Samenvatting
Suikermais werd gedurende ruim 2 maanden opgeslagen in doorstroom containers bij 1-2°C en 5-6°C in lucht en in een combinatie van 10%CO2 + 2%O2. Gedurende deze periode werden op 3 momenten (na 14, 34 en 63 dagen opslag) monsters genomen. Deze werden beoordeeld op uitwendige kwaliteitskenmerken als bladkleur en parasitair bederf van bladeren en zaden. Verder werden aantal inhoudsstoffen (sucrose, fructose, glucose en ethanol) geanalyseerd.
Het kwaliteitsverlies werd m.b.t. de uitwendige kenmerken het beste tegengegaan met d laagste temperatuur in de CA conditie. De reducerende suikers namen tijdens de bewaring toe met uitzondering van de bewaring in lucht bij 5-6°C. Het verlies aan sucrose was minimaal tijdens de bewaring. Het relatief grootste verlies werd geleden bij 5-6°C in lucht. Bij de CA conditie bij beide temperaturen werd ophoping van ethanol gemeten.
Op basis van deze resultaten en eerder wordt geconcludeerd, dat toepassing van CA condities duidelijke voordelen heeft. Het beste bewaarresultaat kan worden verwacht na opslag in 0-1°C, bij 10%CO2 en een verlaagde zuurstofspanning.

Invloed van CA condities op een aantal kwaliteits kenmerken van suikermais.

Inleiding

Suikermais is een produkt, dat zich in een groeiende belangstelling mag verheugen. In Nederland is het nog min of meer een "nieuw" produkt. Het produkt heeft een scherpe aanvoerpiek door de nauwe tijdsgrenzen, waarbinnen de oogst moet worden uitgevoerd. Overvoering van de markt is snel aan de orde. Spreiding van de aanvoer wordt gezocht in het gebruik van rassen en planttijdstippen. Dit laatste kan in principe ook door opslag, hoewel de mogelijkheden beperkt zijn tot hooguit enkele weken bij ongeveer 0°C. Uit de literatuur blijkt, dat CA condities in principe een bijdrage kunnen leveren aan verlenging van de opslag duur (Saltveit 1989).
Onderzoek van ATO-DLO in 1991 (Schouten 1991) bevestigde de principe mogelijkheden van CA bewaring. Vastgesteld kon worden, dat de kleur van het produkt vooral in hoge CO2 concentraties beter behouden bleef en dat de ontwikkeling van parasitair bederf op blad en zaden werd afgeremd. Het ATO-DLO onderzoek, dat werd uitgevoerd in opdracht van een op suikermais gespecialiseerd bedrijf Farm Pack in Kapelle, liet enkele vragen onbeantwoord.
Op de eerste plaats werd vrijwel geen verschil gevonden tussen verhoogde koolzuur spanning met 21% zuurstof of hoge koolzuur gehaltes plus verlaagde zuurstof spanningen. Verder werd ook waargenomen, dat de kolven eenmaal uit de CA condities in de warmte zeer snel bederf vertoonden. Tenslotte bleek, dat het glucose gehalte in de zaden sterk bleek te stijgen. Dit laatste doet denken aan de koude verzoeting zoals die zich bijv. bij aardappelen kan voordoen bij temperaturen tussen 0 en 5°C. Deze waarnemingen ontlokten de vraag, of de geaccepteerde lage temperatuur van 0-1°C wel de juiste is en of dit LTB (Lage Temperatuur Bederf) verschijnselen zouden kunnen zijn. De vraag naar mogelijke interacties tussen temperatuur en CA condities werd naar aanleiding van het onderzoek in 1991 gesteld. Deze vraag werd centraal gesteld in het vervolg onderzoek, dat door ATO-DLO in de zomer van 1992 werd uitgevoerd. Dit rapport behandelt de resultaten van het onderzoek in 1992.

Materiaal en Methoden

Bewaring

De maiskolven werden door Farm Pack te Kapelle aan het ATO voor onderzoek ter beschikking gesteld. Het produkt werd machinaal geoogst en gesorteerd op het bedrijf. Vervolgens werden de kolven naar het ATO gebracht en direct bij de betreffende temperaturen 1-2 en 5-6°C geplaatst. De CA condities waren 0%CO2 + 21%O2 (controle lucht) en 10%CO2 + 2%O2. De laatste luchtsamenstelling was als een van de beste CA condities uit het voorgaande onderzoek gekomen.
De bewaring vond plaats in metalen doorstroom containers. Hierin kan de luchtsamenstelling van de lucht worden beheerst. De afzonderlijke gassen koolzuur, zuurstof en stikstof kunnen nauwkeurig op een bepaalde waarde worden afgesteld met mass flow controllers.
De bewaarcondities werden in duplo in de proef ingebracht. Elke container bevatte 30 kolven, die verdeeld over 3 uitslag tijdstippen werden geruimd na 14, 34 en 63 dagen bewaring.

Bepalingen

Bij elke uitslag werden de volgende bepalingen/beoordelingen uitgevoerd:
- bladkleur bepaling. Dit werd uitgevoerd door het blad van elke individuele kolf te plaatsen in een 4 categorieën systeem als volgt: 0 = geen verkleuring; 1 = lichte geel verkleuring; 2 = matige geel verkleuring en 3 = sterke geelverkleuring.
- parasitair bederf blad. Op dezelfde manier als voor de blad kleur werden de individuele kolven in 4 categorieën ondergebracht. 0 = geen bederf; 1 = lichte mate van bederf; 2 = matig bederf en 3 = ernstig bederf.
- parasitair bederf zaden. Dit werd op dezelfde manier gedaan als voor bederf blad.
- suiker bepalingen. Met behulp van enzym kits werden glucose, fructose en sucrose bepaald volgen Boehringer Mannheim voorschrift (zie bijlage 1).
- ethanol gehalte. Dit werd eveneens gedaan langs enzymatische weg (zie bijlage 1).

De ademhaling werd regelmatig bepaald als volgt. Op een bepaald moment wordt de gasstroom door de container gestopt. Als gevolg van de ademhaling loopt de CO2 spanning in de container op. Door monsters lucht bij het afsluiten van de gasstroom en enkele uren later te analyseren kan uit de ophoping van de koolzuur de CO2 produktie in mg.kg.uur worden berekend.

Tijdschema
Inslag produkt: 24-07-1992.
Uitslagen: 07-08, 27-08 en 25-09-1992.

Resultaten

In de bijlage 2 zijn de gedetailleerde resultaten weergegeven van de bepalingen naar de bladkleur en de ontwikkeling van parasitair bederf op de bladeren en de zaden.

Kleur

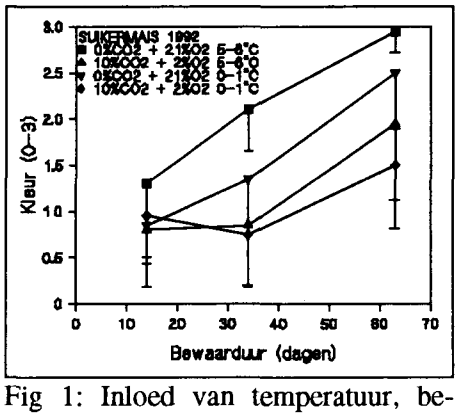

Fig 1: Inloed van temperatuur, bewaarduur en CA conditie op de bladkleur van suikermais.

Een beperking van dit onderzoek is gelegen in de cellen. Het was technisch niet mogelijk de invloed van de temperatuur in herhaling in het onderzoek in te brengen. Hierdoor zijn celeffecten en temperatuur effecten niet van elkaar los te maken. Door deze beperking kan alleen iets gezegd worden van de gemiddelden van de twee temperaturen.
De gemiddelden voor 1-2°C en 5-6°C bedragen resp. 1.32 en 1.66, terwijl de gemiddelden voor lucht en CA bewaring resp. 1.84 en 1.13 zijn. In bijgaande figuur 1 zijn weergegeven de resultaten met betrekking tot de kleur beoordelingen. Er blijkt dus vooral een duidelijke verslechtering van de bladkleur gedurende de opslag. Uit de statistische analyse blijkt, dat de invloed van CA conditie en bewaarduur aantoonbaar zijn evenals de interactie bewaarduur*CA conditie ($p<5\%$). Dit laatste betekent, dat naarmate de bewaarduur langer wordt, de verschillen tussen de lucht en CA bewaring groter worden, zie tabel 1.

Tabel 1: Invloed van de interactie CA conditie * uitslag op de kleur* van suikermais.

CA conditie	Bewaarduur		
%CO2 + %O2	14 dagen	34 dagen	64 dagen
0 + 21	1.075 a	1.725 b	2.725 c
10 + 2	0.875 a	0.800 a	1.725 b

* gemiddelden met dezelfde letter zijn niet significant verschillend ($p<5\%$) L.S.D. = 0.3890

Parasitair bederf blad
De resultaten van de bepalingen naar parasitair bederf op de bladeren zijn weergegeven in figuur 2. De gemiddelden voor de twee temperaturen zijn: 1.617 (5-6°C) en 1.358 (1-2°C), terwijl de gemiddelden voor de luchtsamenstelling 1.817 (lucht) en 1.158 (10%CO2 + 2%O2) bedragen.
Ook voor deze parameter zijn de invloeden van CA conditie en bewaarduur aantoonbaar ($p<5\%$), (zie figuur 2) evenals de interactie bewaarduur * CA conditie. De verschillen tussen lucht en CA bewaring worden dus met het verstrijken van de opslagduur groter, zie tabel 2.

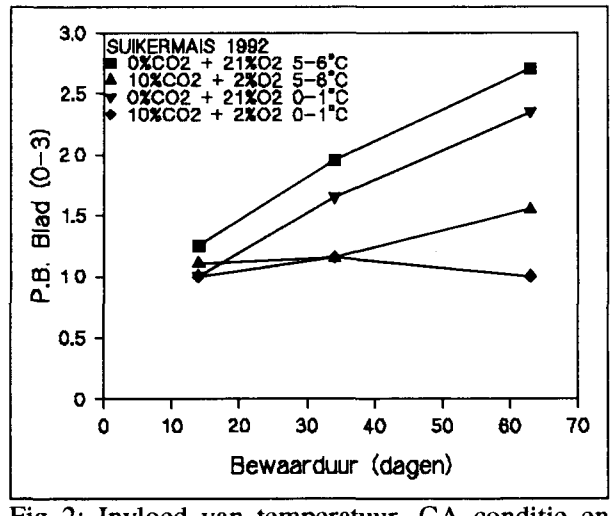

Fig 2: Invloed van temperatuur, CA conditie en bewaarduur op parasitair bederf op het blad van suikermais.

Tabel 2: Invloed van de interactie CA conditie * bewaarduur op het parasitair bederf van suikermais bladeren.

CA conditie %CO2 + %O2	Bewaarduur 14 dagen	34 dagen	64 dagen
0 + 21	1.125 ab	1.800 c	2.525 d
10 + 2	1.050 a	1.150 ab	1.275 b

* zie tabel 1; L.S.D. + 0.2002.

Parasitair bederf zaden

De gemiddelden voor de temperatuur en de luchtsamenstellingen waren als volgt: 1.275 (5-6°C), 1.250 (1-2°C), 1.492 (lucht) en 1.033 (10%CO2 + 2%O2). De invloed van de uitslag en de CA conditie zijn significant evenals de interacties Uitslag * CA conditie en Temperatuur * CA conditie * uitslag. De invloed van de interactie Uitslag * CA conditie is weergegeven in tabel 2.

Tabel 2: Invloed van de interactie Uitslag * CA conditie op parasitair bederf* van de zaden van suikermais.

CA conditie %CO2 + %O2	Bewaarduur 14 dagen	34 dagen	64 dagen
0 + 21	0.925 a	1.550 b	2.000 c
10 + 2	0.825 a	0.775 a	1.500 b

* zie tabel 1, L.S.D. = 0.2956

Ook hier wordt dus weer zichtbaar, dat gedurende de loop van de bewaring het verschil tussen

bewaring in lucht en in 10%CO2 + 2%O2 groter wordt. Dat de gemiddelde verschillen tussen de temperaturen zo gering zijn heeft mogelijk iets te maken met de vrij hoge mate van beschadiging van de kolven. De interactie Temperatuur * CA conditie * bewaarduur is weergegeven in figuur 3.

N.B. Bij de mechanische oogst worden vrij regelmatig zaden beschadigd. Hierdoor komt suikerrijk vocht vrij, waarop zich gemakkelijk schimmels kunnen ontwikkelen. Van de 240 in de proef betrokken kolven bleken er bij de drie uitslagen 129, dus ongeveer 50%, in lichte mate vooral aan de punten beschadigd.

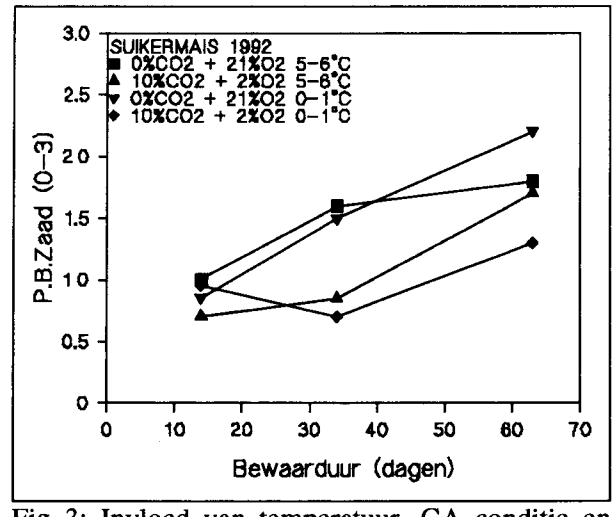

Fig 3: Invloed van temperatuur, CA conditie en bewaarduur op parasitair bederf van de zaden van suikermais.

Inhoudsstoffen

De gedetailleerde gegevens m.b.t. de analyse van inhoudsstoffen zijn weergegeven in bijlage 3. Uit

de statistische analyse van dit cijfermateriaal bleek, dat voor glucose, fructose, sucrose en ethanol de invloeden van uitslag en CA conditie aantoonbaar waren. Ook de interacties Uitslag * Temperatuur, Uitslag * CA conditie en Temperatuur * CA conditie * Uitslag bleken significant. In de figuren 4 t/m 7 is het verloop in de tijd weergegeven van de verschillende inhoudsstoffen.

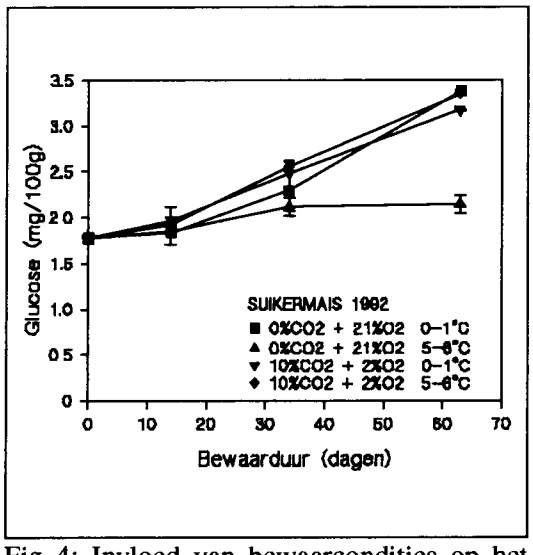

Fig 4: Invloed van bewaarcondities op het glucose gehalte in suikermais.

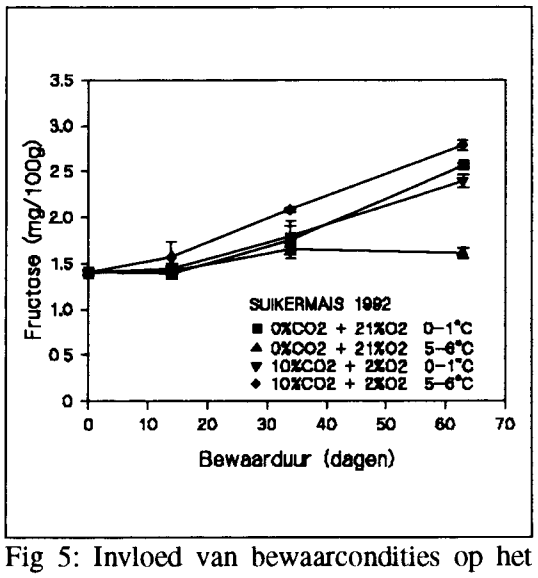

Fig 5: Invloed van bewaarcondities op het fructose gehalte in suikermais.

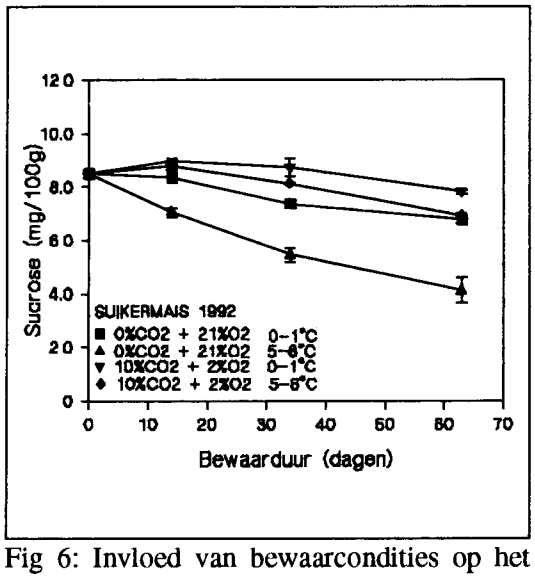

Fig 6: Invloed van bewaarcondities op het sucrose gehalte van suikermais.

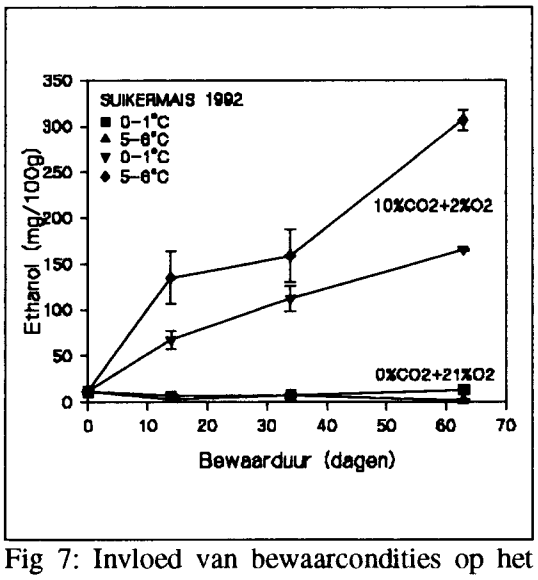

Fig 7: Invloed van bewaarcondities op het ethanol gehalte van suikermais.

Glucose en fructose

Uit de vele aantoonbare invloeden van factoren en interacties volgt niet, dat de onderling verschillen alle erg groot zijn. Het zijn vooral de bij de hoogste temperatuur in lucht bewaarde kolven, die zich afwijkend gedragen. De andere verschillen worden vooral veroorzaakt door het uitslag tijdstip, zie tabel 4.

Tabel 4: Invloed van temperatuur, CA conditie en bewaarduur op de glucose en fructose gehalten (mg/100 g) bij suikermais.

Bewaarduur (dagen)	GLUCOSE*				FRUCTOSE**			
	5-6°C		1-2°C		5-6°C		1-2°C	
	0+21	10+2	0+21	10+2	0+21	10+2	0+21	10+2
14	1.85	1.91	1.84	1.96	1.42	1.57	1.39	1.43
34	2.11	2.56	2.29	2.48	1.66	2.09	1.75	1.79
63	2.14	3.35	3.38	3.18	1.61	2.79	2.57	2.39

* l.s.d. 0.214
** l.s.d. 0.1836

Vanaf de uitslag na 14 dagen is er steeds een toename te constateren naar de latere 2 uitslagen met uitzondering van 5-6°C in lucht.

Sucrose

Een zekere afscheiding van de mais bewaard in lucht bij de hoogste temperatuur t.o.v. de andere bewaaromstandigheden valt waar te nemen. Het gemiddelde voor temperatuur en CA condities is als volgt:6.74 (5-6°C), 8.00 (1-2°C), 6.51 (0%CO + 21%O2) en 8.23 (10%CO2 + 2%O2). Er is dus opnieuw de reeds eerder gemelde tendens van een gunstig effect van de laagste temperatuur en de CA conditie.
Statistische analyse wijst uit, dat de effecten van de uitslag en van de interacties Temperatuur * Uitslag en CA conditie * uitslag aantoonbaar zijn (p<5%). Voor de laatste interactie zijn de gemiddelden weergegeven in tabel 5.

Tabel 5: Invloed van de interactie CA conditie * Uitslag op het sucrose* gehalte van suikermais.

CA conditie %CO2 + %O2	Uitslag 14 dagen	34 dagen	63 dagen
0 + 21	7.70 d	6.41 b	5.44 a
10 + 2	8.87 f	8.44 e	7.38 c

Gemiddelden voor eenzelfde letter zijn niet significant verschillend; L.S.D. = 0.2892

Deze gemiddelden onderstrepen eens te meer reeds na 14 dagen bewaring het veel minder grote verlies aan sucrose door een lage temperatuur en toepassing van de CA conditie.

Ethanol

De gemiddelden voor de twee temperaturen en CA condities zijn: 102.2 , (5-6°C), 62.0 , (1-2°C), 6.5 , (0%CO2 + 21%O2) en 157.6 mg/100 gr vers gewicht (10%CO2 + 2%O2). Verder blijkt uit de statistische analyse, dat de effecten van de uitslag significant zijn evenals van de interacties tussen Temperatuur * Uitslag, CA conditie * Uitslag en Temperatuur * CA conditie * Uitslag (P<5%).
In figuur 7 wordt de invloed van de laatste interactie weergegeven. Bij beide temperaturen in lucht is er nauwelijks sprake van enige ophoping van ethanol. Des te sterker is dit het geval ook bij beide temperaturen voor de CA conditie 10%CO2 + 2%O2.

Ademhaling

In figuur 8 is de invloed van de verschillende bewaarcondities op de ademhaling weergegeven

gedurende de eerste maand van de bewaring. Enkele wel is waar zwakke tendensen zijn uit de figuur te halen. Op de eerste plaats blijkt de bewaring in lucht een redelijk stabiel verloop te vertonen. Mogelijk heeft dit te maken met de manier, waarop ademhaling wordt gemeten. Uit de container worden monsters genomen, die in een stikstofstroom worden ingespoten. Deze stroom doorloopt een CO2 analysator, die kwantitatief de concentratie aangeeft. De 10%CO2 zit echter aan de rand van de mogelijkheden, waardoor het moeilijk is via de ophopingsmethode de ademhaling te meten. Mogelijk is dit een verklaring voor het grillige verloop van de curven voor 10%CO2 + 2%O2. Een tweede punt is, dat in lucht bij beide temperaturen de lijnen vrijwel een horizontaal verloop vertonen i.t.t. de lijnen voor de CA conditie. De laatste vertonen een tendens tot stijgen in de tijd. Tenslotte is te zien, dat er een voortdurend verschil is enerzijds tussen de twee temperaturen en anderzijds tussen de lucht en de CA bewaring. De hoogste temperatuur en de luchtbewaring vertonen voortdurend een hogere activiteit.

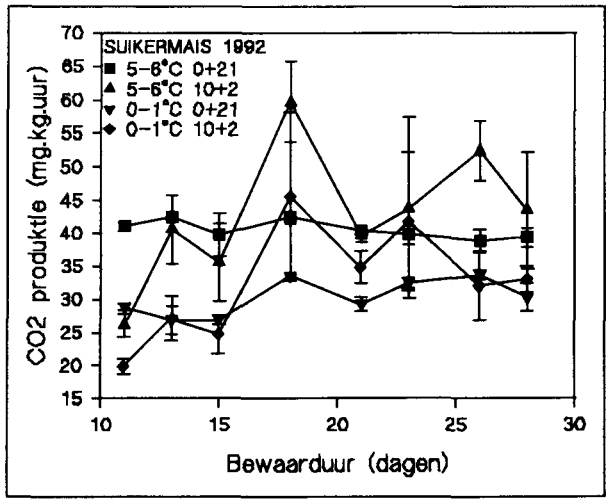

Fig 8: Invloed van temperatuur, CA conditie en bewaarduur op de ademhalingsintensiteit van suikermais.

Discussie

In voorgaand onderzoek (Schouten 1991) werd vastgesteld, dat vooral een verhoging van het koolzuurgehalte gunstig bleek met betrekking tot parasitair bederf en kleur. Er werd evenwel de vraag bij gesteld, of de temperatuur van 0-1°C wel de goede temperatuur is. Uit dit onderzoek blijkt, dat het beste kwaliteitsbehoud steeds wordt gevonden bij de laagste temperatuur. Dit geldt vooral voor de uitwendige (kleur blad, parasitair bederf op bladeren en zaden). Voor de suikers ligt de zaak wat gecompliceerder. Verlies aan sucrose kan met uitzondering van luchtbewaring bij 5-6°C bijna worden verwaarloosd voor alle bewaarcondities. Ook het verschil tussen lucht en CA conditie is opnieuw ten gunste van de laatste. En ander beeld blijkt voor de twee reducerende suikers. Met uitzondering van de luchtbewaring bij de hoogste temperatuur nemen glucose en fructose duidelijk toe. De afbraak van zetmeel in de zaden zorgt voor een zekere hoeveelheid sucrose, dat op zijn beurt weer wordt omgezet in glucose en fructose. Blijkbaar wordt er meer van de reducerende suikers aangemaakt dan er wordt verademd, daar gedurende de gehele bewaarperiode er sprake is van een langzame maar zekere stijging. Dit beeld gaat niet op voor de luchtbewaring bij de hoogste temperatuur. Met betrekking tot de kwaliteit van het produkt zou verdedigd kunnen worden, dat door deze ophoping van reducerende suikers de kwaliteit er op vooruitgaat. Immers een toename in het suikergehalte in het algemeen zal leiden tot een betere smaakwaardering, daar de zoetheid van suikermais een belangrijk sensorisch kwaliteits kenmerk is voor dit produkt. Mogelijk is dit effect gesignaleerd door Spalding et al (1978), die hun verwondering uitspraken over de betere waardering door een panel van CA bewaard produkt. Ondanks een relatief hoog ethanol gehalte werd dit produkt verkozen boven lucht bewaard produkt. Het lijkt waarschijnlijk, dat de toename in de suikers belangrijker is voor de sensorische waardering dan enige ophoping van ethanol.

De ophoping van ethanol bij CA bewaard produkt in dit onderzoek wijst op een minder gewenst effect. Bij extreem lage zuurstof concentraties of extreem hoge koolzuur concentraties gaat het produkt vergisten en wordt er alcohol geproduceerd. Blijkbaar zijn we in dit onderzoek met de combinatie van 10%CO2 + 2%O2 al iets te ver gegaan en heeft er enigermate ethanol ophoping zich voorgedaan. De iets oplopende ademhaling van suikermais bewaard in 10%CO2 + 2%O2 (figuur 9) zou dit beeld kunnen bevestigen.

Spalding et al (1978) toonden aan, dat vooral een sterke verhoging van de koolzuurspanning

ethanol ophoping tot gevolg had. Er deed zich echter ook ethanol ontwikkeling voor in de lucht bewaarde mais. Dit laatste werd in dit onderzoek niet of nauwelijks waargenomen. Mogelijk liggen rasverschillen hieraan ten grondslag.

Samenvattend kan worden gesteld, dat opslag van suikermais in een veranderde luchtsamenstelling voordelen heeft m.b.t. behoud van de uitwendige kwaliteit. Tevens wordt de inwendige kwaliteit bevorderd door het geringe verlies aan sucrose en een duidelijke toename in reducerende suikers. Afbreuk aan de inwendige kwaliteit kan worden veroorzaakt door ophoping van alcohol. CA condities zouden vooral kunnen worden gebruikt voor transport en MAP(Modified Atmosphere Packaging)verpakkingen. Tevens zouden in Nederland CA condities kunnen worden toegepast voor enige verlenging van het seizoen. De condities, waarbij dit dan dient te gebeuren zijn als volgt: temperatuur 0-1°C, ongeveer 10%CO2 en een zuurstof spanning van 2-4%. Met betrekking tot het laatste kan ook een hogere concentratie worden overwogen, daar in onderzoek het verschil tussen koolzuurverhoging bij 21% zuurstof en lage zuurstof concentraties vrij gering bleek (Schouten, 1991).

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Spalding D.H., P.L. Davis and W.F. Reeder Quality of Sweet Corn Stored in Controlled Atmospheres or under Low Pressure. J. Am. Soc. Hort. Sci. 103, 5, 592-595 (1978).

Schouten S.P. CA bewaring suikermais 1991. ATO-DLO Rapport No256, Wageningen The Netherlands (1991).

Bylage II

Not for use in *in vitro* diagnostic procedures for clinical diagnosis

mannheim boehringer **biochemical analysis food analysis**

Ethanol

UV-method

for the determination of ethanol in foodstuffs and other materials

Siemplified procedure for the determination of ethanol in alcoholic beverages see pt. 1.2.

Cat. No. 176 290
Test-Combination for ca. 30 determinations

Recommendations to methods and standardized procedures see references.

Principle (Ref. 1,2)

Ethanol is oxidized to acetaldehyde in the presence of the enzyme alcohol dehydrogenase (ADH) by nicotinamide-adenine dinucleotide (NAD). (1).

(1) Ethanol + NAD^+ $\rightleftharpoons^{ADH}$ acetaldehyde + NADH + H^+

The equilibrium of this reaction lies on the side of ethanol and NAD. It can, however, be completely displaced to the right at alkaline conditions and by trapping of the acetaldehyde formed. Acetaldehyde is oxidized in the presence of aldehyde dehydrogenase (Al-DH) quantitatively to acetic acid (2).

(2) Acetaldehyde + NAD^+ + H_2O $\xrightarrow{Al\text{-}DH}$ acetic acid + NADH + H^+

NADH is determined by means of its absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 100 ml solution, consisting of: potassium diphosphate buffer, pH 9.0; stabilizers.
2. Bottle 2 with approx. 30 tablets, each tablet contains: NAD, approx. 4 mg; aldehyde dehydrogenase, 0.8 U; stabilizers.
3. Bottle 3 with approx. 1.6 ml enzyme suspension, consisting of: ADH, 7000 U; stabilizers.
4. Ethanol standard solution.

Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve **one** tablet of bottle 2 with **3 ml** solution of bottle 1 in a beaker or in a centrifuge tube for each assay (blank or samples) depending on the number of determinations. Use forceps for taking the tablets out of bottle 2. This results in reaction mixture 2*.
3. Use contents of bottle 3 undiluted.

Stability of solutions

Solution 1 is stable for one year at +4°C.
Bring solution 1 to 20–25°C before use.
Reaction mixture 2 is stable for one day at +4°C.
Bring reaction mixture 2 to 20–25°C before use.
Contents of bottle 3 are stable for one year at +4°C.

Procedure

Wavelength[1]: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]: 1 cm light path
Temperature: 20–25°C
Final volume: 3.15 ml
Read against air (without a cuvette in the light path), against water or against blank[3].
Sample solution: 0.5–12 µg ethanol/cuvette[4] (in 0.1–0.5 ml sample volume)

Pipette into cuvettes	blank	sample
reaction mixture 2*	3.00 ml	3.00 ml
redist. water	0.10 ml	–
sample solution**	–	0.10 ml
mix***, after approx. 3 min read absorbances of the solutions (A_1). Start reaction by addition of		
suspension 3	0.05 ml	0.05 ml
mix***, after completion of the reaction (approx. 5–10 min) read absorbances of the solutions immediately one after another (A_2).		

It is absolutely necessary to stopper the cuvettes, e.g. with Parafilm®, during measurement (see "Instructions for performance of assay").

* For simplification of the assay performance it is also possible to pipette directly 3 ml of solution 1 into the cuvette. Afterwards add 1 tablet of bottle 2 and dissolve it *(for solubilisation crush the tablet with a glass rod, if necessary)*. Continue as described in the scheme. The volume error of approx. 1 % (the increase of volume caused by one tablet/3.15 ml final volume) has to be taken into account in the calculation by multiplication of the result with 1.01.
** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution *before dispensing the sample solution.*
*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm® (registered trademark of the American Can Company, Greenwich, Ct., USA).

Determine the absorbance differences (A_2–A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = \Delta A_{sample} - \Delta A_{blank}$$

The absorbance differences measured should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results (see "Instructions for performance of assay").

Calculation

According to the general equation for calculating the concentration in reactions in which the amount of NADH formed is stoichiometric with half the amount of substrate:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 2 \times 1000} \times \Delta A \text{ [g/l], where}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = absorption coefficient of NADH at:
340 nm = 6.3 [l x $mmol^{-1}$ x cm^{-1}]
Hg 365 nm = 3.4 [l x $mmol^{-1}$ x cm^{-1}]
Hg 334 nm = 6.18 [l x $mmol^{-1}$ x cm^{-1}]

It follows for ethanol:

$$c = \frac{3.15 \times 46.07}{\varepsilon \times 1 \times 0.1 \times 2 \times 1000} \times \Delta A = \frac{0.7256}{\varepsilon} \times \Delta A \text{ [g ethanol/l sample solution]}$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{content}_{ethanol} = \frac{c_{ethanol} \text{ [g/l sample solution]}}{c_{sample} \text{ [g/l sample solution]}} \times 100 \text{ [g/100 g]}$$

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; when spectralline photometers equipped with a mercury vapour lamp are used, measurements are taken at a wavelength of 365 nm **or** 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 For example, when using a double-beam photometer.
4 See instructions for performance of the assay.

Instructions for performance of assay

The amount of ethanol present in the cuvette should range between 1 µg and 12 µg (measurement at 365 nm) or 0.5 µg and 6 µg (measurement at 340, 334 nm), respectively. The sample solution must therefore be diluted sufficiently to yield an ethanol concentration between 0.01 and 0.12 g/l or 0.005 and 0.06 g/l, respectively.

Because of the high sensitivity of the method it has to be taken care that ethanol free water is used and it is worked in an ethanol free atmosphere.

Dilution table

estimated amount of ethanol per liter measurements at 340 or 334 nm	365 nm	dilution with water	dilution factor F
< 0.06 g	< 0.12 g	-	1
0.06–0.6 g	0.12–1.2 g	1 + 9	10
0.6 –6.0 g	1.2 –12 g	1 + 99	100
6.0 –60 g	12 –120 g	1 + 999	1000
> 60 g	> 120 g	1 + 9999	10000

Because of the volatility of ethanol, the dilution of samples should be carried out as follows:

Fill the volumetric flask half with water and pipette the sample with an enzyme test pipette or a piston type pipette under the surface of the water. Fill up to the mark with water and mix.

If the absorbance difference measured (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared anew (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 0.5 ml. The volume of solution 1 or reaction mixture 2, respectively, remains the same (3.00 ml). The volume of water pipetted into the blank cuvette must then be increased so as to obtain the same final volume for the sample and blank in the cuvettes. The new sample volume v and the new final volume (V) must be taken into account in the calculation.

1. Instructions for sample preparation

1.1. Liquid foodstuffs

Use clear, colorless or slightly colored solutions directly or after dilution according to the dilution table for the assay. Filter turbid solutions or clarify with Carrez reagents. Strongly colored solutions, which are used undiluted for the assay because of their low ethanol concentration, are to be decolorized with polyamide or polyvinylpolypyrrolidone (PVPP). Carbonic acid containing beverages are to be degassed, beverages with low ethanol content should be adjusted to the alkaline pH range. During the whole procedure it is to be taken care that the ethanol is not evaporated. For example, when diluting an ethanol containing sample, it is to be *pipetted under the surface of the water.*

Examples:

Determination of ethanol in fruit juices

a) Use clear light juices after neutralization or dilution, depending on the ethanol content, for the assay (see dilution table).

b) Decolorize intensely colored juices by addition of 2% polyamide or polyvinylpolypyrrolidone (PVPP) (e.g. 5 ml juice + 100 mg polyamide or PVPP), stir for 2 min (vessel must be stoppered) and filter. Use the mostly clear solution after neutralization for the assay. Decolorization can often be omitted on dilution.

c) Filter turbid juices and clarify with Carrez-solutions, if necessary: Pipette 10 ml of juice into a 25 ml volumetric flask, add 1.25 ml Carrez-I-solution (3.60 g potassium hexacyanoferrate-II, K_4 [$Fe(CN_6)$] · 3 H_2O/100 ml), 1.25 ml Carrez-II-solution (7.20 g zinc sulfate, $ZnSO_4$ · 7 H_2O/100 ml) and 2.50 ml NaOH (0.1 mol/l), shake vigorously after each addition, dilute to 25 ml with water, filter (dilution factor F = 2.5). Use the clear sample solution, which may be weakly opalescent, for the assay directly or diluted, if necessary.

Determination of ethanol in alcohol-deficient and alcohol-free beer

Add solid potassium hydroxide or solid sodium hydroxide to approx. 100 ml sample in a beaker while stirring carefully until a pH value of approx. pH 8–9 is obtained. Use solution, diluted according to the dilution table, if necessary, for the assay.

Determination of ethanol in vinegar

Filter, if necessary and neutralize vinegar. Neutralization can be omitted on dilution.

Determination of ethanol in alcoholic beverages

a) *Wine* (Ref. 14): Dilute wine with redist. water to the appropriate concentration (see dilution table). Decolorization and neutralization are not necessary.

b) *Beer:* To remove carbonic acid, stir approx. 5–10 ml of beer in a beaker for approx. 30 s using a glass rod or filter. Dilute the sample 1 : 1000 (1 + 999) with water and use the diluted sample solution for the assay.

c) *Liqueur:* Pipette liquid liqueurs for dilution into an appropriate volumetric flask and fill up with water to the mark. Weigh approx. 1 g of viscous liqueurs (e.g. egg liqueur) accurately into a 100 ml volumetric flask, fill up to the mark with redist. water, keep it in a refrigerator for separation of fat, and filter. Dilute the clear solution 1 : 100 (1 + 99) with water and use it for the assay.

d) *Brandy:* Take care as mentioned for taking the sample of alcoholic beverages and dilute to a certain concentration (e.g. 1 + 999). Convert the measured values (g ethanol/l solution) into volume percentage (v/v) with the aid of conversion tables.

1.2. Simplified determination of ethanol in beer, wine (Ref. 14) and brandy

Sample preparation

Dilute beer, wine and brandy according to the dilution table.

Reagent solution for 10 determinations

Dissolve 10 tablets of bottle 2 with 30 ml solution from bottle 1, add 0.5 ml suspension from bottle 3, and mix.
(Attention: Prepare reagent solution with alcohol-free water in alcohol-free atmosphere. Store in a container tightly stoppered.)

Stability

The reagent solution is stable for 8 h at 20°C.

Procedure

Pipette 3.00 ml reagent solution into the cuvette and read absorbance A_1. Start reaction by addition of 0.1 ml diluted sample. On completion of the reaction (approx. 5 min) read absorbance A_2. Determine absorbance difference of $A_2 - A_1 = \Delta A$.

Calculation

$$c = \frac{0.714}{\varepsilon} \times \Delta A \times F \text{ [g ethanol/l sample]}$$

F = dilution factor

1.3. Pasty foodstuffs

Homogenize semi-solid samples, extract with water or dissolve, respectively, and filter, if necessary. Clarify with Carrez-solutions or decolorize.

Examples:

Determination of ethanol in chocolates, sweets and other alcohol-containing chocolate products

Chocolates with liquid filling compound (brandy balls, brandy cherries):

Open, e.g., one brandy ball carefully, pipette 0.50 ml of the liquid filling into a 50 ml volumetric flask filled with approx. 25 ml water, taking care that the tip of the pipette dips into the water. Fill up to the mark with water, stopper and mix. Dilute the solution with water in a ratio of 1 : 20 (1 + 19). Use 0.1 ml of the diluted solution for the assay (dilution factor F = 2000).

Chocolate products with highly viscous filling:

Weigh accurately the filling of one or several sweets or chocolates into a 50 ml volumetric flask filled with approx. 5 ml water (when the sample is weighed by means of a pipette, the tip of the pipette must **not** touch the water surface). Fill up to the mark with water, mix, filter, if necessary, and dilute until the alcohol content of the sample is less than 0.12 g/l.

Determination of ethanol in jam

Homogenize sample thoroughly (mixer, etc.) and weigh approx. 10–20 g into a beaker. Add some water, mix and neutralize the mixture with KOH, if necessary. Transfer the mixture quantitatively into a 100 ml volumetric flask and fill up to the mark with redist. water.
Decolorize solution with 2% polyamide or PVPP, if necessary (see "Instructions 1.1.b") and filter. Use the filtrate for the assay undiluted.

Determination of ethanol in honey

Weigh approx. 20 g honey accurately into a 100 ml volumetric flask and dissolve with some water under slightly agitation at approx. 50°C (ascending tube!), cool to room temperature and fill up to the mark with redist. water. Use the solution for the assay, clarify with Carrez-solutions (see „Instructions 1.1.c"), if necessary (dilution factor F = 2.5). Use the clear solution for the assay after filtration.

Determination of ethanol in dairy products (e.g. curds, kefir)

Weigh approx. 10 g of the homogenized sample accurately into a 100 ml volumetric flask, add approx. 50 ml water and keep the flask (ascending tube!) at 50°C for 15 min under slightly agitation. For protein precipitation add 5 ml Carrez-I-solution, 5 ml Carrez-II-solution and 10 ml NaOH (0.1 mol/l) (see "Instructions 1.1.c"), shake vigorously after each addition. Allow to cool to room temperature and fill up to the mark with water. Mix and filter. Use the clear, possibly slightly turbid solution for the assay.

1.4. Solid foodstuffs

Homogenize solid or semi-solid samples (using a mortar, etc.), extract with water or dissolve; filter, if necessary.

Extract fat-containing samples with warm water (approx. 50°C) in a small flask with ascending tube. Allow to cool for separation of fat, rinse the ascending tube with water and filter.

Deproteinize protein-containing sample solutions with perchloric acid (1 mol/l) in a ratio of 1:3 (1+2) and centrifuge. Neutralize with KOH (2 mol/l).

2. Specificity

The influence of aldehydes and ketones is eliminated by the order of reagent addition during the assay. Methanol is not converted because of the unfavourable K_m-values of the used enzymes.
n-Propanol is quantitatively converted under assay conditions, higher primary alcohols lead to sample dependent creep reactions. Secondary, tertiary and aromatic alcohols do not react. Even higher concentrations of glycerol do not disturb the assay.

3. Sources of error

The presence of ethanol in the used redist. water or in air results in increased blanks or in creep reactions, respectively. Therefore it is necessary to cover the cuvette during the assay.

Detection of interferences of the test system

When the enzymatic reaction is complete after the time given in "Procedure" it can be concluded in general that the reaction is not interfered. For assurance of results a re-start of the reaction (qualitatively or quantitatively) by the addition of 'standard material' can be done: a further change of absorbance proves suitability of measurements.

For the detection of gross errors when performing the assays and of interfering substances in the sample material it is recommended to analyze a sample solution in a double determination with two different sample volumes (e.g. 0.10 ml and 0.20 ml): the measured absorbance differences have to be proportional to the sample volumes.

When analyzing solid samples it is recommended to weigh in two different amounts (e.g. 1 g and 2 g) into 100 ml volumetric flasks and to perform the determinations with the same sample volume: the absorbance differences have to be proportional to the amounts weighed in.

4. Further applications (s. References)

The method may also be used in the examination of cosmetics, pharmaceuticals, and in research when analyzing biological samples.

For details of sampling, treatment and stability of the sample see Bernt, E. & Gutmann, I. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd ed. vol. 3, p. 1500, Verlag Chemie, Weinheim, Academic Press, Inc. New York and London.

Examples:

4.1. Determination of ethanol in blood, plasma or serum, respectively (Ref. 2)

Mix 0.5 ml blood with 4.0 ml ice-cold perchloric acid (0.33 mol/l) and centrifuge. Use 0.1 ml for the assay.

The dilution factor F (depending on sample preparation) is obtained from the sample volume (0.5 ml), the perchloric acid volume (4.0 ml), the specific gravity of the sample material (1.06 g/ml blood, 1.03 g/ml plasma or serum) and the fluid content (0.80 in case of blood and 0.92 in case of plasma or serum):

$$F_{blood} = \frac{0.5 \times 1.06 \times 0.80 + 4.0}{0.5} = 8.85$$

$$F_{plasma,\ serum} = \frac{0.5 \times 1.03 \times 0.92 + 4.0}{0.5} = 8.95$$

Calculation:

$$c = \frac{0.7256 \times \Delta A \times F}{\varepsilon}\ \text{[g ethanol/l sample]}$$

$$c = \frac{15.75 \times \Delta A \times F}{\varepsilon}\ \text{[mmol ethanol/l sample]}$$

Ethanol in blood:

Wavelength	Hg 365 nm	340 nm	Hg 334 nm
c [g/l]	1.889 x ΔA	1.019 x ΔA	1.039 x ΔA
c [mmol/l]	41.00 x ΔA	22.13 x ΔA	22.55 x ΔA

Ethanol in plasma or serum, respectively:

Wavelength	Hg 365 nm	340 nm	Hg 334 nm
c [g/l]	1.910 x ΔA	1.031 x ΔA	1.051 x ΔA
c [mmol/l]	41.46 x ΔA	22.38 x ΔA	22.81 x ΔA

4.2. Determination of ethanol in urine (Ref. 12)

Dilute urine with bidest. water according to the dilution table. Use the diluted sample for the assay (dilution factor = F).

Calculation:

$$c = \frac{0.7256 \times \Delta A \times F}{\varepsilon}\ \text{[g ethanol/l sample]}$$

$$c = \frac{15.75 \times \Delta A \times F}{\varepsilon}\ \text{[mmol ethanol/l sample]}$$

Wavelength	Hg 365 nm	340 nm	Hg 334 nm
c [g/l]	0.2134 x ΔA x F	0.1152 x ΔA x F	0.1174 x ΔA x F
c [mmol/l]	4.632 x ΔA x F	2.500 x ΔA x F	2.549 x ΔA x F

4.3. Determination of ethanol in fermentation samples and cell culture media

Place the sample (after centrifugation, if necessary) into a water-bath at 80°C for 15 min (cover the tube because of the volatility of ethanol) to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with perchloric acid or with Carrez-solutions. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

5. Technical instructions

1. Ethanol is very volatile. Therefore it is necessary to be very careful when handling ethanol containing samples, diluting samples and pipetting sample solutions into the assay system.

When filtering solutions the filtrate should not drop into the container but rinse down the wall.

When dispensing ethanol containing solutions, always pipette these solutions under the surface of water (when diluting) or of buffer (when performing the assay).

2. When pipetting highly diluted sample solutions into the assay system, rinse measuring glass pipet (enzyme test pipet) at least 5 times. The tip of the piston type pipet should be rinsed 3 times.

3. Do not use the same piston type pipet for diluting the sample and pipetting the sample solution into the assay system.

4. Always work in alcohol-free atmosphere with ethanol-free water.

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Ethanol standard solution

for the Test-Combination Ethanol
UV-method, Cat. No. 176 290

Concentration*: see bottle label.
Ethanol standard solution is a stabilized aqueous solution of ethanol. It serves as standard solution for the enzymatic analysis of ethanol in foodstuffs and other materials.

Application

1. Addition of ethanol standard solution to the assay mixture:
Instead of sample solution the standard solution is used for the assay.

2. Restart of the reaction, quantitatively:
After completion of the reaction with sample solution and measuring of A_2, add 0.05 ml standard solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 5 min). Calculate the concentration from the difference of (A_3-A_2) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by addition of the standard solution, the result differs insignificantly from the data stated on the bottle label.

3. Internal standard
The standard solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	blank	sample	standard	sample + standard
reaction mixture 2	3.00 ml	3.00 ml	3.00 ml	3.00 ml
redist. water	0.10 ml	–	–	–
sample solution	–	0.10 ml	–	0.05 ml
standard solution	–	–	0.10 ml	0.05 ml
mix, and read absorbances of the solutions (A_1) after approx. 3 min. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.				

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$

BOEHRINGER MANNHEIM GMBH
Biochemica

Sucrose/D-Glucose/ D-Fructose

UV-method

for the determination of sucrose, D-glucose and D-fructose in foodstuffs and other materials

Cat. No. 716 260
Test-Combination for ca. 20 tests, each

Recommendations to methods and standardized procedures see references.

Principle (Ref. 1)

The D-glucose concentration is determined before and after the enzymatic hydrolysis of sucrose; D-fructose is determined subsequently to the determination of D-glucose.

Determination of D-glucose before inversion:

At pH 7.6, the enzyme hexokinase (HK) catalyzes the phosphorylation of D-glucose by adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1). In the presence of glucose-6-phosphate dehydrogenase (G6P-DH) the glucose-6-phosphate (G-6-P) formed is specifically oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).

(1) D-Glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP

(2) G-6-P + $NADP^+$ $\xrightarrow{G6P\text{-}DH}$ gluconate-6-phosphate + NADPH + H^+

The NADPH formed in this reaction is stoichiometric with the amount of D-glucose and is measured by means of its absorbance at 334, 340 or 365 nm.

Determination of D-fructose:

Hexokinase also catalyzes the phosphorylation of D-fructose to fructose-6-phosphate (F-6-P) (3) with the aid of ATP.

(3) D-fructose + ATP $\xrightarrow{HK}$ F-6-P + ADP

On completion of the reaction (3) F-6-P is converted by phosphoglucose isomerase (PGI) to G-6-P (4).

(4) F-6-P $\xrightarrow{PGI}$ G-6-P

G-6-P reacts again with NADP under formation of gluconate-6-phosphate and NADPH (2). The amount of NADPH formed now is stoichiometric with the amount of D-fructose.

Enzymatic inversion:

At pH 4.6, sucrose is hydrolyzed by the enzyme β-fructosidase (invertase) to D-glucose and D-fructose (5).

(5) Sucrose + H_2O $\xrightarrow{\beta\text{-fructosidase}}$ D-glucose + D-fructose

The determination of D-glucose after inversion (total D-glucose) is carried out according to the principle outlined above.
The sucrose content is calculated from the difference of the D-glucose concentrations before and after enzymatic inversion.

The Test-Combination contains

1. Bottle 1 with approx. 0.5 g lyophilisate, consisting of: citrate buffer, pH 4.6; β-fructosidase, 720 U; stabilizers.
2. Bottle 2 with approx. 7.2 g powder mixture, consisting of: triethanolamine buffer, pH 7.6; NADP, 110 mg; ATP, 260 mg; magnesium sulfate; stabilizers.
3. Bottle 3 with 1.1 ml enzyme suspension, consisting of: hexokinase, 320 U; glucose-6-phosphate dehydrogenase, 160 U.
4. Bottle 4 with approx. 0.6 ml phosphoglucose isomerase suspension, 420 U.
5. Sucrose standard material.

Preparation of solutions

1. Dissolve contents of bottle 1 with 10 ml redist. water.
2. Dissolve contents of bottle 2 with 45 ml redist. water.
3. Use contents of bottle 3 undiluted.
4. Use contents of bottle 4 undiluted.

Stability of solutions

Solution 1 and solution 2 are stable for 4 weeks at +4°C, or for 2 months at −20°C.
Bring solutions 1 and 2 to 20–25°C before use.
Contents of bottle 3 and bottle 4 are stable for 1 year at +4°C.

Procedure

Wavelength[1]: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]: 1 cm light path
Temperature: 20–25°C
Final volume: 3.02 ml (3.04 ml, determination of D-fructose)
Read against air (without a cuvette in the light path) or against water.
Sample solution: 5–150 µg sucrose, D-glucose and D-fructose/cuvette[3] (in 0.1–2.0 ml sample volume).

Pipette into cuvettes	blank D-glucose/ D-fructose sample	D-glucose D-fructose sample	blank sucrose sample	sucrose sample
solution 1*	–	–	0.20 ml	0.20 ml
sample solution**	–	0.10 ml	–	0.10 ml
mix*, incubate for 15 min at 20–25°C or for 5 min at 37°C (before pipetting, warm solution 1 to 37°C). Addition of				
solution 2	1.00 ml	1.00 ml	1.00 ml	1.00 ml
redist. water	2.00 ml	1.90 ml	1.80 ml	1.70 ml
mix***, read absorbances of the solutions after approx. 3 min (A_1). Start reaction by addition of				
suspension 3	0.02 ml	0.02 ml	0.02 ml	0.02 ml
mix***, wait for completion of the reaction (approx. 10–15 min) and read absorbances of the solutions (A_2). If the reaction has not stopped after 15 min, continue to read the absorbances at 5 min intervals until the absorbance increases constantly over 5 min. Addition of				
suspension 4	0.02 ml	0.02 ml	–	–
mix, read absorbances of the solutions after 10–15 min (A_3).				

* Pipette solution 1 and sample solution each, onto the bottom of the cuvette and mix by gentle swirling. When using a plastic spatula, remove it from the cuvette only directly before measuring absorbance A_1.
** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.
*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm® (registered trademark of the American Can Company, Greenwich, Ct., USA).

If the absorbance A_2 increases constantly, extrapolate the absorbances A_2 to the time of the addition of suspension 3.

Determine the absorbance differences (A_2–A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = \Delta A_{sample} - \Delta A_{blank}$$

The difference $\Delta A_{total\ D\text{-}glucose}$ (from the sucrose sample) and $\Delta A_{D\text{-}glucose}$ (from the D-glucose sample) yields $\Delta A_{sucrose}$.

It follows for the determination of D-fructose:
Determine the absorbance differences (A_3–A_2) for both, blank and sample (D-glucose/D-fructose sample). Subtract the absorbance difference of the blank from the absorbance difference of the sample. This results in $\Delta A_{D\text{-}fructose}$.

The absorbance differences measured should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results (see "Instructions for performance of assay").

1 The absorption maximum of NADPH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; when spectralline photometers equipped with a mercury vapour lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 See instructions for performance of the assay.
4 Available from Boehringer Mannheim GmbH.

Calculation

According to the general equation for calculating the concentrations:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l], where}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = absorption coefficient of NADPH at
340 nm = 6.3 [l x mmol^{-1} x cm^{-1}]
Hg 365 nm = 3.5 [l x mmol^{-1} x cm^{-1}]
Hg 334 nm = 6.18 [l x mmol^{-1} x cm^{-1}]

It follows for

sucrose:

$$c = \frac{3.02 \times 342.30}{\varepsilon \times 1 \times 0.1 \times 1000} \times \Delta A_{sucrose} = \frac{10.34}{\varepsilon} \times \Delta A_{sucrose} \text{ [g sucrose/l sample solution]}$$

D-glucose:

$$c = \frac{3.02 \times 180.16}{\varepsilon \times 1 \times 0.1 \times 1000} \times \Delta A_{D\text{-glucose}} = \frac{5.441}{\varepsilon} \times \Delta A_{D\text{-glucose}} \text{ [g D-glucose/l sample solution]}$$

D-fructose:

$$c = \frac{3.04 \times 180.16}{\varepsilon \times 1 \times 0.1 \times 1000} \times \Delta A_{D\text{-fructose}} = \frac{5.477}{\varepsilon} \times \Delta A_{D\text{-fructose}} \text{ [g D-fructose/l sample solution]}$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$content_{sucrose} = \frac{c_{sucrose} \text{ [g/l sample solution]}}{c_{sample} \text{ [g/l sample solution]}} \times 100 \text{ [g/100 g]}$$

$$content_{D\text{-glucose}} = \frac{c_{D\text{-glucose}} \text{ [g/l sample solution]}}{c_{sample} \text{ [g/l sample solution]}} \times 100 \text{ [g/100 g]}$$

$$content_{D\text{-fructose}} = \frac{c_{D\text{-fructose}} \text{ [g/l sample solution]}}{c_{sample} \text{ [g/l sample solution]}} \times 100 \text{ [g/100 g]}$$

Instructions for performance of assay

The amount of sucrose, D-glucose and D-fructose present in the cuvette should range between 10 µg and 150 µg (measurement at 365 nm) or 5 µg and 80 µg (measurement at 340, 334 nm), respectively. The sample solution must therefore be diluted sufficiently to yield a sugar concentration between 0.10 and 1.5 g/l or 0.05 and 0.8 g/l, respectively.

Dilution table

estimated amount of sucrose, D-glucose and D-fructose per liter measurements at		dilution with water	dilution factor F
340 or 334 nm	365 nm		
< 0.8 g	< 1.5 g	–	1
0.8–8.0 g	1.5–15.0 g	1 + 9	10
8.0–80 g	15.0–150 g	1 + 99	100
> 80 g	> 150 g	1 + 999	1000

If the absorbance difference measured (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared anew (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 2.0 ml (D-glucose and D-fructose sample), or up to 1.8 ml (sucrose sample). The volume of water added must then be reduced so as to obtain the same final volume for the sample and blank in the cuvettes. The new sample volume v must be taken into account in the calculation.

If the amount of sucrose estimated is below 0.2 g/l, the incubation time stated in the assay scheme, when sucrose is splitted by β-fructosidase, may be reduced from 15 min to 5 min.

If the D-glucose : sucrose (D-glucose : D-fructose) ratio is higher than 10 : 1, the excess of D-glucose must first be destroyed by means of GOD/catalase so that the concentration of sucrose (D-fructose) may be accurately determined. Proceed as described under "honey", see 1.3.

1. Instructions for sample preparation

1.1. Liquid foodstuffs

Use clear, colorless or slightly colored solutions directly or after dilution according to the dilution table for the assay. Filter turbid solutions or clarify with Carrez reagents. Strongly colored solutions which are used undiluted for the assay because of their low sucrose, D-glucose and D-fructose concentration are to be decolorized with polyamide or polyvinylpolypyrrolidone (PVPP). Carbonic acid containing beverages are to be degassed.

Examples:

Determination of sucrose, D-glucose and D-fructose in fruit juices and similar beverages

Filter turbid juices (alternatively clarify with Carrez reagents – s. below) and dilute sufficiently to yield a sucrose, D-glucose and D-fructose concentration of approx. 0.1–1.5 g/l. The diluted sample solution can also be used for the assay if it is colored. Only strongly colored juices which are used undiluted for the assay because of their low sucrose, D-glucose and D-fructose content are to be decolorized. In that case proceed as follows:

Mix 10 ml of juice and approx. 0.1 g of polyamide powder or polyvinylpolypyrrolidone, stir for 1 min and filter. Use the clear, slightly colored solution for the assay.

Determination of sucrose, D-glucose and D-fructose in beer

To remove the carbonic acid, stir approx. 5–10 ml of beer in a beaker for approx. 30 s with a glass rod or filter through a folded filter. The largely CO_2-free sample can be used undiluted for the assay.

Determination of sucrose, D-glucose and D-fructose in sweetened condensed milk

Weigh approx. 1 g of sample accurately into a 100 ml volumetric flask, add approx. 60 ml water and incubate for 15 min at approx. 70°C; shake from time to time. For protein precipitation, add 5 ml Carrez-I-solution (3.60 g potassium hexacyanoferrate-II, $K_4[Fe(CN)_6] \cdot 3\ H_2O$/100 ml), 5 ml Carrez-II-solution (7.20 g zinc sulfate, $ZnSO_4 \cdot 7\ H_2O$/100 ml) and 10 ml NaOH (0.1 mol/l), shake vigorously after each addition, adjust to room temperature and fill up to the mark with water, mix and filter. Use the clear, possibly slightly opalescent solution diluted according to the dilution table for the assay.

1.2. Solid foodstuffs

Mince solid and semi-solid foodstuffs (e.g. bread and pastries, fruit, vegetable, meat and milk products) in an electric mixer, meat grinder or mortar. Weigh out the well mixed sample and extract with water (heated to 60°C, if necessary). Transfer quantitatively into a volumetric flask and fill up to the mark with water. Filter, and use the clear solution, diluted, if necessary, for the assay.

Examples:

Determination of sucrose, D-glucose and D-fructose in potatoes

Homogenize 50 g peeled potatoes with 50 ml water in a homogenizer for 3 min. Transfer quantitatively into a 250 ml beaker. Fill up to approx. 150 ml with water. Add successively 5 ml Carrez-I-solution (preparation see 1.1) and 5 ml Carrez-II-solution (preparation see 1.1) shake vigorously after each addition. Adjust to pH 7.0 to 7.5 (pH meter) with sodium hydroxide (0.1 mol/l). Transfer quantitatively into a 250 ml volumetric flask, rinse with water, add 0.3 ml n-octanol and shake until the foam has disappeared. Fill up to 250 ml with water, mix and filter.

Use the light yellow, occasionally yellow – green solution with v = 0.1 ml or 0.2 ml, if necessary, immediately for the assay.

Determination of sucrose, D-glucose and D-fructose in tobacco (Ref. 8)

Weigh approx. 0.3 g dried, finely ground and sieved tobacco leaves (grain size approx. 0.2 mm) accurately into a 100 ml volumetric flask, add approx. 70 ml water and stir for 1 h (magnetic stirrer). Fill up to the mark with water, mix and filter. In a 25 ml volumetric flask add successively 1.25 ml Carrez-I-solution and 1.25 ml Carrez-II-solution (preparation see 1.1) to 10 ml of the filtrate, shake, and subsequently add 2.5 ml sodium hydroxide (0.1 mol/l) and shake again. Fill up to the mark with water, mix and filter.

Use the clear solution diluted, if necessary, for the assay.

1.3. Pasty products

Homogenize semi-solid samples, extract with water or dissolve, respectively, filter, if necessary, clarify with Carrez reagents or decolorize.

Examples:

Determination of sucrose, D-glucose and D-fructose in jam and ice-cream

Homogenize approx. 10 g sample in a mixer. Weigh approx. 0.5 g of the homogenized sample accurately into a 100 ml volumetric flask, mix with water, dilute to the mark and filter. Discard the first 5 ml of the

filtrate. Use the clear filtrate diluted according to the dilution table, if necessary, for the assay.

Determination of sucrose, D-glucose and D-fructose in honey
Thoroughly stir the honey with a spatula. Take approx. 10 g of the viscous (or crystalline) honey, heat in a beaker for 15 min at approx. 60°C, and stir occasionally with a spatula (there is no need to heat liquid honey). Allow to cool. Weigh approx. 1 g of the liquid sample accurately into a 100 ml volumetric flask. Dissolve first with only a small portion of water, and then fill up to the mark.

a) *Determination of D-glucose and D-fructose*
Dilute the 1% honey solution 1 : 10 (1 + 9) and use for the assay.

b) *Determination of sucrose*
If the estimated sucrose content in the honey lies between 5 and 10%, dilute the 1% solution 1 : 3 (1 + 2) and use for the assay.
If the estimated sucrose content in the honey lies between 0.5 and 5%, the excess of D-glucose should be removed as much as possible before sucrose is determined. D-glucose is oxidized to D-gluconate in the presence of the enzymes glucose oxidase (GOD) and catalase.

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{GOD} \text{D-gluconate} + H_2O_2$$

The hydrogen peroxide is destroyed by catalase:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

Reagents
Glucose oxidase (GOD), Cat. No. 105 139[4]
Catalase, Cat. No. 106 810[4]
Triethanolamine hydrochloride, Cat. No. 127426[4]
$MgSO_4 \cdot 7\ H_2O$
NaOH, 4 mol/l

Preparation of solutions for 10 determinations
Enzyme solution:
Dissolve 5 mg (≙ approx. 1250 U) GOD with 0.75 ml redist. water, add 0.25 ml suspension catalase, and mix.

Buffer solution:
Dissolve 5.6 g triethanolamine hydrochloride and 0.1 g $MgSO_4 \cdot 7\ H_2O$ in 80 ml redist. water, adjust to pH 7.6 with sodium hydroxide (4 mol/l), and fill up with redist. water to 100 ml.

Stability of solutions
The enzyme solution must be prepared fresh daily.
The buffer solution is stable for 4 weeks at 4°C.

Procedure for D-glucose oxidation

Pipette into 10 ml volumetric flask	
buffer solution	2.0 ml
sample solution (up to approx. 0.5% D-glucose)	5.0 ml
enzyme solution	0.1 ml
Pass a current of air (O_2) through the mixture for 1 h; during the oxidation process **check the pH with indicator paper and, if necessary, neutralize the acid formed with NaOH.**	

To inactivate the enzymes GOD and catalase, keep the volumetric flask in a boiling water-bath for 15 min, allow to cool, and dilute to the mark with water. Mix and filter, if necessary. Use 0.5 ml of the clear solution for the determination of sucrose. Determine the residual D-glucose in a parallel assay and subtract as usual.

Further information see instructions of

Test-Combination D-Glucose	**Cat. No. 716 251**
Test-Combination D-Glucose/D-Fructose	**Cat. No. 139 106**
Test-Combination D-Glucose/D-Fructose/Sorbitol	**Cat. No. 724 831**
Test-Combination Sucrose/D-Glucose	**Cat. No. 139 041**
Test-Combination Sorbitol/Xylitol	**Cat. No. 670 057**
Test-Combination Starch	**Cat. No. 207 748**

2. Specificity

β-Fructosidase hydrolyzes the β-fructosidic bonds in sucrose and other glycosides. If the sample only contains sucrose it will be measured specifically via D-glucose. Even in the presence of fructosanes, sucrose can be measured specifically if after enzymatic hydrolysis with β-fructosidase, D-glucose and D-fructose are determined and the ratio of these monosaccharides is 1:1. If the D-fructose part dominates the sample contains 2 β-fructosanes.
The measuring of the D-glucose and D-fructose is specific.

3. Further applications

The method may also be used in the examination of pharmaceuticals, paper (Ref. 4) and in research when analyzing biological samples.

Determination of sucrose, D-glucose and D-fructose in fermentation samples and cell culture media
Place the sample (after centrifugation, if necessary) into a water-bath at 80°C for 15 min to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with Carrez-solutions. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

4. Detection of interferences of the test system

When the enzymatic reaction is complete after the time given in "Procedure" it can be concluded in general that the reaction is not interfered. For assurance of results a re-start of the reaction (qualitatively or quantitatively) by the addition of 'standard material' (e.g. D-glucose or D-fructose) can be done: a further change of absorbance proves suitability of measurements.

For the detection of gross errors when performing the assays and of interfering substances in the sample material it is recommended to analyze a sample solution in a double determination with two different sample volumes (e.g. 0.10 ml and 0.20 ml): the measured absorbance differences have to be proportional to the sample volumes.

When analyzing solid samples it is recommended to weigh in two different amounts (e.g. 1g and 2g) into 100 ml volumetric flasks and to perform the determinations with the same sample volume: the absorbance differences have to be proportional to the amounts weighed in.

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Note

for Test-Combination Sucrose/D-Glucose/ D-Fructose

Sucrose is supplied in this pack as reference material. It may be used for the preparation of a standard solution (concentration e.g. 1 g/l) which is pipetted (v = 0.1 ml) instead of the sample according to the pipetting scheme.

Furthermore, the sucrose may also be used for performing the so-called Swiss Sucrose Test in order to check performance of the test.

The Swiss Sucrose Test
A standard solution is prepared and the concentration is measured enzymatically. The results are used for the evaluation of accuracy and precision.

Reagents
Prepare solutions according to the instructions in the Test-Combination.

Sample solution (standard solution)
Weigh 1.6 g of sucrose (accuracy 0.1 mg) and dissolve with redist. water in a 1 liter volumetric flask, fill up to the mark and mix thoroughly.

Procedure
For details of performing the test and calculating the results see instructions in the Test-Combination.

Run 2 blanks and 6 samples.

Pipetting scheme

pipette into cuvettes	blanks		samples					
	blank 1	blank 2	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6
solution 1*	0.20 ml	0.20 ml	0.20 ml	0.20 ml	0.20 ml	0.20 ml	0.20 ml	0.20 ml
sample solution*	–	–	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml
mix**, incubate for 15 min at 20–25°C. Mix in								
solution 2	1.00 ml	1.00 ml	1.00 ml	1.00 ml	1.00 ml	1.00 ml	1.00 ml	1.00 ml
redist. water	1.80 ml	1.80 ml	1.70 ml	1.70 ml	1.70 ml	1.70 ml	1.70 ml	1.70 ml
mix, read absorbances of the solutions after approx. 3 min (A_1). Start reaction by addition of								
suspension 3	0.02 ml	0.02 ml	0.02 ml	0.02 ml	0.02 ml	0.02 ml	0.02 ml	0.02 ml
mix, incubate for 15 min at 20–25°C. Read the absorbances of the solutions (A_2).								

* Pipette the solutions onto the bottom of the cuvettes.
** Mix by gentle shaking the cuvettes. If a mixing spatula is used, remove the spatula from the cuvette before reading A_1, not earlier.

Readings

A_1:							
A_2:							
$A_2 - A_1$:							
Mean of the blanks:							
$(A_2 - A_1)_{sample}$ – mean of the blanks = ΔA:							

Calculation
Calculate the absorbance differences (A_2–A_1) for each blank and samples. Subtract the mean absorbance difference of the blanks from the absorbance differences of the samples. It follows:

$\Delta A_{sample\ 1, 2, \ldots, 6}$

Calculate the concentration of sucrose in g/l:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A_{sample\ 1, 2, \ldots, 6}$$

$$c = \frac{3.02 \times 342.3}{\varepsilon \times 1 \times 0.1 \times 1000} \times \Delta A_{sample\ 1, 2, \ldots, 6}$$

$$c = \frac{10.34}{\varepsilon} \times \Delta A_{sample\ 1, 2, \ldots, 6}$$

$c_{sucrose}$ (g/l):							

From the 6 results c_1, c_2, ... , c_6 calculate the mean $\bar{c}$ and the standard deviation s_c.

mean $c_{sucrose}$ ($\bar{c}$): [illegible] g/l

standard deviation s_c: [illegible] g/l

Calculation of the mean yield $\bar{Y}$ and its standard deviation s_Y:

$$\bar{Y} = \frac{\bar{c}\ [g/l] \times 100}{\text{weighed sucrose } [g/l]} = \frac{[illegible] \times 100}{[illegible]} = [illegible]\ g/100\ g$$

$$s_Y = \frac{s_c\ [g/l] \times 100}{\text{weighed sucrose } [g/l]} = \frac{[illegible] \times 100}{[illegible]} = [illegible]\ g/100\ g$$

Evaluation of the standard deviation
Standard deviation $s_Y \leqq 0.79$ g/100 g:
The precision of the determination is perfect.

Standard deviation $s_Y > 0.79$ g/100 g:
The standard deviation is too high. This may result either from the use of unsuitable equipment (balance, photometer, cuvettes, pipettes) or from their wrong handling. Something should be done to overcome these difficulties (e. g. control of balance, photometer, cuvettes and pipettes).

Evaluation of yield
Deviation of the mean yield ($\bar{Y}$) from the theoretical yield (≙ 100 g/100 g) = ΔY

$\Delta Y = |100 - \bar{Y}| \leqq 0.42$ g/100 g:
The accuracy of the determination is perfect.

$\Delta Y = |100 - \bar{Y}| = 0.43$ to 1.42 g/100 g:
Systematic errors are evident. This has to be accepted because they lie within the specifications of most photometers.

$\Delta Y = |100 - \bar{Y}| > 1.42$ g/100 g:
The deviation of the mean yield from the theoretical yield is too high. The reason is also either the use of unsuitable equipment (balance, photometer, cuvettes, pipettes) or due to their wrong handling. Something should be done to overcome these difficulties (e. g. control of balance, photometer, cuvettes and pipettes).

References
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(Elaborated from the members of the 26th Sub-commission, edited from the "Eidgenössische Drucksachen- und Materialzentrale", CH-Bern.)

Bijlage 2.1

...ber mais proef 1992
Meting ademhaling, ... en beder ... d ...
bij 0-1 en 5-6C in ... en in 10-7
Uitslagen: 7
Ademhaling veel frequenter
Inslag product: 24-07-1992
Uitslagen: 07-08, 27-08 en 25-09-1992

Beoordeling met schaal: 0-1-2-3 voor bladkleur en parasitair beder
0 = geen verkleuring of bederf;
3 = in sterke mate geelverkleuring or bederf.

Cont.		Parasit bederf No's	uit1	uit2	uit 3	Fleur blad	uit1	uit2	uit3	Parasit bederf s	uit1	uit2	uit3
		Kolf											
13	0-21	1	1	2	2		1	3	2		1	1	1
5C		2	1	2	3		1	2	3		1	1	3
		3	2	3	2		1	3	3		1	3	0
		4	1	2	2		1	2	3		0	1	3
		5	1	2	3		2	2	3		2	2	1
		6	1	2	3		2	2	3		1	2	2
		7	1	2	3		1	2	3		0	1	1
		8	1	2	3		1	2	3		2	1	3
		9	1	1	3		1	2	3		1	2	2
		10	1	2	3		2	2	3		0	2	3
16	0-21	1	1	2	3		1	2	3		2	1	0
5C		2	2	2	2		2	3	3		1	2	1
		3	1	2	2		0	1	3		1	2	2
		4	2	3	3		3	2	3		1	2	2
		5	1	1	3		1	2	3		1	2	1
		6	2	2	3		2	2	3		1	1	1
		7	1	1	2		1	2	3		0	1	2
		8	2	2	3		1	2	3		3	1	2
		9	1	2	3		1	2	3		1	2	3
		10	1	2	3		0	2	3		0	2	3
14	10-2	1	1	1	1		1	1	1		0	0	1
5C		2	1	1	1		1	1	2		1	0	0
		3	1	2	1		2	2	1		0	1	1
		4	1	1	0		1	1	1		1	1	1
		5	1	1	1		1	1	2		0	1	1
		6	2	1	2		1	0	3		1	2	2
		7	2	1	2		1	0	2		1	1	3
		8	1	1	1		1	1	3		1	1	2
		9	1	1	3		1	1	3		1	0	3
		10	1	1	2		2	0	3		1	1	1

36	10-2	1	1	1	2	1	2	1
1C		2	1	2	2	1	1	2
		3	1	1	2	1	2	2
		4	1	1	2	1	2	2
		5	1	1	2	1	2	2
		6	2	1	2	1	2	2
		7	1	1	2	1	2	2
		8	1	1	2	1	2	2
		9	1	1	2	1	2	2
		10	1	1	2	1	2	2
37	0-21	1	1	3	2	1	2	1
1C		2	1	2	2	1	1	1
		3	1	1	2	0	1	3
		4	1	2	2	1	2	2
		5	1	2	2	2	1	2
		6	1	1	2	0	2	2
		7	1	3	2	1	1	2
		8	1	1	2	0	1	2
		9	1	1	2	1	2	2
		10	1	1	2	0	2	2
40	0-21	1	1	2	2	1	1	2
1C		2	1	2	2	1	2	2
		3	1	1	2	1	2	2
		4	1	2	2	0	2	2
		5	1	2	2	0	1	2
		6	1	1	2	0	2	2
		7	1	3	2	1	1	2
		8	1	1	2	2	1	2
		9	1	1	2	2	2	2
		10	1	1	2	1	1	2
38	10-2	1	1	1	1	1	1	3
1C		2	1	2	1	1	1	0
		3	1	1	1	0	1	1
		4	1	1	1	1	0	2
		5	1	1	0	1	1	2
		6	1	1	0	1	1	2
		7	0	1	1	1	1	1
		8	2	1	2	0	1	2
		9	1	1	1	1	1	2
		10	1	1	1	1	0	1
39	10-2	1	1	2	1	0	2	1
1C		2	1	1	1	1	0	2
		3	1	1	0	1	0	2
		4	1	1	1	1	1	1
		5	1	1	1	1	1	1
		6	1	1	2	1	0	2
		7	1	1	1	1	1	2
		8	1	1	1	2	1	1
		9	1	1	2	1	1	3
		10	1	2	1	2	1	3

Mongst	Temp	Cond	Duur (dgn)	Gemiddeld gluc	fruc	suc
1	0-1	0-21	14	1.85	1.428	6.4
2			14	1.83	1.35	8.2
3			34	2.445	1.895	7.265
4			34	2.14	1.61	7.46
5			63	3.385	2.55	6.845
6			63	3.77	2.59	6.68
7	5-6	0-21	14	1.88	1.445	8.935
8			14	1.81	1.29	7.16
9			34	2.045	1.61	5.255
10			34	2.18	1.7	5.65
11			63	3.215	1.845	4.45
12			63	2.07	1.57	3.77
13	0-1	10-2	14	1.99	1.475	8.995
14			14	1.91	1.4	8.95
15			34	2.37	1.715	8.515
16			34	2.58	1.87	8.97
17			63	3.19	2.34	7.775
18			63	3.17	2.44	7.88
19	5-6	10-2	14	2.06	1.685	8.85
20			14	1.77	1.46	8.7
21			34	2.59	2.1	8.12
22			34	2.52	2.07	8.17
23			63	3.355	2.83	6.87
24			63	3.35	2.75	6.99

Mongaster	Temp	Cond	Occur (dgr)		Dep
1	0-1	0-21		15	7.98
2				15	5.145
3				28	7.065
4				28	7.205
5				43	11.36
6				43	14.93
7	5-6	0-21		15	3.48
8				15	3.32
9				28	4.86
10				28	8.46
11				43	1.82
12				43	2.53
13	0-1	10-2		15	74.69
14				15	80.31
15				28	102.3
16				28	122.28
17				43	164.77
18				43	165.6
19	5-6	10-2		15	155.425
20				15	115.095
21				28	179.35
22				28	138.525
23				43	298.685
24				43	314.77