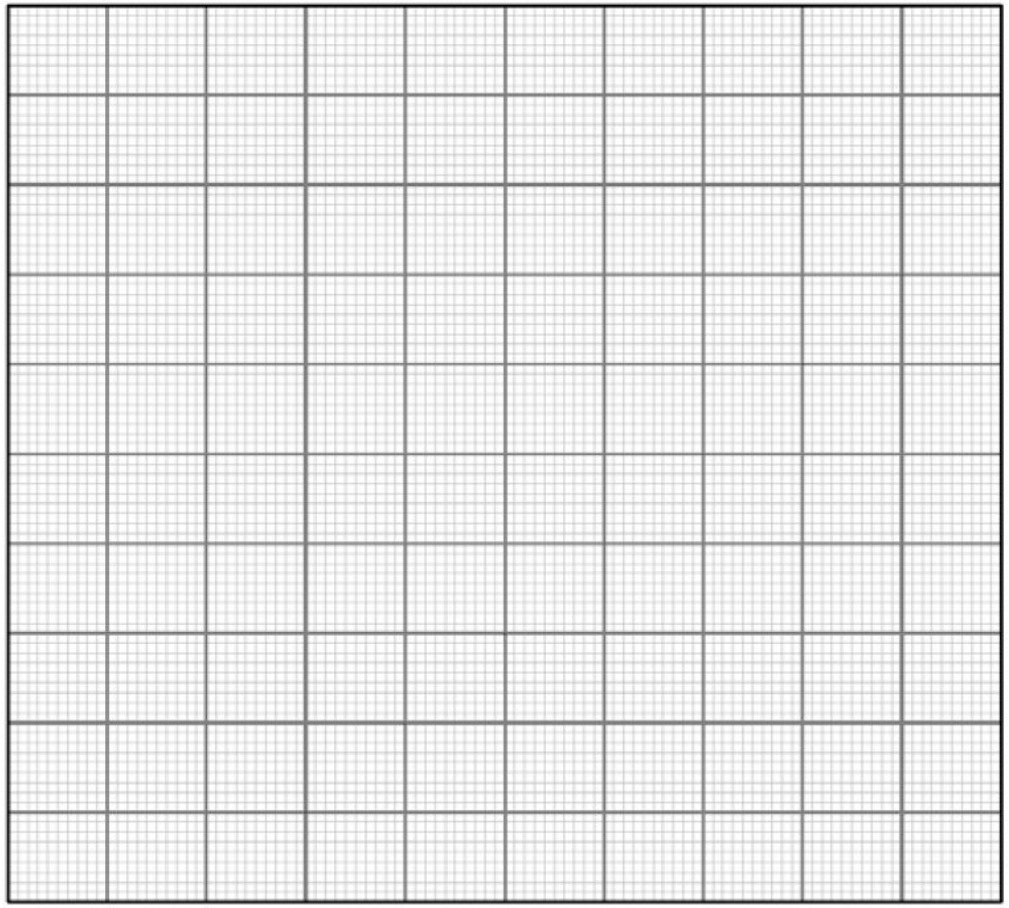
V_a = ……..... V_b = ……..... V_c = …… $c_{Na_2S_2O_3}$ = ……

Further data can be found in the description of the experimental procedure.

Calculations and graph

$n_{Na_2S_2O_3\ glucose}$ =

$n_{Na_2S_2O_3\ all\ reduc\ sugars}$ =



Dependence of reducing sugar mass on $Na_2S_2O_3$ amount

The mass of glucose in 25 mL of diluted sample read from the graph at calculated value of $n_{Na_2S_2O_3\ glucose}$ = …….

$m_{glucose}$ in 250 mL of diluted sample =

$m_{glucose}$ in the sample =

The mass of all reducing sugars in 25 mL of hydrolysate read from the graph at calculated value of $n_{Na_2S_2O_3\ all\ reduc\ sugars}$ = ……..

$m_{all\ reduc\ sugars}$ in 100 mL of hydrolysate =

$m_{all\ reduc\ sugars}$ in 250 mL of diluted sample =

$m_{all\ reduc\ sugars}$ in the sample =

$m_{sucrose}$ in the sample =

Result

sample number ……

$m_{glucose}$ in the sample =

$m_{sucrose}$ in the sample =

DETERMINATION OF TRYPSIN INHIBITOR ACTIVITY IN SOY

Trypsin inhibitors

Enzyme inhibitors are various substances that reduce the activity of enzymes and occur naturally or are added to foods. From a nutritional point of view, inhibitors of proteolytic digestive enzymes, the proteases, are particularly important.

Protease inhibitors are found in plants (mainly in legumes, but also in cereals, potatoes, tomatoes) where they are supposed to fulfill various tasks - protecting the plant from endogenous proteases that are released when the plant tissue is damaged; protecting against proteases from microorganisms, insects, …. The highest content of these inhibitors was found in seeds, leaves and tubers. Protease inhibitors of microbial or animal origin are also known.

Protease inhibitors are often proteins or polypeptides. Depending on the catalytic type of protease they inhibit, protease inhibitors are classified into four groups.

The soybean serine protease inhibitors include:

- Kunitz-type inhibitors contain few cysteine residues and form two disulfide bridges. They are less thermally stable.
- Bowman-Birk type inhibitors contain several cysteine residues and form several disulfide bridges. They are more thermally stable. Compared with Kunitz-type inhibitors, they have lower molar mass.

Both types of serine protease inhibitors are potent trypsin inhibitors.

Trypsin is an endoprotease that catalyses the cleavage of the peptide bond in proteins and peptide substrates on the carboxyl side of the amino acids lysine or arginine. The inhibition of trypsin is the result of the formation of a very stable complex between the inhibitor and the enzyme.

In humans, the inhibition of trypsin leads to incomplete protein digestion, which reduces the bioavailability of amino acids from proteins. Inhibition of trypsin leads to excessive production and secretion of trypsin, which in extreme cases can lead to hypertrophy (enlargement) of the pancreas. In addition, the increased synthesis of pancreatic enzymes impairs synthesis of other proteins in the body, resulting in slower growth.

The activity of trypsin inhibitors is reduced by thermal treatment, as denaturation occurs at elevated temperatures due to disruption of the native structure. In the presence of reducing agents, where the disulfide bonds in the inhibitors are reduced, thermal treatment leads to even more effective denaturation.

Since the use of legumes, which are particularly rich in trypsin inhibitors (especially soy), is quite widespread in the diet, care must be taken to ensure that they are sufficiently thermally treated. The method, time and temperature of thermal treatment, the particle size and the moisture content of the seeds influence the extent to which the activity of the trypsin inhibitors is reduced. Cooking in boiling water and the use of microwaves have been shown to be effective. Sufficient pre-soaking of the seeds (~24 hours) is important, as the effect is greater in seeds with a higher moisture content.

Objective

Determine trypsin inhibitor activity in yellow and red soy.

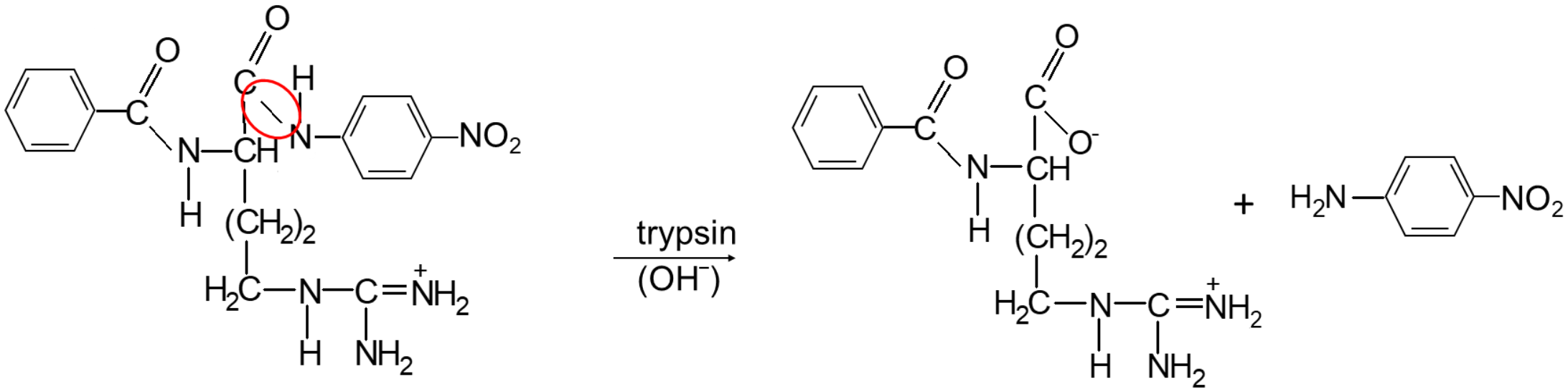
Determine the effect of thermal treatment of soy (cooking in boiling water for 3 h (A); 3 h in ventilator oven at 104 °C (B); 6 h in ventilator oven at 104 °C (C)) on trypsin inhibitor activity.

Principle of method

The determination of trypsin inhibitor activity is based on the fact that trypsin catalyses the cleavage of *p*-nitroaniline from the synthetic substrate N-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA).

The trypsin inhibitor in the soybean extract reduces the activity of trypsin so that less product *p*-nitroaniline is formed.

How much less product is formed in a given time is proportional to the activity of the trypsin inhibitor or the content of the trypsin inhibitor in the reaction mixture.



N-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA)

N-benzoyl-D,L-arginine

p-nitroaniline

The content of the *p*-nitroaniline reaction product is determined spectrophotometrically.

Experimental procedure

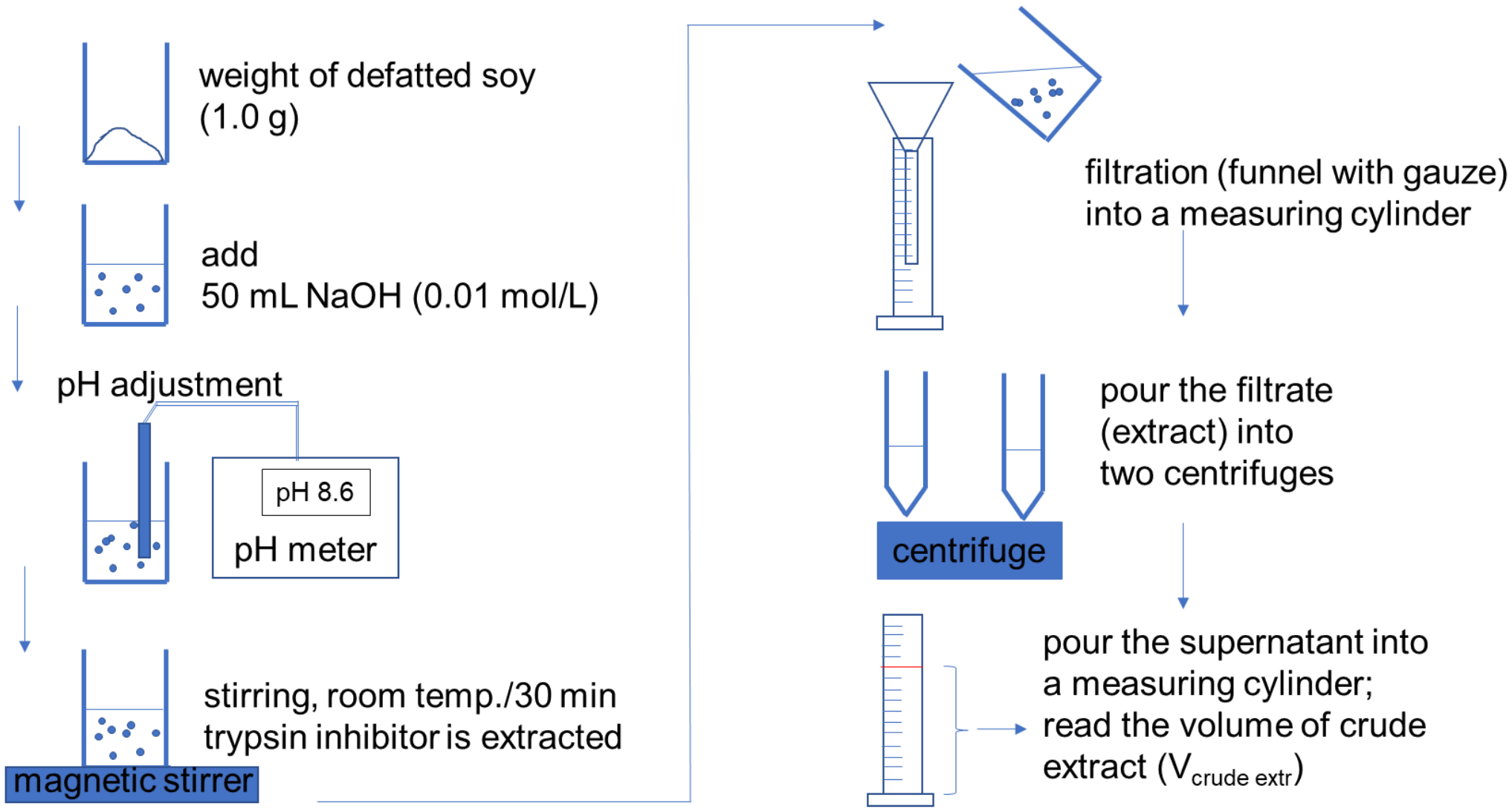
a) Extraction of trypsin inhibitor from soy (we perform the procedure of determining trypsin inhibitor activity in yellow and red soy, and in yellow soy that has been thermally treated in three different ways)

b) Spectrophotometric determination of trypsin inhibitor activity

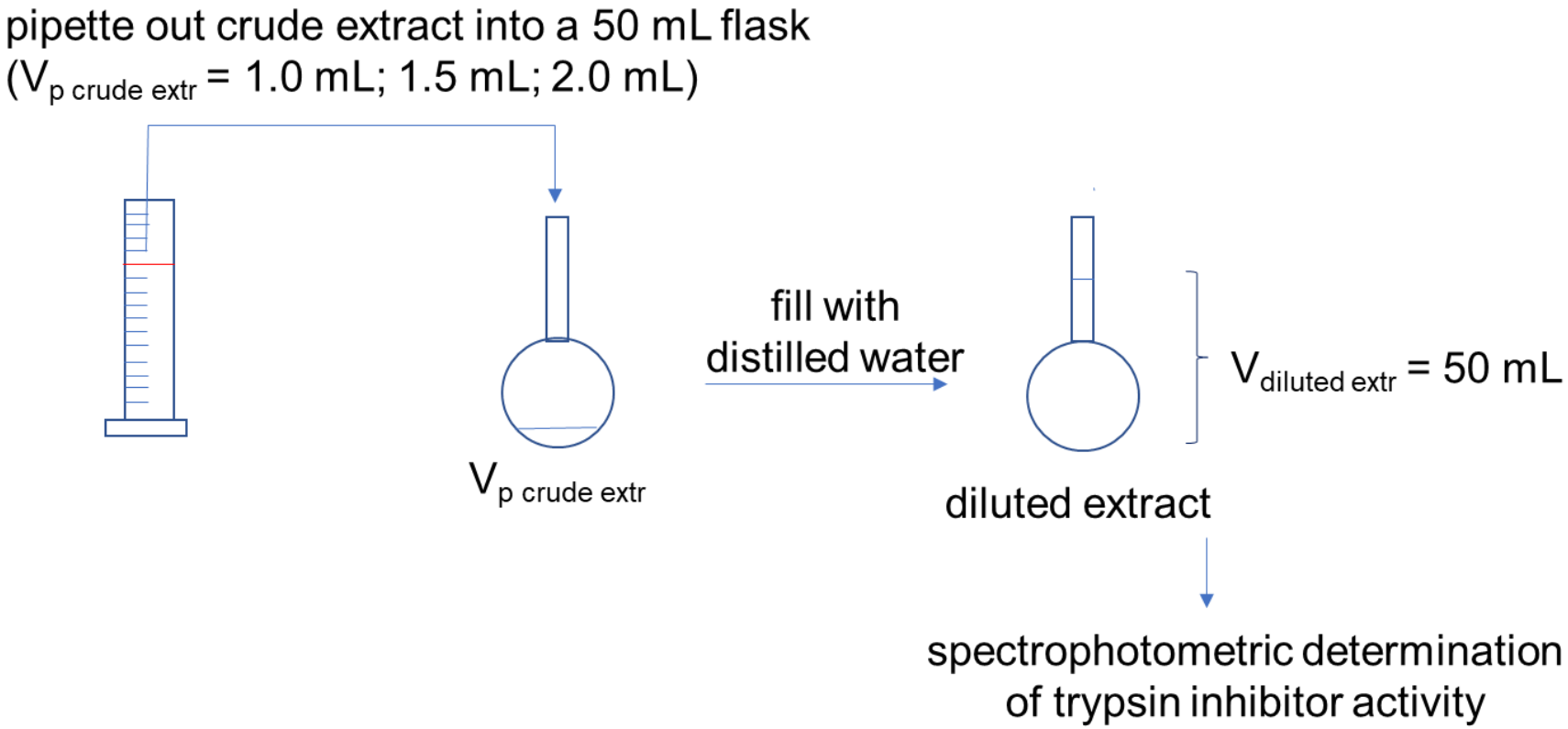
a) Extraction of trypsin inhibitor from soy (extract preparation)

- Weigh 1.0 g of ground and defatted soy ($m_{defatted\ soy}$) into a 100 mL beaker; soy is defatted with diethyl ether.
- Add 50 mL of the extraction solvent (NaOH solution (0.01 mol/L)); adjust the pH (HCl and NaOH solutions) to 8.6 (pH meter).
- Stirring on a magnetic stirrer for 30 min at room temperature; the trypsin inhibitor is extracted from the soy.
- Filter through a funnel with gauze into a measuring cylinder; the filtrate (trypsin inhibitor extract) is turbid; we need a clear extract, as the determination of activity is done spectrophotometrically; pour the filtrate (extract) into two centrifuges; make sure that the two centrifuges (together with their contents) are of equal weight; centrifugation follows.
- The supernatant (the clear part above the sediment after centrifugation in a centrifuge) is carefully poured into the measuring cylinder and the volume of the crude extract is read ($V_{crude\ extr}$).
- Prepare a diluted extract: measure a certain volume of crude extract into each individual 50 mL flask ($V_{p\ crude\ extr}$ = 1.0 mL; 1.5 mL; 2.0 mL), fill to the mark with distilled water, close the flasks and mix the contents; the volume of diluted extract ($V_{diluted\ extr}$) is 50 mL.

SCHEME OF TRYPSIN INHIBITOR EXTRACTION PROCEDURE



DILUTED EXTRACT PREPARATION SCHEME



Different $V_{p\ crude\ extr}$ mean different amounts of trypsin inhibitor in the reaction mixture in spectrophotometric determination.

b) Spectrophotometric determination of trypsin inhibitor activity

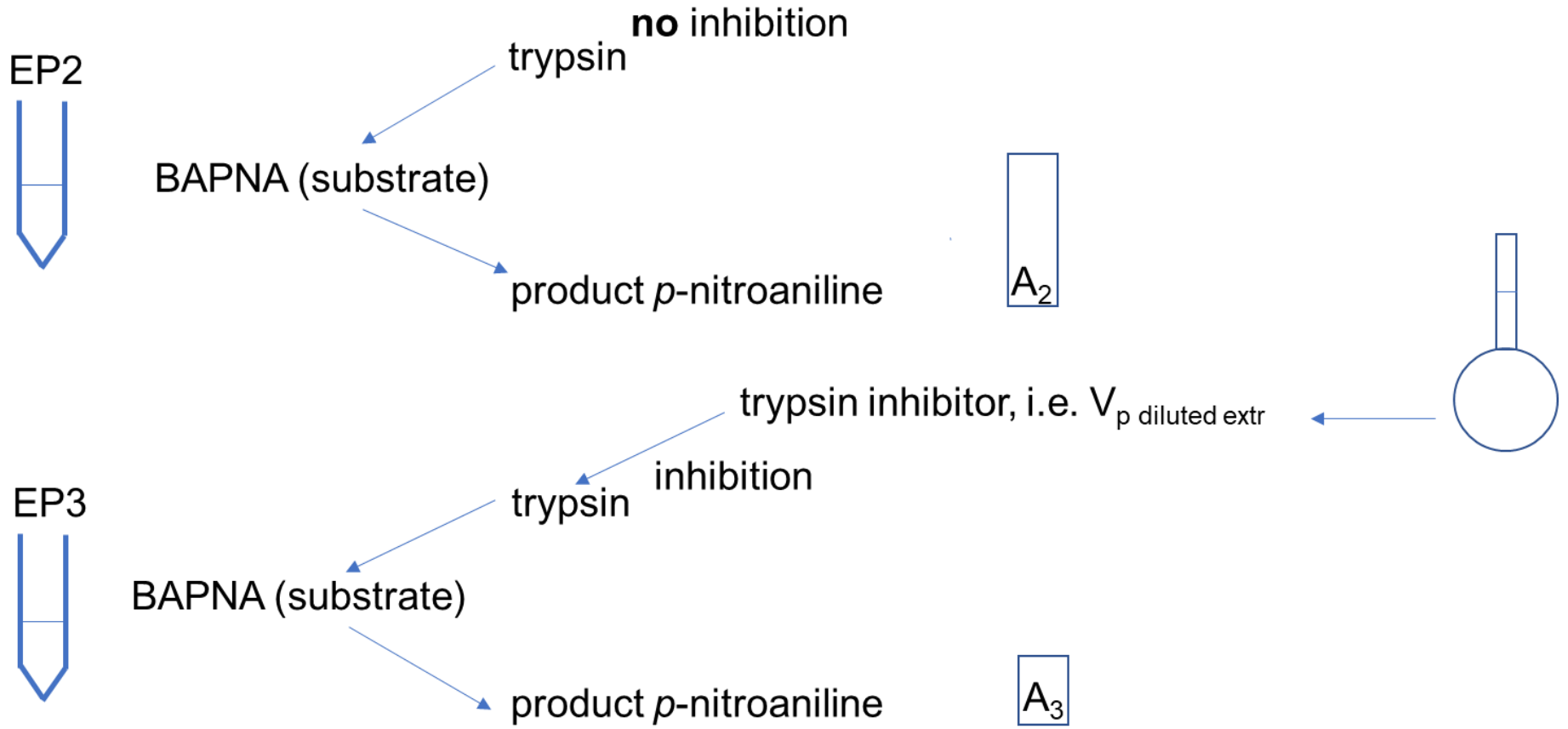
- Prepare three test tubes for each individual diluted extract: EP1 (blank sample), EP2 (without tryps. inh.), EP3 (contains tryps. inh.).
- Measure into a test tube EP1 (blank sample): 2.0 mL of the diluted extract, 1.0 mL of acetic acid solution (30%), 5.0 mL of BAPNA solution and 2.0 mL of trypsin solution (why this specified order?); mix with a vortex mixer.
- Measure a certain volume of diluted extract ($V_{p\ diluted\ extr}$), water and trypsin (Table 1 on p. 154) into test tubes EP2 and EP3; mix with a vortex mixer.

Table 1: Volume of diluted extract ($V_{p\ diluted\ extr}$), water ($V_{p\ water}$) and trypsin ($V_{p\ trypsin}$) for transfer to EP2 in EP3.

test tube	$V_{p\ diluted\ extr}$ (mL)	$V_{p\ water}$ (mL)	$V_{p\ trypsin}$ (mL)
EP2	-	2.0	2.0
EP3	2.0	-	2.0

- The BAPNA solution and EP1, EP2 and EP3 with the described contents are placed in the water bath at 37 °C for approx. 5 min.
- Then pipette 5.0 mL of BAPNA solution (substrate) into EP2 and EP3.
- The reaction in EP2 and EP3 is allowed to run for exactly 10 min (water bath at 37 °C).
- Stop the reaction in EP2 and EP3 by adding 1.0 mL of acetic acid solution (30%) (why acetic acid solution?); mix with a vortex mixer.
- Pour the contents of the test tubes into cuvettes and measure the absorbance of the solution in EP2 (A_2) and EP3 (A_3) against a blank sample (EP1) at 410 nm.

SCHEME OF SPECTROPHOTOMETRIC DETERMINATION



Concentration of the product *p*-nitroaniline in EP2 > concentration of the product *p*-nitroaniline in EP3; therefore: $A_2 > A_3$

Calculations

Trypsin inhibitor activity is proportional to ΔA ($\Delta A = A_2 - A_3$).

The greater the inhibition of trypsin, the less product is produced and the lower is A_3, the greater is ΔA.

Trypsin inhibitor activity is expressed as the number of trypsin inhibition units (TIU).

By definition: 1 trypsin inhibition unit (1 TIU) corresponds to $\Delta A = 0.010$, determined under the conditions described.

$$\text{tryps. inh. activity in reaction mixture} = \frac{\Delta A}{0.010}$$

According to the experiment description (Table 1 on p. 154): a certain $V_{\text{p diluted extr}}$ was measured into the reaction mixture (in EP3), therefore:

$$\text{tryps. inh. activity in } V_{\text{p diluted extr}} = \text{tryps. inh. activity in reaction mixture}$$

$$\text{tryps. inh. activity / mL of diluted extr.} = \frac{\text{tryps. inh. activity in } V_{\text{p diluted extr}}}{V_{\text{p diluted extr}}}$$

tryps. inh. activity in total $V_{diluted\ extr}$ = $V_{diluted\ extr}$ × tryps. inh. activity / mL of diluted extr.

According to the experiment description (p. 151, 152): $V_{diluted\ extr}$ = 50 mL; the diluted extract is prepared by measuring a certain $V_{p\ crude\ extr}$ and adding water, therefore:

tryps. inh. activity in $V_{p\ crude\ extr}$ = tryps. inh. activity in total $V_{diluted\ extr}$

$$\text{tryps. inh. activity / mL of crude extr.} = \frac{\text{tryps. inh. activity in } V_{p\ crude\ extr}}{V_{p\ crude\ extr}} \qquad \text{(unit: TIU/mL)}$$

According to the experiment description (p. 151, 153): the trypsin inhibitor was extracted from soybeans and after filtration and centrifugation, the $V_{crude\ extr}$ was read, therefore:

tryps. inh. activity in total $V_{crude\ extr}$ = $V_{crude\ extr}$ × tryps. inh. activity / mL of crude extr.

tryps. inh. activity in defatted soy = tryps. inh. activity in total $V_{crude\ extr}$

$$\text{tryps. inh. activity / mg of defatted soy} = \frac{\text{tryps. inh. activity in defatted soy}}{m_{defatted\ soy}} \qquad \text{(unit: TIU/mg)}$$

Example: an extract ($V_{crude\ extr}$ = 42 mL) was prepared from defatted soybeans ($m_{defatted\ soy}$ = 1000 mg) and according to the experiment procedure described ($V_{p\ crude\ extr}$ = 1.5 mL, $V_{diluted\ extr}$ = 50 mL, $V_{p\ diluted\ extr}$ = 2.0 mL) $\Delta A = 0.050$ was determined.

$$\text{tryps. inh. activity in reaction mixture} = \frac{\Delta A}{0.010} = \frac{0.050}{0.010} = 5.0\ \text{TIU}$$

tryps. inh. activity in $V_{p\ diluted\ extr}$ = tryps. inh. activity in reaction mixture = 5.0 TIU

$$\text{tryps. inh. activity / mL of diluted extr.} = \frac{\text{tryps. inh. activity in } V_{p\ diluted\ extr}}{V_{p\ diluted\ extr}} = \frac{5.0\ \text{TIU}}{2.0\ \text{mL}} = 2.5\ \text{TIU/mL}$$

tryps. inh. activity in total $V_{diluted\ extr}$ = $V_{diluted\ extr}$ × tryps. inh. activity / mL of diluted extr. =

= 50 mL × 2.5 TIU/mL = 125 TIU

tryps. inh. activity in $V_{p\ crude\ extr}$ = tryps. inh. activity in total $V_{diluted\ extr}$ = 125 TIU

$$\text{tryps. inh. activity / mL of crude extr.} = \frac{\text{tryps. inh. activity in } V_{\text{p crude extr}}}{V_{\text{p crude extr}}} = \frac{125\ \text{TIU}}{1.5\ \text{mL}} =$$

$$= 83.3\ \text{TIU/mL}$$

$$\text{tryps. inh. activity in total } V_{\text{crude extr}} = V_{\text{crude extr}} \times \text{tryps. inh. activity / mL of crude extr.} =$$

$$= 42\ \text{mL} \times 83.3\ \text{TIU/mL} = 3500\ \text{TIU}$$

$$\text{tryps. inh. activity in defatted soy} = \text{tryps. inh. activity in total } V_{\text{crude extr}} = 3500\ \text{TIU}$$

$$\text{tryps. inh. activity / mg of defatted soy} = \frac{\text{tryps. inh. activity in defatted soy}}{m_{\text{defatted soy}}} =$$

$$= \frac{3500\ \text{TIU}}{1000\ \text{mg}} = 3.5\ \text{TIU/mg}$$

Report

Experimental data

soy sample investigated: ……...

$m_{defatted\ soy}$ = …… $V_{crude\ extr}$ = ……..… $V_{p\ crude\ extr}$ = …… A_2 = …… A_3 = ……

Further data can be found in the description of the experimental procedure.

Calculations

ΔA =

tryps. inh. activity in reaction mixture =

tryps. inh. activity in $V_{p\ diluted\ extr}$ =

tryps. inh. activity / mL of diluted extr. =

tryps. inh. activity in total $V_{diluted\ extr}$ =

tryps. inh. activity in $V_{p\ crude\ extr}$ =

tryps. inh. activity / mL of crude extr. =

tryps. inh. activity in total $V_{crude\ extr}$ =

tryps. inh. activity in defatted soy =

tryps. inh. activity / mg of defatted soy =

Complete the table and comment on the trypsin inhibitor activity in soy depending on the variety and thermal treatment.

Table 2: Trypsin inhibitor activity in soy.

	red soy			yellow soy			therm. treat. yellow soy (A)			therm. treat. yellow soy (B)			therm. treat. yellow soy (C)		
$V_{p\ crude\ extr}$ (mL)	1.0	1.5	2.0	1.0	1.5	2.0	1.0	1.5	2.0	1.0	1.5	2.0	1.0	1.5	2.0
ΔA															
tryps. inh. activity in reaction mixture (TIU)															
tryps. inh. activity in $V_{p\ diluted\ extr}$ (TIU)															
tryps. inh. activity / mL of diluted extr. (TIU/mL)															
tryps. inh. activity in total $V_{diluted\ extr}$ (TIU)															
tryps. inh. activity in $V_{p\ crude\ extr}$ (TIU)															
tryps. inh. activity / mL of crude extr. (TIU/mL)															
tryps. inh. activity in total $V_{crude\ extr}$ (TIU)															
tryps. inh. activity in defatted soy (TIU)															
tryps. inh. activity / mg of defatted soy (TIU/mg)															

Comment

QUANTITATIVE DETERMINATION OF STARCH IN FLOUR

Starch

Starch is a mixture of two different polymers: amylose and amylopectin.

Amylose consists of several 100 to several 1000 glucose units linked by an α-(1→4)-glycosidic bond. The amylose molecule has one reducing end. The polymer forms a helix. Hydrogen bonds are formed between –OH groups of the glucose units in the polymer, which stabilize the helical structure. The molar mass of amylose depends on the botanical source (species, varieties). Amylose in wheat starch contains on average 1000-2000 glucose units and in potato starch up to 4500.

The amylopectin polymer is larger than amylose and consists of 60000 to 3000000 glucose units. The polymer is branched. A branch occurs approximately every 10 to 100 glucose units in the chain. In the polymer, the glucose units are linked by an α-(1→4)-glycosidic bond. At the branch point, the glucose molecule from C1 is linked to the main chain by an α-(1→6)-glycosidic bond. The side chains of amylopectin form double helices. The amylopectin molecule has one reducing end in the main chain.

Plants usually contain more amylopectin. The weight ratio between amylopectin and amylose depends on the botanical source and is generally 3:1.

Starch synthesis takes place in the organelles of the plant cells, in the chloroplasts of the leaves and in the amyloplasts, where it is stored in the form of starch granules. This so-called reserve starch, which is mainly found in the endosperm of seeds, and in roots and tubers, represents an energy store. The size and shape of the starch granules depend on the botanical source (species, varieties). The diameter of the starch granules in corn is 15 µm, in wheat 25 µm and in potatoes 40 µm.

Due to the close arrangement of the starch polymers in the granules, hydration in cold water is quite limited, which results in poor solubility. The structure of the granules does not change significantly. In cold water, the starch forms a suspension with low viscosity. Noticeable changes occur at higher temperatures (55-70 ºC), which weakens the strength of the hydrogen bonds between the polymer segments. This allows the polymers to separate from each other. The structure becomes more amorphous. As a result, more and more water penetrates the granules, which leads to increased hydration and swelling. Amylose molecules, and to a lesser extent, amylopectin molecules are released from the granules and the granules disintegrate.

The main sources of starch in the human diet are tubers, cereals, legumes, root vegetables, chestnuts, nuts and fruit. As the fruit ripens, the starch content decreases. Ripe fruit (with a few exceptions, such as bananas) contain no starch.

Starch is an important nutrient in the human diet. The digestion of starch begins in the mouth in the presence of the enzyme α-amylase in saliva, continues in the small intestine in the presence of α-amylase from the pancreas and then under the influence of maltase and isomaltase, which break down the resulting oligosaccharides and maltose into glucose.

Starch is an important industrial raw material. Modified starch is obtained from native starch which is chemically, physically and/or enzymatically modified to improve its functionality (more suitable technological and sensory properties). Native and modified starches are used in the food industry as thickeners, stabilizers of foams, emulsions and suspensions, as emulsifiers, gelling agents, humectants, fillers, cryostabilizers, encapsulation agents, carriers of aromas and fat substitutes.

Starch hydrolysates are products of starch modification, where glycosidic bonds between glucose units are cleaved by acid (HCl) and suitable enzymes. The products of hydrolysis are various dextrins, oligosaccharides, maltose and glucose. Acid hydrolysis is less specific than enzymatic hydrolysis. At industrial level, endoamylases (e.g. α-amylase) and exoamylases (e.g. β-amylase and amyloglucosidase) are used for the enzymatic hydrolysis of starch.

Objective

Determine the mass fraction of starch (w_{starch}) in wheat flour.

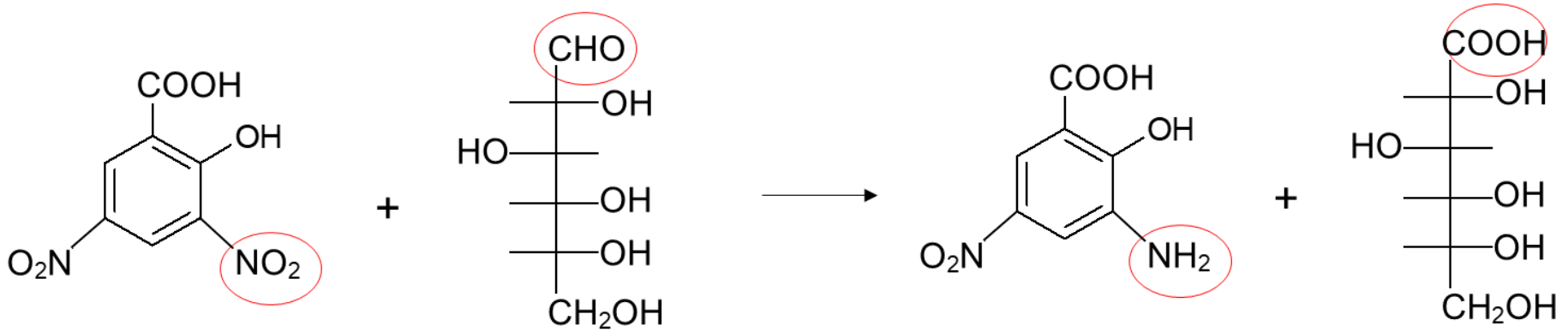
Determine the influence of the starch degradation procedure on the glucose content in the starch hydrolysate.

Principle of method

Soaking starch in boiling water enables better hydration and swelling of the granules, making them more susceptible to acids and enzymes and allowing more efficient starch degradation.

The end product of starch degradation by acid hydrolysis (it is less specific) and hydrolysis by the enzymes α-amylase (cleaves α-(1→4)-glycosidic bonds within the chain) and amyloglucosidase (cleaves α-(1→4)-glycosidic bonds from the non-reducing end of oligosaccharides and α-(1→6)-glycosidic bonds (more slowly)) is glucose.

The determination of glucose in the hydrolysate is based on the fact that glucose is a strong reducing agent and reduces the nitro group in 3,5-dinitrosalicylic acid to the amino group.



3,5-dinitrosalicylic acid (yellow)

glucose

3-amino-5-nitrosalicylic acid (orange-red)

gluconic acid

The content of the reaction product 3-amino-5-nitrosalicylic acid is determined spectrophotometrically. The absorbance is proportional to the concentration of the reaction product. The concentration of the reaction product is proportional to the glucose content in the starch hydrolysate.

To evaluate how the starch degradation procedure (we omit certain steps) affects the glucose content in the hydrolysate, we perform the experiment in 8 different ways (Table 1 on p. 171).

Table 1: Starch degradation procedure.

	acid	heating	enzymes
1A	✓	✓	✓
1B	✓	✓	x
2A	✓	x	✓
2B	✓	x	x
3A	x	✓	✓
3B	x	✓	x
4A	x	x	✓
4B	x	x	x

Experimental procedure

a) Acid hydrolysis

b) Enzymatic hydrolysis

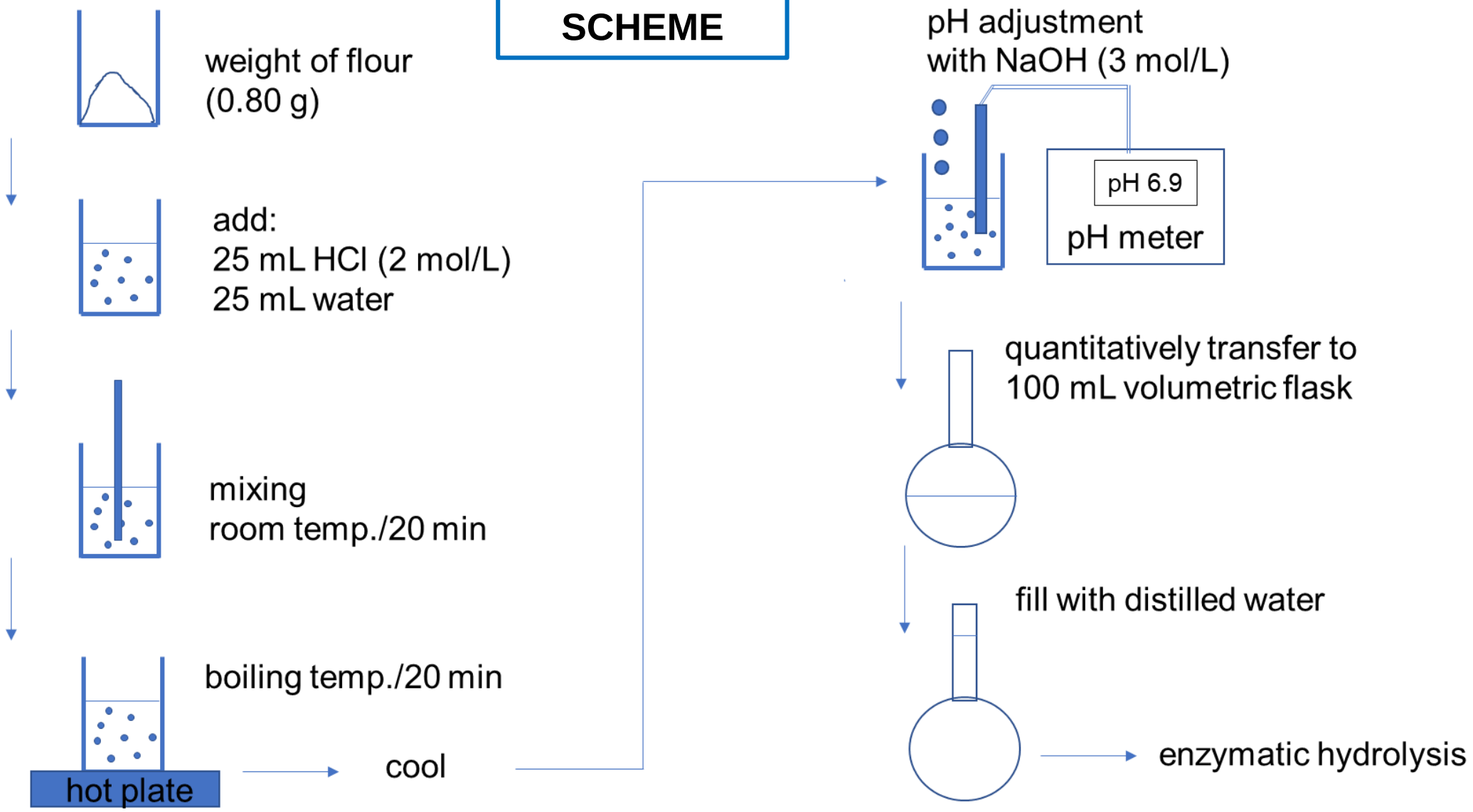
c) Spectrophotometric determination of glucose in starch hydrolysate

d) Calibration curve for determination of glucose in starch hydrolysate

a) Acid hydrolysis

- Weigh 0.80 g of flour (m_{flour}) in a 100 mL beaker.
- Add 25 mL of HCl solution (2 mol/L) and 25 mL of distilled water.
- Mixing with a glass rod at room temperature for 20 min.
- Place the beaker on a hot plate and wait for the solution to boil; boiling for 20 min.
- Cool to room temperature.
- Adjust the pH of the solution to 6.9 with NaOH solution (3 mol/L).
- Transfer the solution from the beaker quantitatively to a 100 mL volumetric flask, fill to the mark with distilled water, close the flask and mix the contents.

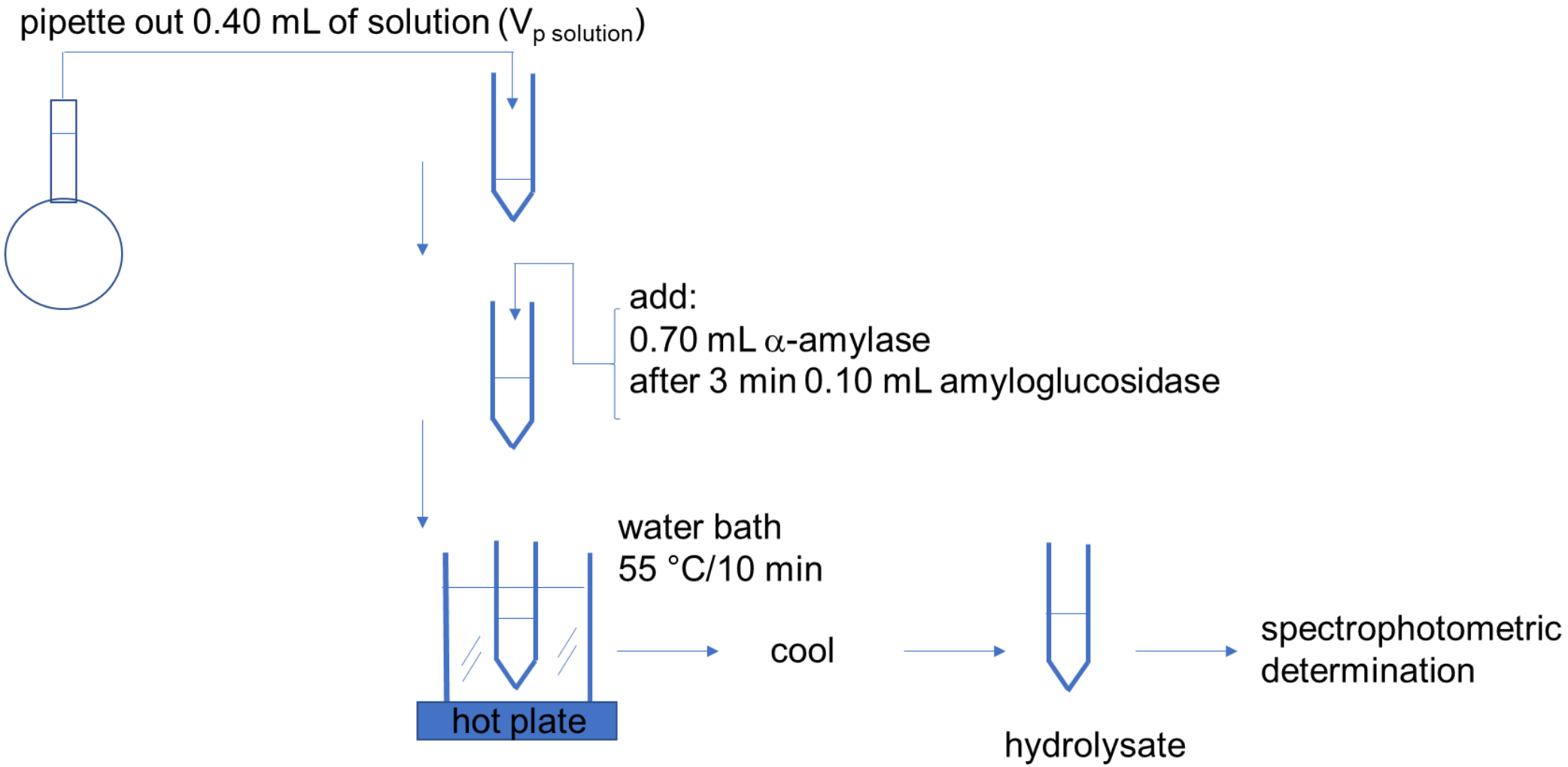
PROCEDURE SCHEME



b) Enzymatic hydrolysis

- Transfer 0.40 mL of the solution ($V_{p\ solution}$) from the 100 mL flask to a test tube.
- Add 0.70 mL of the α-amylase solution.
- Leave for exactly 3 min.
- Add 0.10 mL of the amyloglucosidase solution.
- Incubation for 10 min in a water bath at 55 °C.
- Cool to room temperature.

SCHEME OF ENZYMATIC HYDROLYSIS PROCEDURE



Influence of starch degradation procedure on glucose content in hydrolysate:

1A and 1B: weight, add 25 mL acid, 25 mL water, room temp./20 min, boiling/20 min, cooling, neutralization, quantitatively to 100 mL volumetric flask, then:

1A: 0.40 mL into test tube, add 0.70 mL α-amylase, 0.10 mL amyloglucosidase, water bath
1B: 0.40 mL into test tube, add 0.80 mL water, water bath.

2A and 2B: weight, add 25 mL acid, 25 mL water, room temp./40 min, no cooling, neutralization, quantitatively to 100 mL volumetric flask, then:

2A: 0.40 mL into test tube, add 0.70 mL α-amylase, 0.10 mL amyloglucosidase, water bath
2B: 0.40 mL into test tube, add 0.80 mL water, water bath.

3A and 3B: weight, add 50 mL water, room temp./20 min, boiling/20 min, cooling, no neutralization, quantitatively to 100 mL volumetric flask, then:

3A: 0.40 mL into test tube, add 0.70 mL α-amylase, 0.10 mL amyloglucosidase, water bath
3B: 0.40 mL into test tube, add 0.80 mL water, water bath.

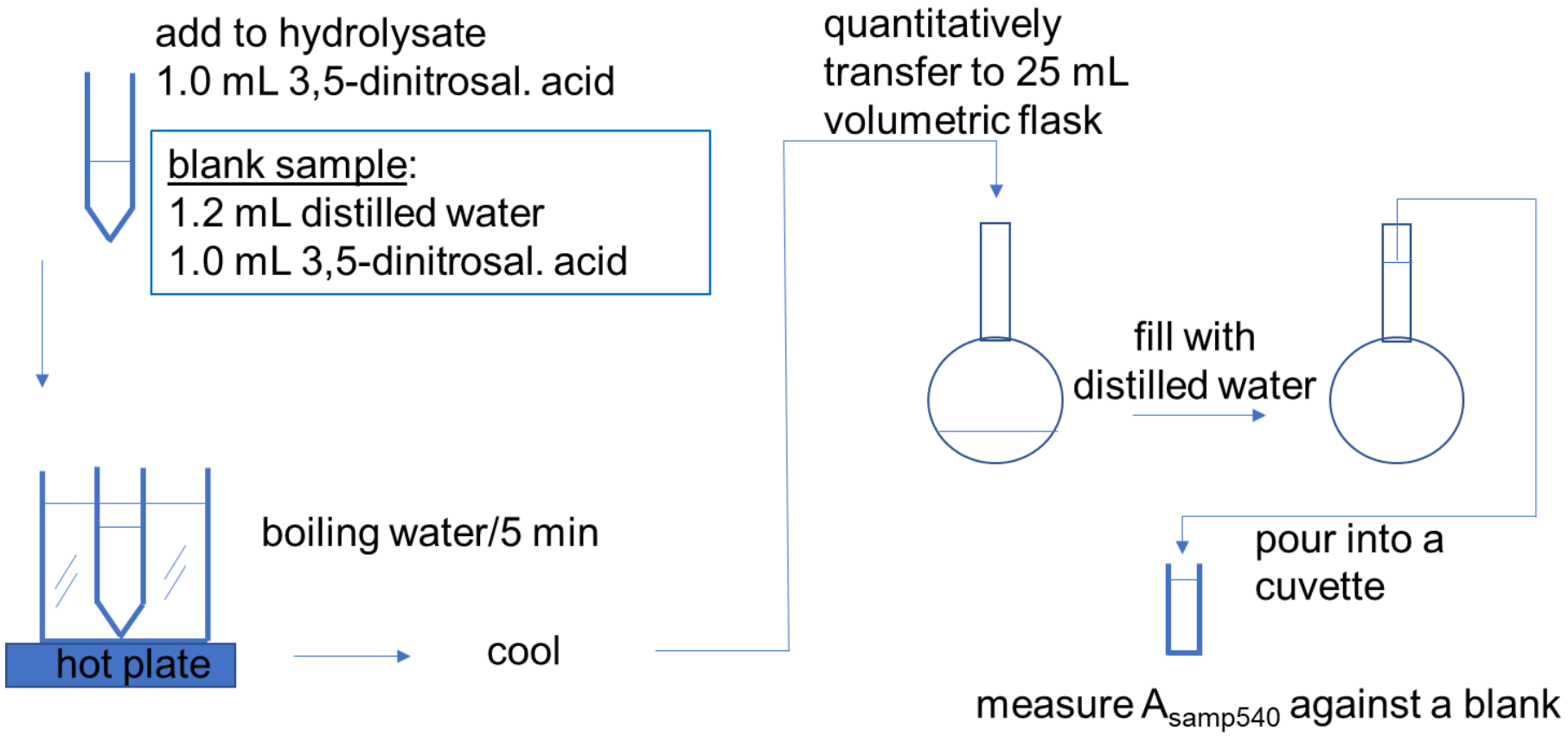
4A and 4B: weight, add 50 mL water, room temp./40 min, no cooling, no neutralization, quantitatively to 100 mL volumetric flask, then:

4A: 0.40 mL into test tube, add 0.70 mL α-amylase, 0.10 mL amyloglucosidase, water bath
3B: 0.40 mL into test tube, add 0.80 mL water, water bath.

c) Spectrophotometric determination of glucose in starch hydrolysate

- After complete hydrolysis, add 1.0 mL of 3,5-dinitrosalicylic acid solution (1 g/100 mL) to the starch hydrolysate in a test tube and mix.
- Prepare a blank sample in another test tube: pipette 1.2 mL of distilled water and 1.0 mL of 3,5-dinitrosalicylic acid solution into a test tube, mix.
- Place both test tubes in a boiling water bath for 5 min, then cool to room temperature.
- Transfer the contents of each test tube quantitatively to a 25 mL volumetric flask, fill to the mark with distilled water, close and mix the contents.
- Prepare two cuvettes: one cuvette for the sample (CU_{sample}) and one cuvette for the blank sample (CU_{blank}).
- Pour the contents of the flasks into a suitable cuvette.
- Measure the absorbance of the sample against a blank sample at 540 nm ($A_{samp540}$).

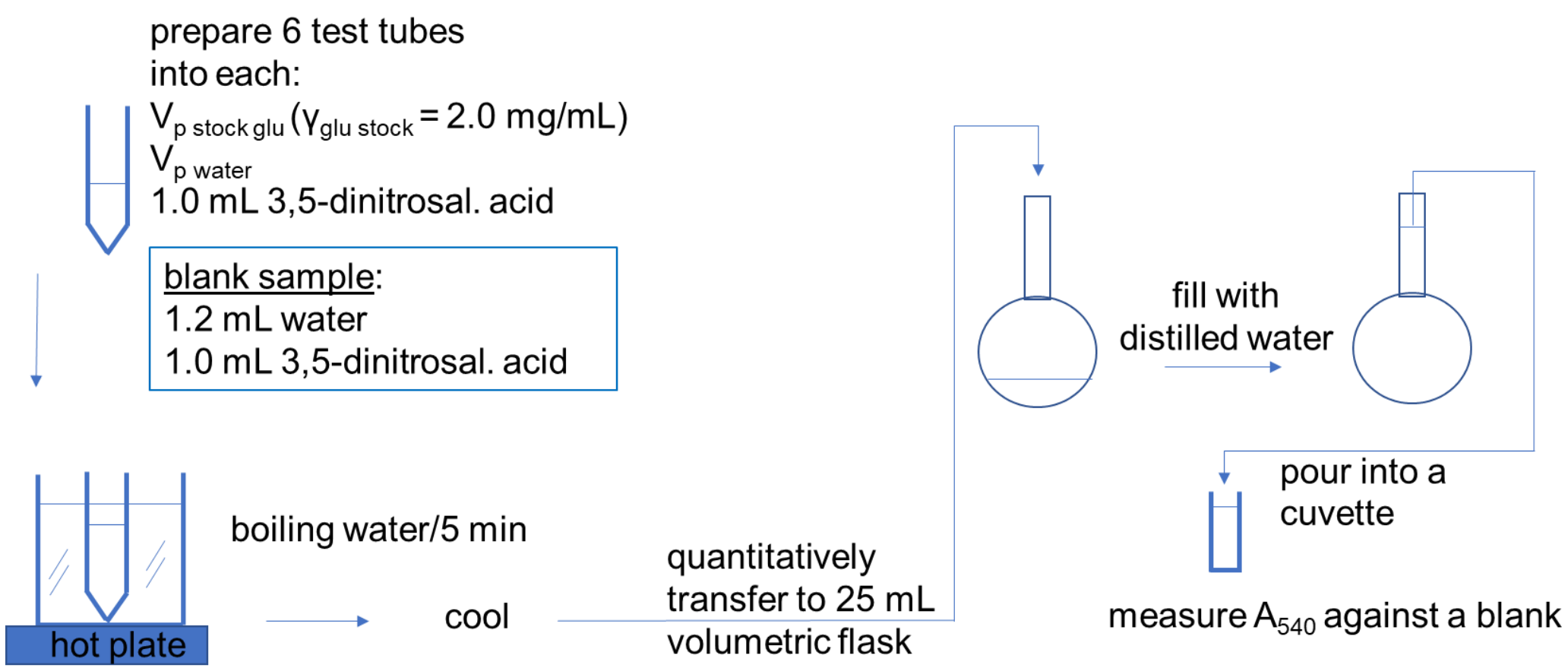
SCHEME OF SPECTROPHOTOMETRIC DETERMINATION OF GLUCOSE IN STARCH HYDROLYSATE



d) Calibration curve for determination of glucose in starch hydrolysate

- Use a glucose stock solution with a mass concentration of 2.0 mg/mL ($\gamma_{glu\ stock}$).
- Prepare 6 test tubes; pipette the appropriate volume of glucose stock solution ($V_{p\ stock\ glu}$) and distilled water ($V_{p\ water}$) into each test tube as indicated in Table 2 on p. 186.
- Add 1.0 mL of 3,5-dinitrosalicylic acid solution to each test tube, mix, place the test tubes in a boiling water bath for 5 min; then cool to room temperature.
- Prepare six 25 mL volumetric flasks; transfer the contents of each test tube quantitatively to the flask, fill to the mark with distilled water, close the flask and mix the contents.
- Prepare 6 cuvettes; pour the contents of each flask into a cuvette and measure A_{540} of all solutions against a blank sample; enter the data in Table 2 on p. 186 ($m_{glucose}$ in 25 mL flask is calculated from $\gamma_{glu\ stock}$).

SCHEME OF CALIBRATION CURVE PREPARATION



Calculations

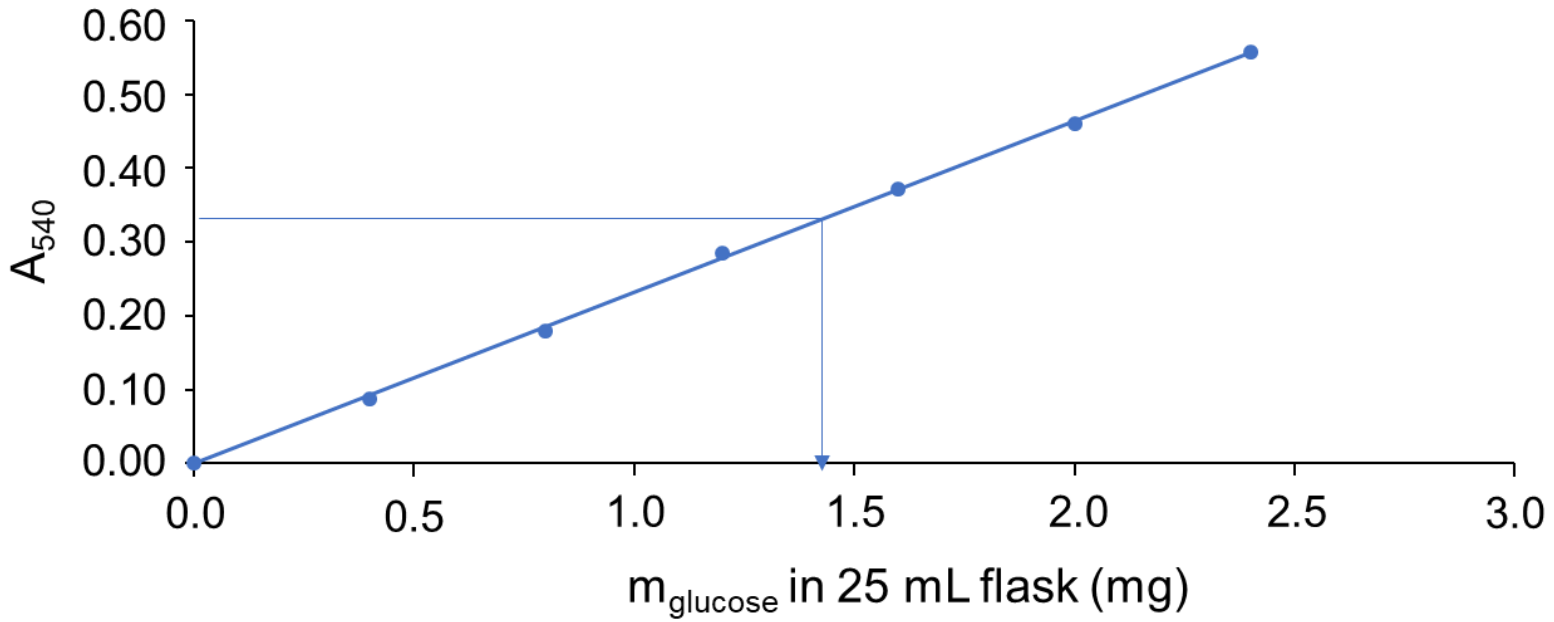
Taking into account the values in Table 2 on p. 186, we draw a graph of the dependence of A_{540} on $m_{glucose}$ in 25 mL flask. Read from the graph the corresponding value of $m_{glucose}$ in 25 mL flask at measured $A_{samp540}$ for the investigated starch hydrolysate.

For example, if we determine the value $A_{samp540} = 0.316$ for the starch hydrolysate, we read $m_{glucose}$ in 25 mL flask as shown on p. 183.

The dependence of A_{540} on $m_{glucose}$ in 25 mL flask can also be described by the equation of a straight line: $A_{540} = k \times m_{glucose}$ in 25 mL flask.

The slope (k) is determined by linear regression analysis of the values of A_{540} and $m_{glucose}$ in 25 mL flask (Table 2 on p. 186). For the investigated starch hydrolysate, we calculate the corresponding value of $m_{glucose}$ in 25 mL flask at measured $A_{samp540}$. The linear regression analysis is carried out using appropriate computer programs (MS Excel or Origin).

How to calculate the mass of starch in the weight of flour (m_{starch}) from the $m_{glucose}$ in the starch hydrolysate in 25 mL flask is explained on p. 184, 185.



Dependence of A_{540} on $m_{glucose}$ in 25 mL flask

$m_{glucose}$ in starch hydrolysate in 25 mL flask = $m_{glucose}$ in starch hydrolysate in test tube

$m_{glucose}$ in starch hydrolysate in test tube = $m_{glucose}$ in $V_{p\ solution}$

According to the experiment description: 0.40 mL of solution is measured from 100 mL flask ($V_{p\ solution}$ = 0.40 mL) into a test tube, therefore:

$$m_{glucose} \text{ in 100 mL flask} = \frac{m_{glucose} \text{ in } V_{p\ solution}}{V_{p\ solution}} \times 100 \text{ mL}$$

We take into account the fact that water is released when a glycosidic bond is formed between glucose units in the polysaccharide ($M_{glucose}$ = 180 g/mol, M_{water} = 18 g/mol), therefore:

$M_{glucose\ unit}$ in polysaccharide = $M_{glucose} \times 0.9$ or: $m_{glucose\ units}$ in starch polymer = $m_{glucose} \times 0.9$

$m_{glucose\ units}$ in 100 mL flask = $m_{glucose}$ in 100 mL flask $\times$ 0.9

According to the experiment description: when preparing the hydrolysate, the contents of the beaker are transferred quantitatively to a 100 mL volumetric flask, therefore:

$$m_{\text{glucose units}} \text{ in 100 mL flask} = m_{\text{glucose units}} \text{ in starch polymer in flour} =$$

$$= m_{\text{starch}} \text{ in flour}$$

$$w_{\text{starch}} \text{ in flour} = \frac{m_{\text{starch}} \text{ in flour}}{m_{\text{flour}}} \times 100\ \%$$

Report

Analysis of starch hydrolysate

starch degradation process: ……

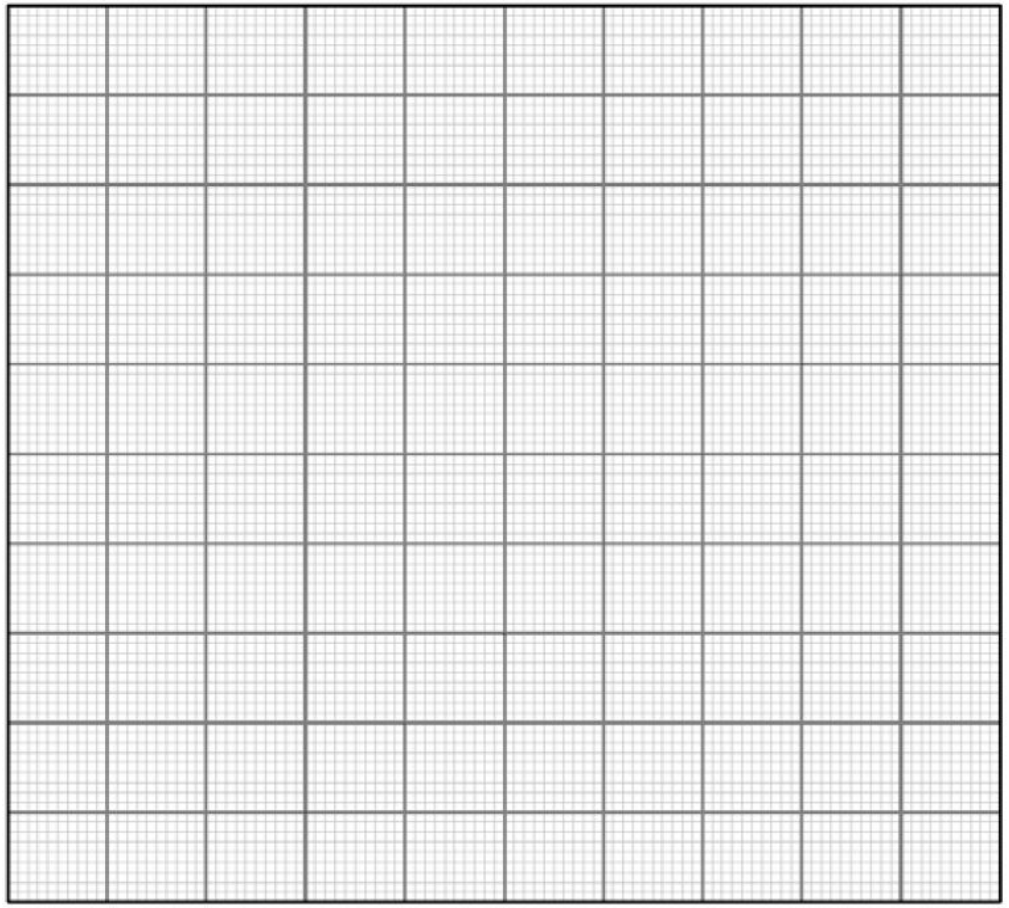
m_{flour} = …….. $A_{samp540}$ = ……..

Further data can be found in the description of the experimental procedure.

Calibration curve

Table 2: Dependence of A_{540} on $m_{glucose}$ in 25 mL flask.

test tube	$V_{p\ stock\ glu}$ (mL)	$m_{glucose}$ in 25 mL flask (mg)	$V_{p\ water}$ (mL)	A_{540}
1	0.20	0.40	1.00	
2	0.40	0.80	0.80	
3	0.60	1.20	0.60	
4	0.80	1.60	0.40	
5	1.00	2.00	0.20	
6	1.20	2.40	0.00	



Dependence of A_{540} on $m_{glucose}$ in 25 mL flask

Calculation of $m_{glucose}$ in starch hydrolysate and the mass fraction of starch in the flour (w_{starch})

The value of $m_{glucose}$ in the starch hydrolysate in 25 mL flask read from the calibration curve or calculated using k (k = …….) at measured $A_{samp540}$: ……..

Calculate w_{starch}; write the complete calculation with numbers and units.

$m_{glucose}$ in starch hydrolysate in test tube =

$m_{glucose}$ in 100 mL flask =

$m_{glucose\ units}$ in starch polymer =

m_{starch} in flour =

w_{starch} in flour =

Complete the table and comment on the effect of the starch degradation procedure on the glucose content in the starch hydrolysate and the calculated mass fraction of starch.

Table 3: Glucose content in prepared starch hydrolysates and calculated mass fraction of starch (w_{starch}).

starch degradation procedure	$A_{samp540}$	$m_{glucose}$ in starch hydrolysate in 25 mL flask (mg)	w_{starch} (%)
1A			
1B			
2A			
2B			
3A			
3B			
4A			
4B			

Comment

QUANTITATIVE DETERMINATION OF CHOLESTEROL IN MILK

Cholesterol

Cholesterol belongs to a group of lipids called steroids. Sterols are compounds with an −OH group attached to the steroid skeleton.

Cholesterol is a component of cell membranes and influences the fluidity or stiffness of the membranes. It is present in large quantities in nervous tissue, especially in the brain. It is also a precursor for the synthesis of bile acids, vitamin D3 (cholecalciferol) and steroid hormones. Humans can synthesize cholesterol themselves (mainly in the liver) or consume it with food. Due to its poor water solubility, it is transported through the body in the blood and other extracellular fluids as part of lipid complexes with proteins, i.e. lipoproteins.

Cholesterol belongs to the so-called animal sterols and is found in foods such as meat, fat, egg yolk, milk and dairy products. In milk, cholesterol is found in lipoprotein complexes, mainly in the phospholipid monolayer that surrounds the fat globules, and to a lesser extent (as esters with fatty acids) in their interior. The cholesterol content in milk is related to the milk fat content. If dietary intake of cholesterol is too high or metabolic disorders occur, the concentration of cholesterol in the blood rises, which represents an increased risk of cardiovascular disease. This underlines the importance of determining cholesterol in food.

Plants synthesize various so-called plant sterols, phytosterols (β-sitosterol, stigmasterol, ...). They are mainly found in vegetable oils. When ingested, phytosterols are supposed to reduce the absorption of cholesterol in the digestive tract and thus lower the concentration of cholesterol in the blood. For this reason, they are added to some foods.

Objective

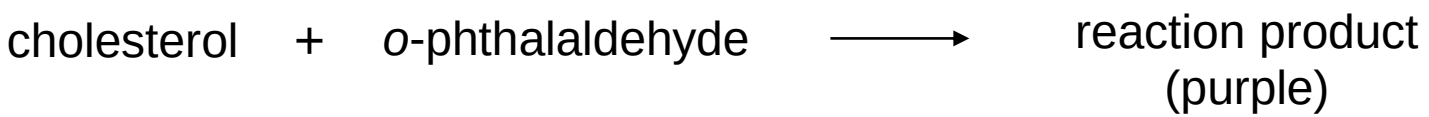
Determine the mass concentration of cholesterol in milk samples (γ_{cholM}) with 0.5%, 1.5% or 3.5% milk fat.

Find out how the cholesterol concentration in milk is related to the content of milk fat.

Principle of method

In the milk sample, the triacylglycerols are degraded by saponification and the cholesterol is released from cholesterol esters and the lipoprotein complex.

After the free cholesterol has been extracted into the organic phase, the product formed in the following reaction in the presence of a strong acid is determined spectrophotometrically:



The absorbance is proportional to the concentration of the reaction product and the concentration of the reaction product is proportional to the cholesterol content.

Experimental procedure

a) Saponification with KOH in ethanol
b) Extraction of cholesterol into hexane and hexane evaporation
c) Spectrophotometric determination of cholesterol
d) Calibration curve for cholesterol determination

a) Saponification with KOH in ethanol

- Measure 2 mL of milk ($V_{p\ milk}$) into a glass-stoppered test tube, add 3 mL of ethanol (95%) and 2 mL of KOH solution (50%); close the test tube and mix the contents.
- Heat for 15 min at 60 °C in a water bath; the triacylglycerols are degraded by saponification and the cholesterol is released from the lipoprotein complex.
- Cool to room temperature.

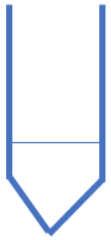
b) Extraction of cholesterol into hexane and hexane evaporation

- Add 5 mL of hexane ($V_{hex\ ph}$) and 3 mL of water.
- Close the test tube and shake it; the cholesterol is extracted from the aqueous phase into the hexane.
- Then place the test tube in a test tube rack and wait until the aqueous and hexane phases are separated; the aqueous phase is in the lower part of the test tube, the hexane phase is above it; the interface between the two phases is visible.
- Transfer the hexane phase to the next clean test tube using a dropper; make sure that you do not catch the aqueous phase with the dropper.
- Transfer the appropriate volume of the hexane phase ($V_{p\ hex\ ph}$) to the next clean test tube using a measuring pipette; for milk with 0.5% fat, $V_{p\ hex\ ph}$ = 3 mL; for milk with 1.5% or 3.5% fat, $V_{p\ hex\ ph}$ = 2 mL.
- Remove the hexane by evaporation in a vacuum dryer (30 min, 40 °C, 30 mbar).

c) Spectrophotometric determination of cholesterol

- To the dry residue after hexane evaporation (contains cholesterol), add 4 mL of *o*-phthalaldehyde solution in concentrated acetic acid, mix carefully; after 10 min, add 2 mL of concentrated H_2SO_4 solution (be careful when working!).
- After 30 min, measure the absorbance at 550 nm ($A_{samp550}$) against a blank sample.
- The blank sample consists of 4 mL of *o*-phthalaldehyde solution and 2 mL of concentrated H_2SO_4 solution.

PROCEDURE SCHEME



2 mL milk ($V_{p\ milk}$)
3 mL ethanol (95%)
2 mL KOH (50%)

close test tube
water bath
60 °C/15 min

hot plate

cool

add:
5 mL hexane ($V_{hex\ ph}$)
3 mL water

shaking

cholesterol is extracted into hexane

after shaking phases separate

hexane ph. - contains cholesterol

aqueous ph.

transfer hexane ph. (dropper)

transfer (pipette) $V_{p\ hex\ ph}$

hexane evaporation

add:
4 mL *o*-phthalaldehyde
after 10 min, 2 mL conc. H_2SO_4

dry residue after hexane evaporation - contains cholesterol

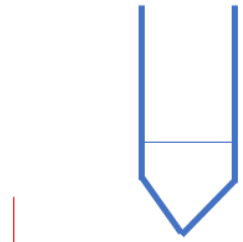
pour into a cuvette

after 30 min, measure $A_{samp550}$

d) Calibration curve for cholesterol determination

- Use a cholesterol stock solution in 95% ethanol with a mass concentration of 0.10 mg/mL ($\gamma_{chol\ stock}$).
- Prepare 5 glass-stoppered test tubes; pipette the appropriate volume of cholesterol stock solution ($V_{p\ chol\ stock}$) and 95% ethanol ($V_{95\%\ ethanol}$) into each test tube as indicated in Table 1 on p. 206.
- Add 2 mL of KOH solution (50%) to each test tube, heat for 15 min at 60 °C in a water bath, and proceed similarly to the determination of cholesterol in milk.
- The only difference is that we transfer 4 mL of the hexane phase ($V_{p\ hex\ ph}$ = 4 mL) before the hexane evaporation and not 2 mL or 3 mL as in the determination of cholesterol in milk.
- Measure A_{550} of all solutions and enter the data in Table 1 on p. 206; how to calculate the cholesterol concentration in the reaction mixture ($\gamma_{chol\ react\ mixt}$) for the calibration curve is explained on p. 203.

SCHEME OF CALIBRATION CURVE PREPARATION



prepare 5 test tubes
into each:
$V_{p\ chol\ stock}$ ($\gamma_{chol\ stock}$ = 0.10 mg/mL)
$V_{95\%\ ethanol}$,
2 mL KOH (50%)

close test tube
water bath
60 °C/15 min

hot plate

cool

add:
5 mL hexane ($V_{hex\ ph}$)
3 mL water

shaking

cholesterol is extracted into hexane

after shaking phases separate

hexane ph. - contains cholesterol

aqueous ph.

transfer hexane ph. (dropper)

transfer (pipette) $V_{p\ hex\ ph}$ = 4 mL

heksane evaporation

dry residue after hexane evaporation - contains cholesterol

add:
4 mL *o*-phthalaldehyde
after 10 min, 2 mL conc. H_2SO_4

pour into a cuvette

after 30 min,
measure A_{550}

Calculations

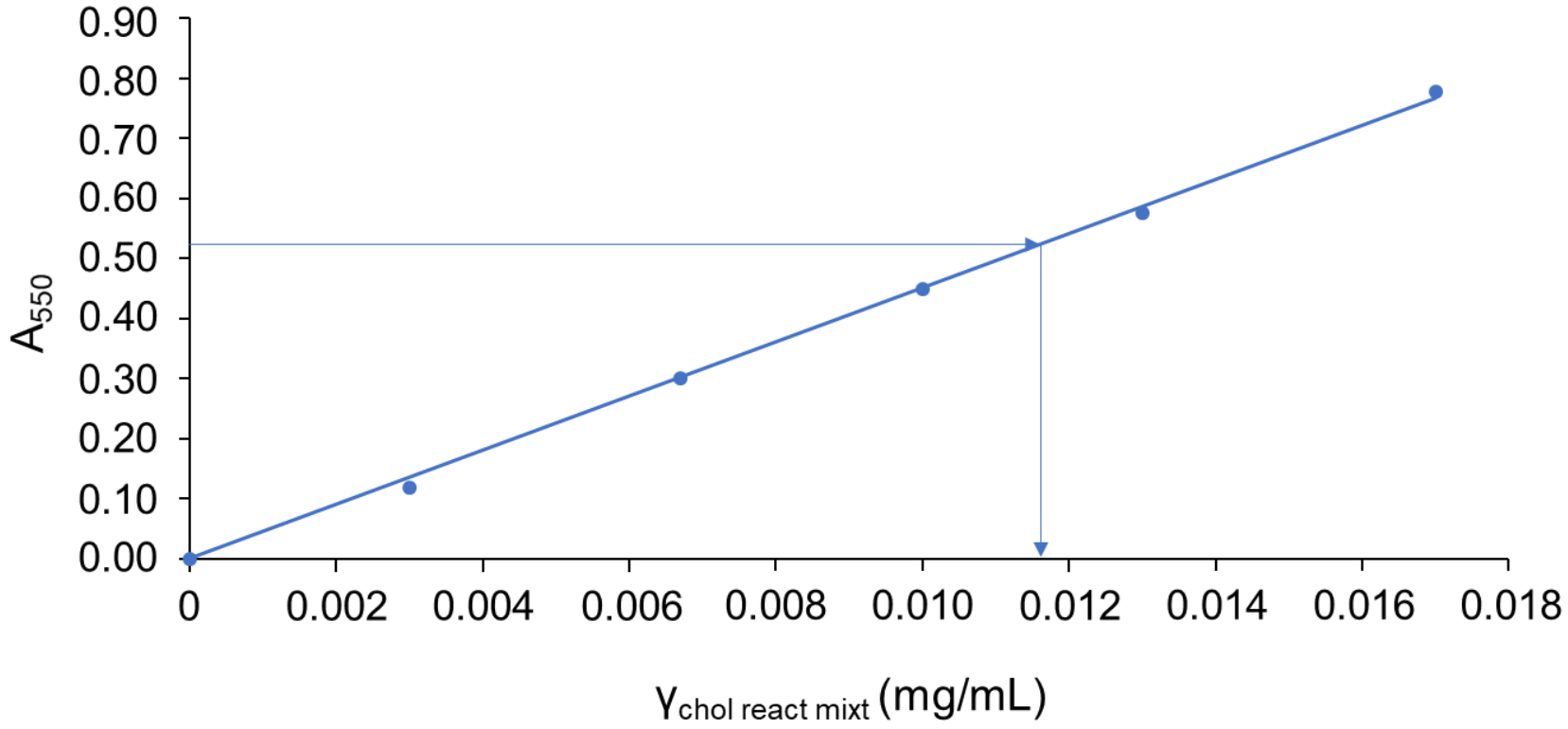
Taking into account the values in Table 1 on p. 206, we draw a graph of the dependence of A_{550} on $\gamma_{chol\ react\ mixt}$. Read from the graph the corresponding value of $\gamma_{chol\ react\ mixt}$ for the milk sample at measured $A_{samp550}$.

For example, if we determine the value $A_{samp550} = 0.525$, we read the value of $\gamma_{chol\ react\ mixt}$ as shown on p. 202.

The dependence of A_{550} on $\gamma_{chol\ react\ mixt}$ can also be described by the equation of a straight line: $A_{550} = k \times \gamma_{chol\ react\ mixt}$.

The slope (k) is determined by linear regression analysis of the values of A_{550} and $\gamma_{chol\ react\ mixt}$ (Table 1 on p. 206). We calculate the corresponding value of $\gamma_{chol\ react\ mixt}$ for the milk sample at measured $A_{samp550}$. The linear regression analysis is carried out using appropriate computer programs (MS Excel or Origin).

How to calculate γ_{cholM} for the investigated milk sample from the $\gamma_{chol\ react\ mixt}$ is explained on p. 204, 205.



Dependence of A_{550} on $\gamma_{chol\ react\ mixt}$

Calculation of cholesterol concentration in the reaction mixture ($\gamma_{chol\ react\ mixt}$) for the calibration curve

$$m_{chol} \text{ in } V_{p\ chol\ stock} = \gamma_{chol\ stock} \times V_{p\ chol\ stock}$$

Values for $V_{p\ chol\ stock}$ are presented in Table 1 on p. 206; $\gamma_{chol\ stock} = 0.10$ mg/mL.

Cholesterol is extracted from the stock solution into hexane, therefore:

$$m_{chol} \text{ in total volume of hexane phase} = m_{chol} \text{ in } V_{p\ chol\ stock}$$

$$\gamma_{chol\ hex\ ph} = \frac{m_{chol} \text{ in total volume of hexane phase}}{V_{hex\ ph}}$$

According to the experiment description: $V_{hex\ ph} = 5$ mL; when preparing the calibration curve, $V_{p\ hex\ ph} = 4$ mL is transferred to a new test tube.

$$m_{chol} \text{ in } V_{p\ hex\ ph} = \gamma_{chol\ hex\ ph} \times V_{p\ hex\ ph}$$

m_{chol} in $V_{p\ hex\ ph}$ = m_{chol} before hexane evaporation = m_{chol} after hexane evaporation = m_{chol} in reaction mixture, therefore:

$$\gamma_{chol\ react\ mixt} = \frac{m_{chol} \text{ in reaction mixture}}{V_{react\ mixt}}$$

According to the experiment description: $V_{react\ mixt} = 6$ mL.

Calculation of cholesterol concentration in milk (γ_{cholM})

Read from the calibration curve or use k to calculate the corresponding value of $\gamma_{chol\ react\ mixt}$ for the investigated milk sample at the measured $A_{samp550}$.

$$m_{chol} \text{ in reaction mixture} = \gamma_{chol\ react\ mixt} \times V_{react\ mixt}$$

According to the experiment description: $V_{react\ mixt} = 6$ mL.

m_{chol} in reaction mixture = m_{chol} after hexane evaporation = m_{chol} before hexane evaporation = m_{chol} in $V_{p\ hex\ ph}$, therefore:

$$\gamma_{chol\ hex\ ph} = \frac{m_{chol} \text{ in } V_{p\ hex\ ph}}{V_{p\ hex\ ph}}$$

According to the experiment description: for milk with 0.5% fat, $V_{p\ hex\ ph} = 3$ mL; for milk with 1.5% or 3.5% fat, $V_{p\ hex\ ph} = 2$ mL.

$$m_{chol} \text{ in total volume of hexane phase} = \gamma_{chol\ hex\ ph} \times V_{hex\ ph}$$

According to the experiment description: $V_{hex\ ph} = 5$ mL.

The cholesterol is extracted from the milk into hexane, therefore:

$$m_{chol} \text{ in } V_{p\ milk} = m_{chol} \text{ in total volume of hexane phase}$$

$$\gamma_{cholM} = \frac{m_{chol} \text{ in } V_{p\ milk}}{V_{p\ milk}}$$

Report

Milk sample analysis

milk fat content = …….

$V_{p\ hex\ ph}$ = ……. $A_{samp550}$ = …….

Further data can be found in the description of the experimental procedure.

Calibration curve

Table 1: Dependence of A_{550} on $\gamma_{chol\ react\ mixt}$.

test tube	$V_{p\ chol\ stock}$ (mL)	$V_{95\%\ ethanol}$ (mL)	$\gamma_{chol\ react\ mixt}$ (mg/mL)	A_{550}
1	0.25	2.75	0.0033	
2	0.50	2.50	0.0067	
3	0.75	2.25	0.0100	
4	1.00	2.00	0.0133	
5	1.25	1.75	0.0167	

Calculation of cholesterol concentration in the reaction mixture ($\gamma_{chol\ react\ mixt}$) for the calibration curve

Calculate the $\gamma_{chol\ react\ mixt}$ for each of the tubes, and check whether your calculated value agrees with the value in Table 1 on p. 206.

m_{chol} in $V_{p\ chol\ stock}$ =

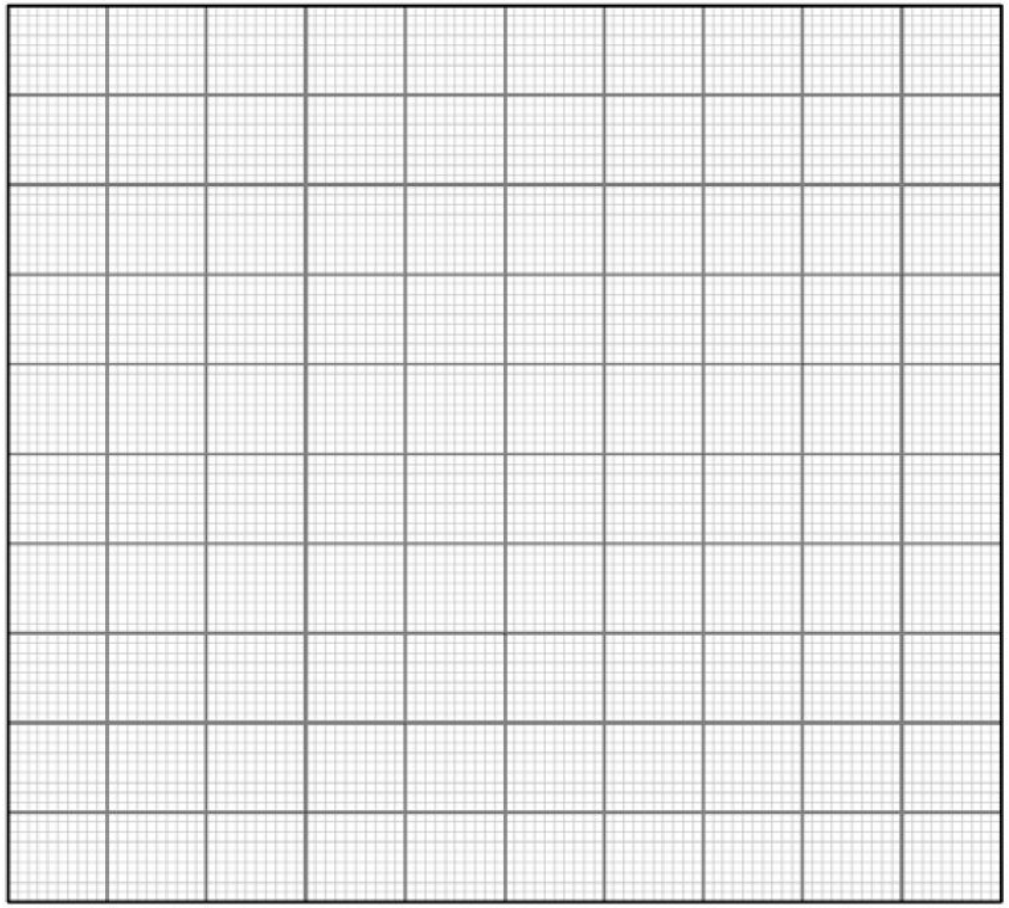
m_{chol} in total volume of hexane phase =

$\gamma_{chol\ hex\ ph}$ =

m_{chol} in $V_{p\ hex\ ph}$ =

m_{chol} in reaction mixture =

$\gamma_{chol\ react\ mixt}$ =



Dependence of A_{550} on $\gamma_{chol\ react\ mixt}$

Calculation of cholesterol concentration in milk (γ_{cholM})

The value of $\gamma_{chol\ react\ mixt}$ for the investigated milk sample read from the calibration curve or calculated using k (k = …….) at measured $A_{samp550}$: ……..

Calculate γ_{cholM}; write the complete calculation with numbers and units.

m_{chol} in reaction mixture =

m_{chol} in $V_{p\ hex\ ph}$ =

$\gamma_{chol\ hex\ ph}$ =

m_{chol} in total volume of hexane phase =

m_{chol} in $V_{p\ milk}$ =

γ_{cholM} =

Complete the table and comment on the cholesterol concentration determined in relation to the milk fat content.

Table 2: Dependence of cholesterol concentration on milk fat content.

milk fat content (%)	0.5	1.5	3.5
$\gamma_{chol\ react\ mixt}$ (mg/mL)			
m_{chol} in reaction mixture (mg)			
m_{chol} in $V_{p\ hex\ ph}$ (mg)			
$\gamma_{chol\ hex\ ph}$ (mg/mL)			
m_{chol} in total volume of hexane phase (mg)			
m_{chol} in $V_{p\ milk}$ (mg)			
γ_{cholM} (mg/mL)			

Comment

DETERMINATION OF POLYPHENOL OXIDASE ACTIVITY IN POTATOES

Phenol oxidases

Phenol oxidases are enzymes that catalyse the aerobic oxidation of phenolic compounds, which leads to the formation of various brown-coloured high molecular weight products.

The first reaction is the hydroxylation of monophenols to *o*-diphenols:

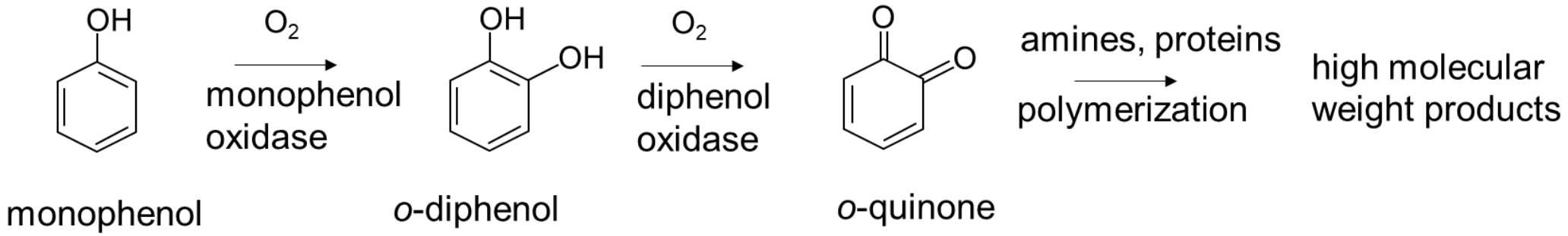
monophenol + O_2 + $2H^+$ → *o*-diphenol + H_2O

the second reaction is the oxidation of *o*-diphenols to *o*-quinones:

o-diphenol + ½ O_2 → *o*-quinone + H_2O

Monophenols are compounds with one –OH group attached to a benzene ring. *o*-diphenols (catechol) are compounds where two –OH groups are attached to a benzene ring in the *ortho* position.

Enzymes that catalyse the hydroxylation of monophenols to *o*-diphenols belong to the monophenol oxidases. Enzymes that catalyse the oxidation of *o*-diphenols to *o*-quinones belong to the diphenol (catechol) oxidases. Enzymes that catalyse both, the hydroxylation of monophenols to *o*-diphenols and their oxidation to *o*-quinones belong to the tyrosinases.



Copper ions are involved in the catalytic activity of these enzymes.

The resulting *o*-quinones are reactive molecules. Brown-coloured products with high molecular weight are formed by the polymerization of *o*-quinones. Amines and proteins are involved in the polymerization reaction, which is not enzyme-catalysed.

Phenol oxidases are found in microorganisms as well as in plant and animal cells, where their activity leads to the formation of melanin pigments. The role of mentioned enzymes in plants is believed to be for protection against insects and pathogens. In a plant cell, they are found in the chloroplasts and chromoplasts. In the event of tissue damage, they are released from these organelles, come into contact with the substrate (phenolic compounds) and are activated by various factors. The resulting *o*-quinones can deactivate enzymes in an invading organism. Polymer products of *o*-quinones may also provide physical protection.

In the food industry, we pay attention to phenol oxidases because they cause enzymatic browning of fruit and vegetables during processing and storage. The browning occurs on the surface of the tissue that is exposed to mechanical processing or damage (peeling, cutting, grinding, ...). Browning can also occur in fruit or vegetable juice, puree, ... Due to the deterioration of sensory properties (appearance), such foods are less acceptable to the consumer.

The resulting waste causes economic losses and represents a burden on the environment. In some cases, such as raisins, prunes, cocoa, black tea and coffee, enzymatic browning is desirable.

Optimal conditions for enzyme activity are a pH range between 4.0 and 7.0 and a temperature between 30 and 50 °C. The enzyme is also relatively stable in the temperature range from 55 to 80 °C. It is therefore possible that the catalytic activity of phenol oxidases can also be demonstrated during a shorter thermal treatment, as the cell membranes are damaged at ~60 °C, so that the enzyme can come into contact with the substrate.

Enzymatic browning is mitigated/prevented by various physical and chemical treatments.

Physical treatments: dehydration, freezing, thermal treatment (blanching) to deactivate the enzyme; packaging in modified atmosphere, coating with sugar syrup, use of protective films to limit O_2 availability.

Chemical treatments to inhibit the enzymes include the use of acids (citric acid, malic acid, phosphoric(V) acid) to lower the pH, as phenol oxidases are weakly active at pH < 3; the addition of chelators (EDTA, oxalic acid, citric acid) that bind copper ions; the addition of reducing agents (sulfite, ascorbic acid, cysteine) that reduce *o*-quinones to diphenols.

Phenolic compounds

This is a large group of structurally diverse compounds. Phenolic compounds include: simple phenols or benzoquinones, phenolic acids, naphthoquinones, xanthones, stilbenes, flavonoids, lignans, biflavonoids, lignins, coumarins and condensed tannins. Nutritionally, the most important are phenolic acids, flavonoids and tannins. Flavonoids are the largest group of phenolic compounds.

The distribution of these compounds in the cell/plant is not uniform. They are located in the vacuole (free, conjugated) and are bound to the cell wall. They accumulate in the outer layers and in the epidermal tissue of the plant. These compounds offer the plant protection against external stress factors, serve as visual markers and are important for growth and reproduction.

Phenolic compounds influence the sensory properties (colour, taste, aroma) of foods. Spices, berries, tea and olive oil are particularly rich in phenolic compounds.

Objective

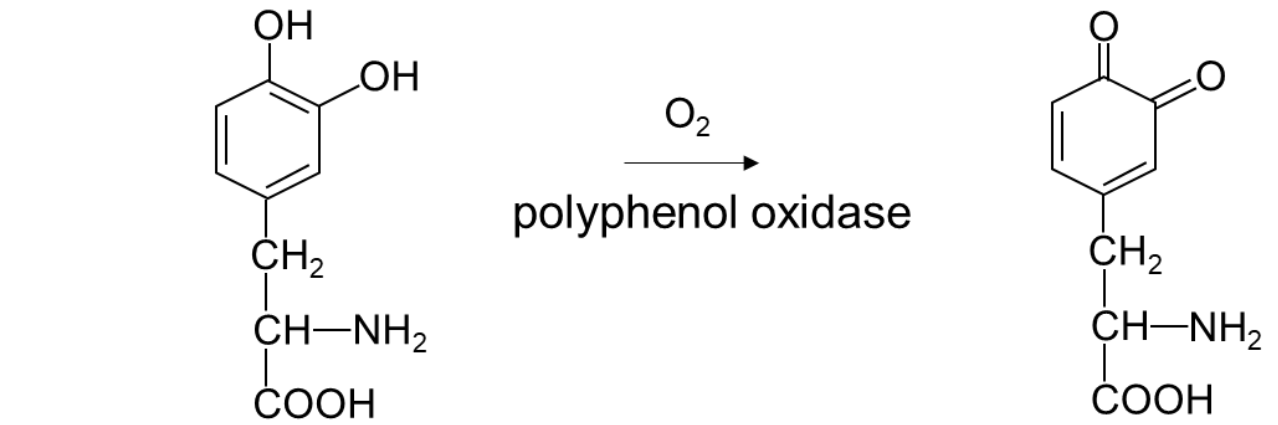
Determine the polyphenol oxidase activity in old and new potatoes.

Determine the effect of thermal treatment of potatoes (cooking in boiling water for 45 min) on polyphenol oxidase activity.

Principle of method

The polyphenol oxidase (PPO) in potato extract catalyses the aerobic oxidation of the phenolic compound 3,4-dihydroxyphenylalanine (DOPA).

The rate of formation of the reaction product (*o*-quinone) is proportional to the activity of the polyphenol oxidase and to the content of polyphenol oxidase in the reaction mixture.



3,4-dihydroxyphenylalanine

o-quinone

The concentration of the reaction product (*o*-quinone) and the rate of its formation are monitored spectrophotometrically.

Experimental procedure

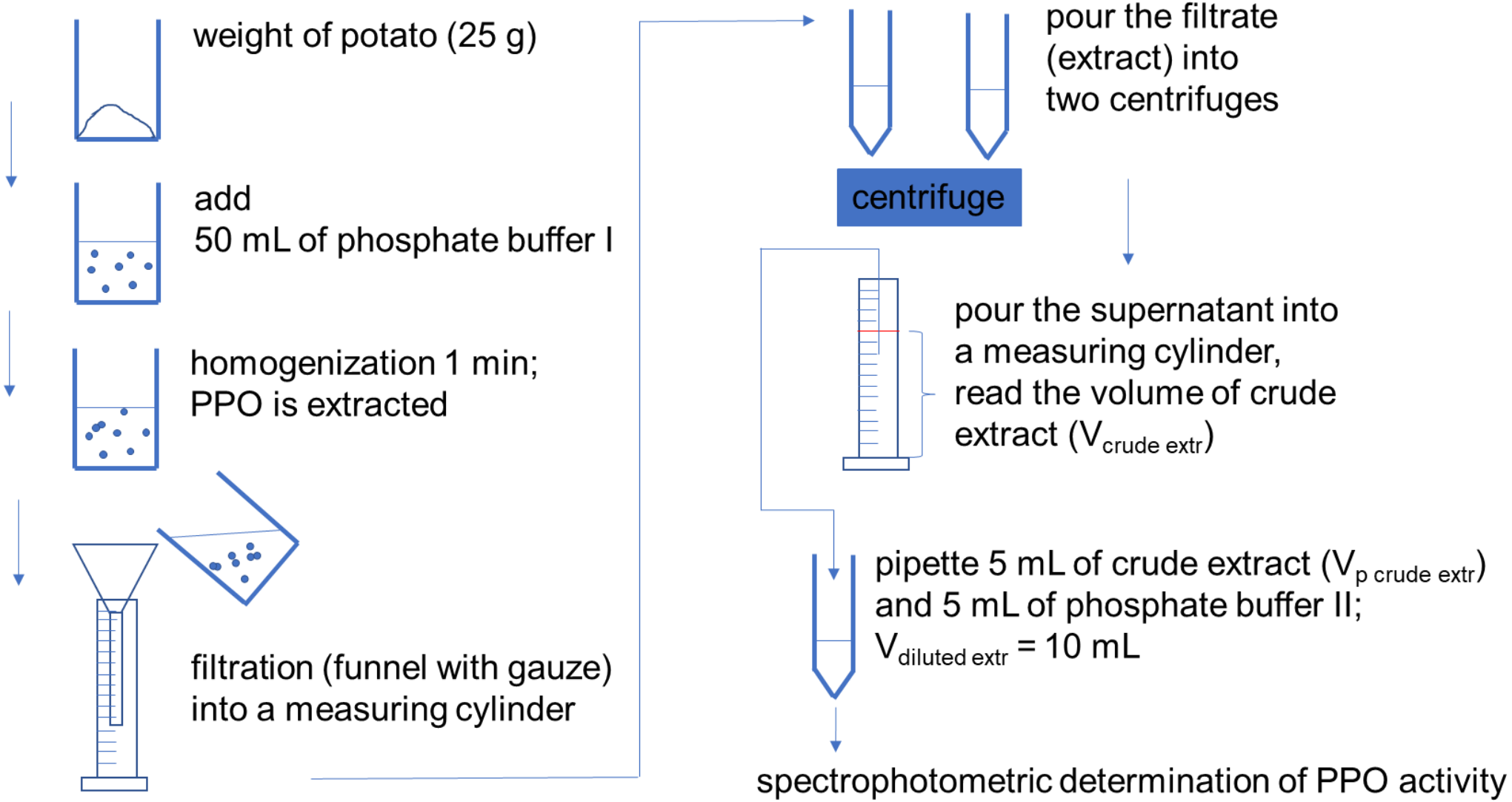
a) Extraction of polyphenol oxidase from potatoes (we perform the procedure of determining polyphenol oxidase activity in old and new potatoes and in thermally-treated new potatoes)

b) Spectrophotometric determination of polyphenol oxidase activity

a) Extraction of polyphenol oxidase from potato (extract preparation)

- Peel the potatoes, cut them and weigh 25 g (m_{potato}) in a chopper.
- Add 50 mL of ice-cold phosphate buffer I (0.1 mol/L, pH 6.8; contains NaF).
- Homogenization for 1 min.
- Filter through a funnel with gauze into a measuring cylinder; the filtrate (PPO extract) is turbid; we need a clear extract, as the activity is determined spectrophotometrically; pour the filtrate (extract) into two centrifuges; make sure that the two centrifuges (together with their contents) are of equal weight; centrifugation follows.
- The supernatant (the clear part above the sediment after centrifugation in a centrifuge) is carefully poured into the measuring cylinder and the volume of the crude extract is read ($V_{crude\ extr}$).
- Prepare a diluted extract: measure 5 mL of the crude extract (that is $V_{p\ crude\ extr}$) into a test tube, add 5 mL of phosphate buffer II (0.1 mol/L, pH 6.8) and mix with a vortex mixer; the volume of the diluted extract ($V_{diluted\ extr}$) is 10 mL.

SCHEME OF POLYPHENOL OXIDASE EXTRACTION PROCEDURE



b) Spectrophotometric determination of polyphenol oxidase activity

- For each diluted extract prepare: sample cuvettes (CU_{sample}), control sample cuvette (CU_{contr}) and blank sample cuvette (CU_{blank}).
- The cuvette CU_{blank} contains water.
- Add buffer II and DOPA solution (Table 1 on p. 224) into the cuvette CU_{contr}, mix and measure the absorbance at 475 nm (A_{contr}) against a blank sample.
- Add buffer II, DOPA solution and a certain volume of diluted extract ($V_{p\ diluted\ extr}$) into cuvettes CU_{sample} (Table 1 on p. 224); the extract is added at the spectrophotometer shortly before starting the measurements.
- After adding the extract, start the stopwatch, mix the contents well, insert the cuvette into the spectrophotometer and start measuring the absorbance at 475 nm against a blank sample ($A_{measured}$).
- $A_{measured}$ is monitored every 15 s until t = 90 s, then every 30 s until t = 300 s.

Table 1: Volume of buffer II ($V_{p\ buffer\ II}$), DOPA solution ($V_{p\ DOPA}$) and diluted extract ($V_{p\ diluted\ extr}$) for transfer to cuvettes.

cuvette	$V_{p\ buffer\ II}$ (mL)	$V_{p\ DOPA}$ (mL)	$V_{p\ diluted\ extr}$ (mL)
CU_{contr}	2.00	1.00	-
Cu_{sample} 1	1.95	1.00	0.05
Cu_{sample} 2	1.90	1.00	0.10
CU_{sample} 3	1.80	1.00	0.20
CU_{sample} 4	1.70	1.00	0.30

Different $V_{p\ diluted\ extr}$ mean different amounts of enzyme in the reaction mixture.

Calculations

The absorbance of the reaction product (A_{sample}) is calculated by subtracting the A_{contr} value from the $A_{measured}$ value ($A_{sample} = A_{measured} - A_{contr}$). Enter the data in Table 2 on p. 231 and draw a graph of the A_{sample} dependence on the incubation time.

To determine the initial rate, draw a tangent to the curve at time t = 0, as shown on p. 226, and read A_{sample} at 60 sec (1 min); this is ΔA_{sample} / min.

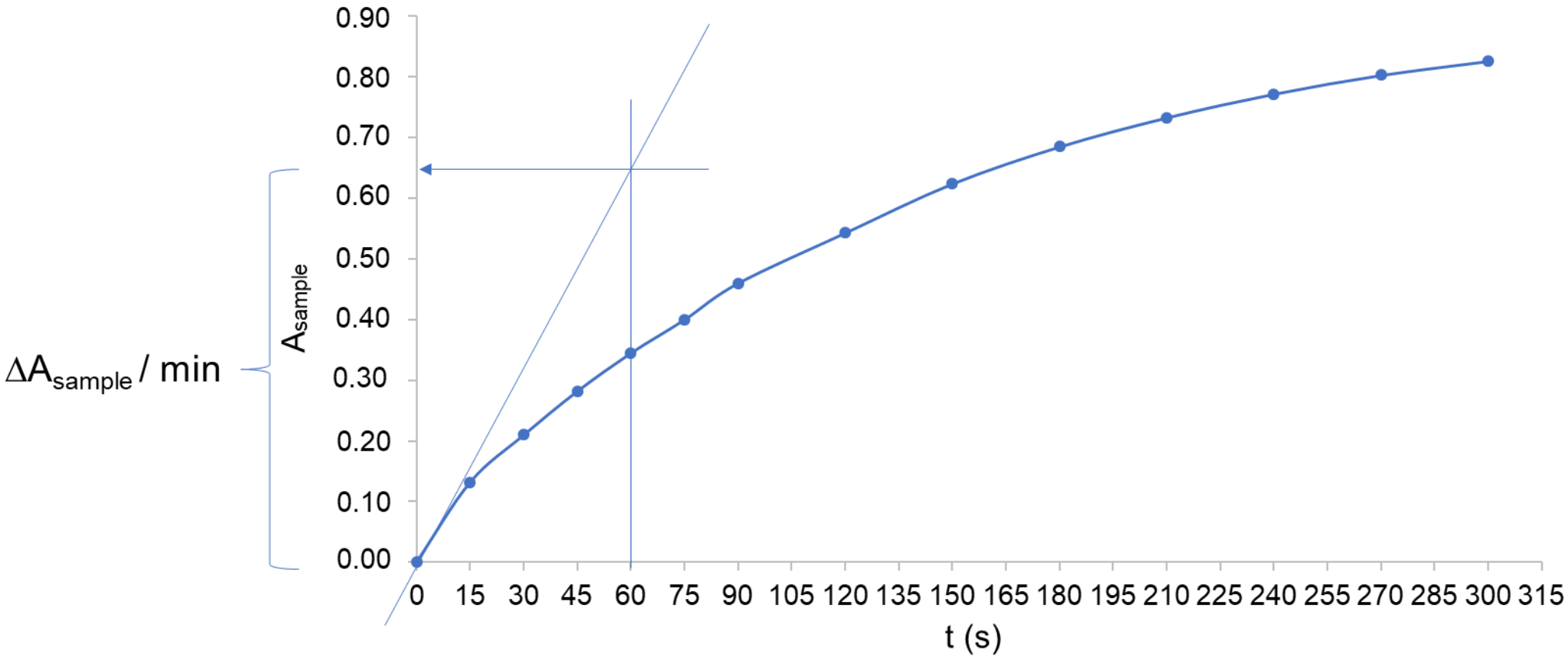
PPO activity is proportional to the amount of a product in a given volume of the reaction mixture ($V_{react\ mixt}$) formed in one minute ($n_{product}$ / min), and $n_{product}$ / min is proportional to ΔA_{sample} / min.

$n_{product}$ / min is calculated taking into account the Beer-Lambert law ($A = \varepsilon \times l \times c$).

The concentration of the product in the reaction mixture ($c_{product}$) is given as:

$$c_{product} = \frac{n_{product}}{V_{react\ mixt}} = \frac{A_{sample}}{\varepsilon \times l} \quad \text{or:} \quad \frac{n_{product} / \text{min}}{V_{react\ mixt}} = \frac{\Delta A_{sample} / \text{min}}{\varepsilon \times l}$$

ε is molar absorption coefficient (5012 $L \times mol^{-1} \times cm^{-1}$); l is the length of the optical path (dimension of the cuvette 1 cm).



Dependence of A_{sample} on incubation time

$$n_{product} / \min = \frac{\Delta A_{sample} / \min}{\varepsilon \times l} \times V_{react\ mixt}$$

The volume of the reaction mixture ($V_{react\ mixt}$) is the sum of the volumes pipetted into a cuvette CU_{sample}; according to the experiment description: $V_{react\ mixt} = 3$ mL.

$n_{product}$ express in µmol (1 µmol = 1×10^{-6} mol)

The polyphenol oxidase activity, which is proportional to $n_{product} / \min$, is expressed as the number of enzyme units (U).

By definition: 1 unit of enzyme activity (1 U) catalyses the formation of 1 µmol of a product in 1 min, determined under the conditions described, therefore:

$$\text{PPO activity in reaction mixture} = \frac{1\ \text{U} \times n_{product} / \min}{1\ \mu\text{mol/min}}$$

For example, if we calculate that 0.02 µmol of product was formed in 1 min ($n_{product} / \min$ = 0.02 µmol/min), the PPO activity in the reaction mixture = 0.02 U.

According to the experiment description (Table 1 on p. 224): a certain $V_{p\ diluted\ extr}$ was measured into a cuvette CU_{sample} (reaction mixture), therefore:

PPO activity in $V_{p\ diluted\ extr}$ = PPO activity in reaction mixture

$$\text{PPO activity / mL of diluted extr.} = \frac{\text{PPO activity in } V_{p\ diluted\ extr}}{V_{p\ diluted\ extr}}$$

PPO activity in total $V_{diluted\ extr}$ = $V_{diluted\ extr}$ × PPO activity / mL of diluted extr.

According to the experiment description (p. 221, 222): $V_{diluted\ extr}$ = 10 mL; diluted extract is prepared by measuring 5 mL of crude extract ($V_{p\ crude\ extr}$) + 5 mL of buffer II, therefore:

PPO activity in $V_{p\ crude\ extr}$ = PPO activity in total $V_{diluted\ extr}$

$$\text{PPO activity / mL of crude extr.} = \frac{\text{PPO activity in } V_{p\ crude\ extr}}{V_{p\ crude\ extr}} \qquad \text{(unit: U/mL)}$$

According to the experiment description (p. 221, 222): PPO was extracted from the potato and after filtration and centrifugation, the $V_{crude\ extr}$ was read, therefore:

PPO activity in total $V_{crude\ extr}$ = $V_{crude\ extr}$ $\times$ PPO activity / mL crude extr.

PPO activity in potato = PPO activity in total $V_{crude\ extr}$

$$\text{PPO activity / mg of potato} = \frac{\text{PPO activity in potato}}{m_{potato}} \qquad \text{(unit: U/mg)}$$

Report

Experimental data

potato sample investigated: ……..

m_{potato} = …….

$V_{crude\ extr}$ = …….

$V_{p\ crude\ extr}$ = …….

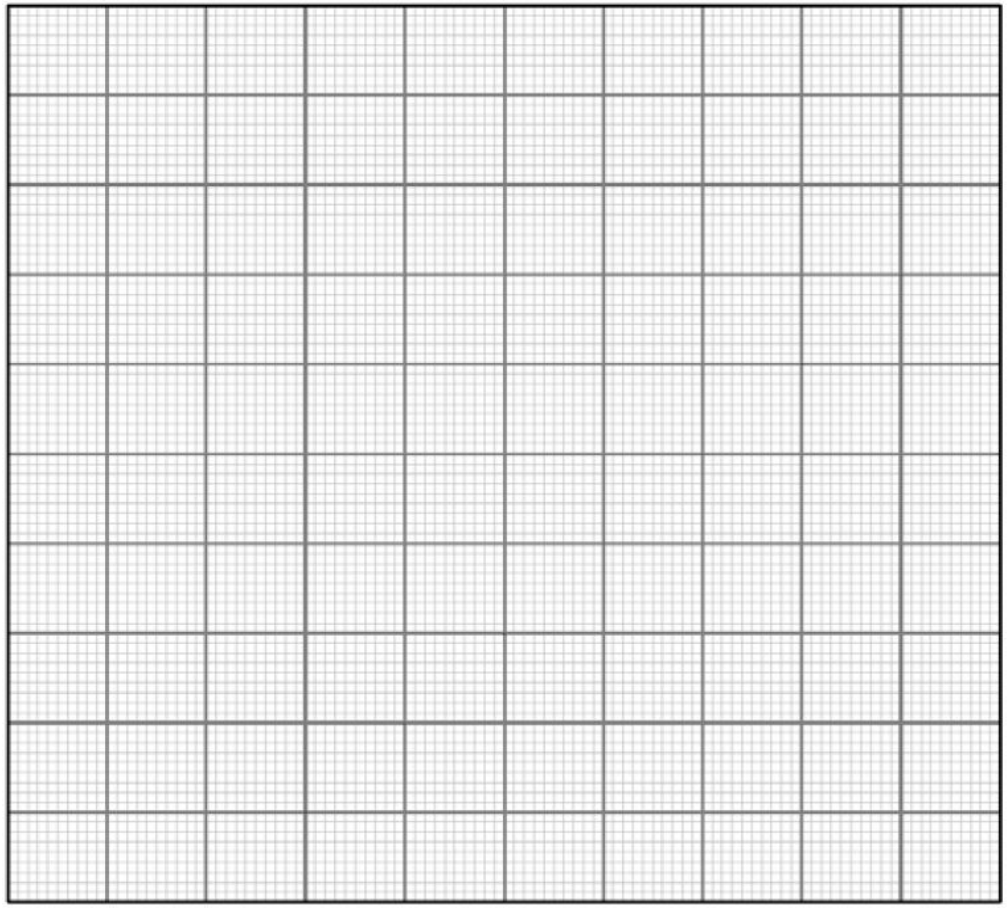
A_{contr} = …….

Further data can be found in the description of the experimental procedure.

Table 2: Dependence of A_{sample} on incubation time.

$V_{p\ diluted\ extr}$ (mL)	old potato				new potato				therm. treated new potato			
	0.05	0.10	0.20	0.30	0.05	0.10	0.20	0.30	0.05	0.10	0.20	0.30
t(s)	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}	A_{sample}
0												
15												
30												
45												
60												
75												
90												
120												
150												
180												
210												
240												
270												
300												

Calculations and graph



Dependence of A_{sample} on incubation time

ΔA_{sample} / min =

$n_{product}$ / min =

PPO activity in reaction mixture =

PPO activity in $V_{p\ diluted\ extr}$ =

PPO activity / mL of diluted extr. =

PPO activity in total $V_{diluted\ extr}$ =

PPO activity in $V_{p\ crude\ extr}$ =

PPO activity / mL of crude extr. =

PPO activity in total $V_{crude\ extr}$ =

PPO activity in potato =

PPO activity / mg of potato =

Complete the table and comment on the PPO activity depending on the selected potato and thermal treatment.

Table 3: PPO activity in potato.

	old potato				new potato				therm. treated new potato			
$V_{p\ diluted\ extr}$ (mL)	0.05	0.10	0.20	0.30	0.05	0.10	0.20	0.30	0.05	0.10	0.20	0.30
ΔA_{sample} / min												
$n_{product}$ / min (µmol/min)												
PPO activity in reaction mixture (U)												
PPO activity in $V_{p\ diluted\ extr}$ (U)												
PPO activity / mL of diluted extr. (U/mL)												
PPO activity in total $V_{diluted\ extr}$ (U)												
PPO activity in $V_{p\ crude\ extr}$ (U)												
PPO activity / mL of crude extr. (U/mL)												
PPO activity in total $V_{crude\ extr}$ (U)												
PPO activity in potato (U)												
PPO activity / mg of potato (U/mg)												

Comment

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