MN8201 606 C

The mechanism of cloud loss phenomena in orange juice

BIBLIOTHEEK
DER
LANDBOUWHOGESCHOOL
WAGENINGEN

J.J.P. Krop

NN08201.606

The mechanism of cloud loss phenomena in orange juice

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus, dr. ir. H.A. Leniger,
hoogleraar in de technologie,
in het openbaar te verdedigen
op donderdag 12 december 1974 des namiddags te vier uur
in de aula van de Landbouwhogeschool te Wageningen

Centre for Agricultural Publishing and Documentation

Wageningen – 1974

Abstract

Krop, J.J.P. (1974) The mechanism of cloud loss phenomena in orange juice. Doctoral thesis, Wageningen. (x) + 103 p., 51 figs, 15 tables, 148 refs, Dutch and Eng. summaries.
Also: Agric. Res. Rep. (Versl. landbouwk. Onderz.) 830.

The importance of many factors for the cloud stability of orange juice was investigated. By the determination of methanol, cloud loss of orange juice could be ascribed directly to the action of pectin esterase. However, clarification only occurs if calcium ions are available to precipitate the low methoxy pectin formed by pectin esterase. This mechanism was confirmed when the addition of pectic acid clarified the orange juice artificially.

Clarification did not occur if the formation of high polymer calcium pectate was prevented either by degrading the juice pectin by a purified pectin lyase before substantial action of pectin esterase, or by enzymic hydrolysis by a yeast polygalacturonase of the low methoxy pectin formed. Methanol determinations showed that cloud stabilization had been achieved in spite of normal action of pectin esterase.

Methods to determine cloud stability have been discussed. Literature on the research of cloud stability of fruit juices, on pectic substances and pectic enzymes, and on methods to determine pectin esterase have been reviewed.

This thesis will also be published as Agricultural Research Reports 830.

© Centre for Agricultural Publishing and Documentation, Wageningen, 1974.

No part of this book may be reproduced or published in any form, by print, photoprint, microfilm or any other means without written permission from the publishers.

Stellingen

1. 'Cloud stable' is een relatief begrip.

   Dit proefschrift, paragraaf 5.2.

2. Als de troebelheid van een vruchtesap spectrofotometrisch of colorimetrisch wordt bepaald, kan men deze beter in extinctie-waarden dan in transmissie-waarden uitdrukken.

   Dit proefschrift, paragrafen 4.3 en 5.2.

3. Een beoordelingstijd van 48 uur voor de troebelingsstabiliteit van citrus-sap, zoals toegepast door Biggs & Pollard, is te enen male te kort om enigerlei conclusie ten aanzien van de stabiliteit of stabilisatie van het sap te kunnen trekken.

   R.H. Biggs & J.E. Pollard, Proc. Fla St. hort. Soc. 83 (1970): 314-318.

4. De usance om in wetenschappelijke publikaties slechts de positieve resultaten van onderzoek te vermelden, kan leiden tot nodeloze herhalingen van vruchteloos onderzoek en dient derhalve verlaten te worden.

5. Het promotiereglement dient zodanig gewijzigd te worden, dat een promovendus niet gehouden wordt aan een minimum aantal stellingen.

6. Het chloren van effluenten kan een belangrijke bijdrage leveren tot het onderbreken van de cyclus van infectieziekten, zoals Salmonellose en Listeriose.

7. De conclusie van Pokorný & Forman dat de instabiliteit van koffielipiden hoofdzakelijk aan triglyceriden en niet aan de vetzuuresters van diterpenen is toe te schrijven, wordt niet gestaafd door de experimentele gegevens die zij zelf verstrekken.

   J. Pokorný & L. Forman, Die Nahrung 14 (1970) 631-632.

Abstract

Krop, J.J.P. (1974) The mechanism of cloud loss phenomena in orange juice. Doctoral thesis, Wageningen. (x) + 103 p., 51 figs, 15 tables, 148 refs, Dutch and Eng. summaries.
Also: Agric. Res. Rep. (Versl. landbouwk. Onderz.) 830.

The importance of many factors for the cloud stability of orange juice was investigated. By the determination of methanol, cloud loss of orange juice could be ascribed directly to the action of pectin esterase. However, clarification only occurs if calcium ions are available to precipitate the low methoxy pectin formed by pectin esterase. This mechanism was confirmed when the addition of pectic acid clarified the orange juice artificially.

Clarification did not occur if the formation of high polymer calcium pectate was prevented either by degrading the juice pectin by a purified pectin lyase before substantial action of pectin esterase, or by enzymic hydrolysis by a yeast polygalacturonase of the low methoxy pectin formed. Methanol determinations showed that cloud stabilization had been achieved in spite of normal action of pectin esterase.

Methods to determine cloud stability have been discussed. Literature on the research of cloud stability of fruit juices, on pectic substances and pectic enzymes, and on methods to determine pectin esterase have been reviewed.

This thesis will also be published as Agricultural Research Reports 830.

© Centre for Agricultural Publishing and Documentation, Wageningen, 1974.

No part of this book may be reproduced or published in any form, by print, photoprint, microfilm or any other means without written permission from the publishers.

8. Bij verbouw en nieuwbouw van woningen in woonwijken besteden de bevoegde autoriteiten onvoldoende aandacht aan het handhaven, respectievelijk scheppen van nestelgelegenheid voor gierzwaluwen (*Apus apus*).

W.J. van der Weijden, Het Vogeljaar 22 (1974): 765-770.

9. Er wordt nog al te veel gras voor de voeten weggemaaid.

Proefschrift van J.J.P. Krop
Wageningen, 12 december 1974

Curriculum vitae

J.J.P. Krop behaalde in 1964 het diploma Gymnasium-β aan het Revius-Lyceum te Doorn. In aansluiting hierop begon hij zijn studie aan de Landbouwhogeschool te Wageningen, waar hij in januari 1969 het kandidaatsexamen aflegde in de levensmiddelentechnologie met chemisch-biologische specialisatie. In april 1971 behaalde hij het doctoraal diploma met lof. Het vakkenpakket bestond uit het hoofdvak kennis van levensmiddelen en de bijvakken voedingsmiddelenmicrobiologie, technische microbiologie en biochemie.

Na zijn afstuderen was hij, dank zij een stipendium van de Coca-Cola Company, in staat dit promotieonderzoek uit te voeren als gastmedewerker op het Laboratorium voor levensmiddelenchemie en -microbiologie van de Landbouwhogeschool.

Sinds 1 augustus 1974 is hij werkzaam als leraar scheikunde bij de Rijks-Middelbare Tuinbouwschool te Nijmegen.

Acknowledgments

My thanks are due to:
- my promotor Prof. Dr W. Pilnik who stimulated me all the time by his enthusiasm for the subject,
- Dr A.W. Noltes of the Coca-Cola Company for discussions,
- the Coca-Cola Company for financial support and for providing the orange concentrate,
- Miss Corrie Spaansen for her cooperation as technical assistant,
- the staff of the Department of Food Science who made this study possible,
- Ir Klaske de Boer, Ir J.M. Faddegon and Mr W.G.F.M. Hendriks for their cooperation and assistance during their graduate-work,
- Mr S. Henstra and Mr H.G. Elerie of the Technical and Physical Engineering Research Service, Wageningen for their assistance with the electron microscopy and for taking the electron micrographs,
- Mr C. Rijpma for drawing the figures,
- my wife Maartje for typing the manuscript,
- Dr A.W. Noltes and Dr J. Rosenbaum of the Coca Cola Company for correcting the English,
- Mr R.J.P. Aalpol for editing the manuscript,
- and last but not least to my parents for giving me the opportunity to study.

Samenvatting

Het doel van deze studie was, meer inzicht te krijgen in de factoren die een rol spelen bij de destabilisatie van troebele vruchtesappen, waardoor de troebeling bezinkt. Alle onderzoekingen werden gedaan aan sinaasappelsap omdat dit het voornaamste troebele vruchtesap is.

De hoofdstukken 2 en 3 zijn literatuuroverzichten. Hoofdstuk 2 geeft achtereenvolgens een overzicht van de oudere literatuur betreffende de destabilisatieproblemen bij sinaasappelsap, de samenstelling van de troebelingsdeeltjes en de meer recente ontwikkelingen bij het onderzoek van troebele vruchtesappen. Hierna wordt het klaringsmechanisme bij appel- en druivesap beschreven.

In hoofdstuk 3 wordt aandacht besteed aan de structuur en de eigenschappen van pectinestoffen en aan pectolytische enzymen die deze stoffen kunnen aantasten. Pectolytische enzymen worden behandeld omdat pectine-esterase (PE) van nature in de sinaasappel voor komt, terwijl enkele pectine-depolymerasen toegepast zijn in het experimentele gedeelte.

Paragraaf 3.3 geeft een overzicht van de verschillende mogelijkheden om PE-activiteit aan te tonen en kwantitatief te bepalen. In deze studie was het vaak belangrijker te weten wat de feitelijke werking van PE was dan het aantal enzym-eenheden; daarom werd onderscheid gemaakt tussen de begrippen 'werking' en 'activiteit'. De werking van het enzym kan gemeten worden door de methanol die er bij vrijkomt, kwantitatief te bepalen. Daarom zijn ook enkele bepalingsmethoden voor methanol beschreven.

Hoofdstuk 4 gaat over het meten van de troebelingsstabiliteit. Eerst wordt een theoretische beschouwing gegeven over de sedimentatie van deeltjes in een vloeibaar medium. Vervolgens wordt een literatuuroverzicht gegeven van methoden om de hoeveelheid bezonken pulpdeeltjes te meten. De conclusie wordt getrokken dat deze methoden niet geschikt zijn om de troebelingsstabiliteit te bepalen. Hierna wordt ingegaan op de theoretische achtergronden van troebelingsmetingen. Dit leidt tot de conclusie dat men de troebeling beter in extinctie-waarden kan uitdrukken dan in transmissie-waarden.

In hoofdstuk 5 wordt een overzicht gegeven van de gebruikte materialen

en toegepaste onderzoeksmethoden. Aan het eind van paragraaf 5.2 worden verschillende troebelingsmeetmethoden met elkaar vergeleken naar aanleiding van enige experimenten. Voor deze studie wordt gekozen voor extinctie-metingen in de supernatant van gecentrifugeerde sapmonsters. Hoofdstuk 5 wordt besloten met discussies van de samenstelling van het gebruikte sinaasappelconcentraat en van de toegepaste gaschromatografische methanolbepalingsmethode. Hoewel dit in principe een gevoelige en geriefelijke methode is, geven de waterige monsters toch vaak problemen.

Hoofdstuk 6 beschrijft het eigenlijke experimentele gedeelte. In paragraaf 6.1 werden verschillende factoren onderzocht die de troebelingsstabiliteit 'van buitenaf' zouden kunnen beïnvloeden. Sommige bleken geen invloed op de resultaten te hebben, hoewel het bleek dat wanneer aan sinaasappelsap na pasteurisatie PE werd toegevoegd, de klaring sneller verliep dan in een vergelijkbaar sap zonder die extra warmtebehandeling. Een reeds geklaard sap bleek weer redelijk troebel gemaakt te kunnen worden door de combinatie van een warmtebehandeling met een centrifugerings- en een suspenderingsbehandeling. Deze resultaten wijzen er op dat resuspensie-experimenten voor het onderzoek van de troebelingsstabiliteit ongeschikt zijn.

Paragraaf 6.2 beschrijft de intrinsieke factoren die van invloed zijn op de troebelingsstabiliteit. De troebeling in sinaasappelsap wordt in belangrijke mate pas enige tijd na het persen gevormd. De klaring van sinaasappelsap bleek sterk te correleren met de werking van PE. Bij een geringer gehalte aan dit enzym duurt het langer voor klaring optreedt, maar het bleek dat het aanwezige pectine gemiddeld minder ver verzeept behoeft te worden om klaring te bewerkstelligen dan bij hogere concentraties enzym. Klaring bleek echter alleen te kunnen optreden als er calcium-ionen in het sap beschikbaar zijn om het gevormde laag veresterde pectine te precipiteren. Klaring van sinaasappelsap kan kunstmatig bewerkstelligd worden door de toevoeging van pectinezuur. Zowel de pectinefractie die aan de deeltjes in het sap is gebonden, als het in het serum opgeloste pectine, worden door PE verzeept. Een suspensie van de afgecentrifugeerde deeltjes van een stabiel sap kan in de aanwezigheid van calcium-ionen door PE geklaard worden. Onderzoek met een electronenmicroscoop toonde dat in een geklaard sap alle soorten deeltjes als het ware in pakketjes verpakt zijn. Het verpakkingsmateriaal is naar alle waarschijnlijkheid het geprecipiteerde calciumpectaat.

Paragraaf 6.3 beschrijft enige eigenschappen van een gist-polygalacturonase (PG). Met dit enzym bleek de klaring van sinaasappelsap door PE voorkomen te kunnen worden, wel is hierbij een minimum verhouding PG/PE vereist.

Een dergelijk gunstig effect op de troebelingsstabiliteit bleek een gezuiverde pectine-lyase (PL) te hebben. In dit geval is afbraak van het sappectine door deze lyase vóór toevoeging van PE reeds voldoende om klaring te voorkomen. PG moet in elk geval nog actief aanwezig zijn tijdens de werking van PE om effectief te zijn. Daar PE het substraat voor PG in gunstige zin en voor PL in ongunstige zin verandert, is PG in PE actief sap effectiever dan PL. De invloed van geconcentreerde suikeroplossingen en van geconcentreerd sinaasappelserum op de activiteit van PG en PL worden beschreven in paragraaf 6.4. Klaring van sinaasappelsap bleek in een modelproef met PG en PL in Ca-bevattende pectineoplossingen goed te correleren met gelering van deze oplossingen; voorkoming van gelering met stabiele sappen (paragraaf 6.5). Tenslotte werd nagegaan of proteasen invloed hebben op de troebelingsstabiliteit, wat niet het geval bleek (paragraaf 6.6).

Tenslotte wordt in hoofdstuk 7 een mechanisme voor de klaring van sinaasappelsap beschreven aan de hand van de gevonden resultaten. De term 'troebelingsstabiliteit' wordt kritisch beschouwd.

Contents

List of abbreviations 3

1 Introduction 5

2 Literature on cloud stability of fruit juices 7
- 2.1 Research until 1960 7
- 2.2 Recent research 8
  - 2.2.1 Composition of orange cloud 8
  - 2.2.2 Clarification and stabilization 9
- 2.3 Processing techniques 12
- 2.4 Clarification mechanism of apple and grape juice 12

3 Pectic substances and pectic enzymes 15
- 3.1 Pectic substances 15
- 3.2 Pectic enzymes 19
- 3.3 Pectin esterase in orange juice; activity and action 23

4 The measuring of cloud changes 27
- 4.1 Sedimentation rate of particles in a fluid 27
- 4.2 The quantity of settled pulp 28
- 4.3 Turbidity in the supernatant phase 30

5 Materials and methods 34
- 5.1 Materials 34
- 5.2 Methods 38
- 5.3 Discussion 49

6 Experiments and results 52
- 6.1 Influence of experimental factors on cloud stability 52
  - 6.1.1 Cloud change assay methods 52
  - 6.1.2 Juice making-up methods 56

6.2 Influence of intrinsic factors on cloud stability 59
6.2.1 Clarification behaviour of unpasteurized orange juices of different varieties 59
6.2.2 Clarification behaviour of mixtures of pasteurized and unpasteurized juices 60
6.2.3 Relation between clarification and pectin esterase action 60
6.2.4 Confirmation of pectin esterase inhibition by pectic acid 62
6.2.5 Effect of addition of pectic acid and bivalent cations on cloud loss 63
6.2.6 The respective role of cloud pectin and serum pectin in cloud loss 65
6.2.7 The possible role of hesperidin in cloud loss as seen by electron microscopic investigation 67
6.3 Influence of pectin and pectate depolymerases on the cloud stability of orange juice 71
6.3.1 Cloud stabilization by a yeast polygalacturonase 71
6.3.2 Cloud stabilization by a pectin lyase 78
6.3.3 Comparison of polygalacturonase and pectin lyase as pectin depolymerases and as cloud stabilizers 84
6.4 Activity of pectic enzymes and clarification in concentrated systems 85
6.4.1 Inhibition of citrus pectin esterase, yeast polygalacturonase and U20 pectin lyase by sugars and concentrated depectinized serum 85
6.4.2 Preliminary experiments on cloud stability of juice stored as 40°Bx concentrate 86
6.5 A model experiment with pectic enzymes in a calcium pectinate solution 86
6.6 Effect of commercial protease preparations on cloud loss 88

7 Discussion and conclusions 92

Summary 98

References 101

List of abbreviations

AGA = anhydrogalacturonic acid
AIS = alcohol insoluble solids
Bx = Brix (density expressed as percentage by wheight sucrose solution)
DE = degree of esterification
DEAE = diethylamino-ethyl
DP = number average degree of polymerization
DU = depolymerase units of enzyme
E = extinction ($lg(I_o/I)$)
EDTA = ethylene diamine tetraacetate
g = acceleration due to gravity (m/s^2)
g.l.c. = gas-liquid chromatography
g.s.c. = gas-solid chromatography
I = intensity of transmitted light
Io = intensity of incident light
K_i = Michaelis constant for inhibitor
K_m = Michaelis constant
LMPL = low methoxy pectin lyase
o.d. = outside diameter
PAL = pectate lyase
PE = pectin esterase
PG = polygalacturonase
PL = pectin lyase
PMG = polymethylgalacturonase
PMR = proton magnetic resonance
ppm = parts pro million
Q = light scattering quotient
%T = percentage light transmission
U = units of enzyme
U20 = Ultrazym 20 (CIBA - GEIGY AG, Basel, Switzerland)
λ = wave length
ρ = dimensionless particle size diameter

1 Introduction

The orange or sweet orange (*Citrus sinensis*) is a subspecies of the species *Citrus* belonging to the family of *Rutaceae*. Many varieties of each subspecies are known. Although citrus originates in the tropical regions of South-East Asia the largest cultivation areas are nowadays found in the subtropics. World production is second only to that of grape. Ten years ago world production of oranges and mandarins amounted to 20 x 10^6 tons. About 25% were produced in the USA, nearly 30% in the Mediterranean Basin and over 10% in Brazil. A large part of the production of citrus fruit is industrially processed to single-strength juices, concentrates and soft drinks: about 2/3 in the USA, in the whole world about 1/3 (Monselise, 1973).

An important quality aspect of a citrus juice or citrus juice containing beverage is its cloudy appearance. Much of the flavour and colour are associated with the cloud. Moreover a cloudy aspect of the juice reminds the consumer of the fruits used. However, this cloud is not stable by nature and from its inception the citrus industry has encountered problems with this instability. These problems present themselves in single-strength juices or beverages as clarification or cloud loss and in concentrates also as gelation. If clarification occurs the supernatant layer in a bottle may become crystal clear. Upon resuspension by shaking the destabilized cloud will resettle rapidly and such products are in fact unsalable. Gelation of concentrates due to destabilization of the cloud makes impossible their reconstitution to single-strength juice with the consequence that they become worthless.

This study aims to make a contribution toward the elucidation of the mechanism of cloud loss phenomena in orange juice. In order to study these cloud change phenomena I started from the PE hypothesis (Section 2.1): Cloud loss was provoked by adding PE to PE inactive orange juice and these changes were studied in relation to PE action and to various treatments and additions before, during or after the PE addition. The experiments therefore consist mainly in making up orange juice samples with standardized PE activity and following cloud change and pectin deesterification against time. The results

are also of value in understanding similar phenomena in cloudy juices and juice containing beverages from different origin, e.g. other citrus subspecies, apricots, peaches, pears and carrots.

2 Literature on cloud stability of fruit juices

2.1 RESEARCH UNTIL 1960

The literature on cloud stability until ca. 1960 was reviewed by Pilnik (1958) and Joslyn & Pilnik (1961). Many of the publications from that period consider gelation problems in 42°Bx frozen orange concentrates. The frozen orange concentrate industry was established in the USA after World War II and assumed large proportions. Pasteurized orange juice is concentrated up to 60°Bx after which the concentrate is cut back with fresh unpasteurized juice to about 42°Bx. This 1:4 concentrate is then deep frozen. This concentrate is easily reconstituted with three volumes of water and a juice obtained with the flavour of fresh juice. A disadvantage of the 'cut back method' is the introduction of enzymes and microorganisms into the concentrate. If not stored at sufficient low temperatures clarification of the reconstituted juice or gelification of the concentrate occurs.

Many of the data discussed in the papers reviewed by Joslyn & Pilnik (1961) concern the fact that a heat treatment improves the cloud stability of orange juice and that clarification changes the pectic substances so that upon fractional extraction the low methoxy pectin fractions increase. This led to a hypothesis with the following main features: Active PE saponifies the native pectin which is believed to stabilize and support the cloud particles as a protective colloid. The resulting low methoxy pectin reacts with bivalent cations present in the juice to form insoluble pectinates or pectates which deposit carrying the cloud particles along. PE can be inactivated by pasteurization, the degree of heat treatment required depending on pH and pulp content. PE is most active at about 40°Bx, 65°Bx concentrates are far more stable. To prevent gelation and/or clarification in unpasteurized juices or in cut back concentrate storage temperatures of -18°C or below are indispensable since slight abuses already cause some cloud loss.

2.2 RECENT RESEARCH

2.2.1 Composition of orange cloud

Scott et al. (1965) first published data on the composition of orange cloud. About one quarter of the cloud is made up of lipids, the remainder is insoluble in ethanol, acetone and n-hexane and consists for about 80% of pectic substances, the remainder being cellulose, hemicellulose and protein. From the analytical figures of the various fruit parts the authors concluded that the cloud originates from the juice cells rather than from mechanical disintegration of the tissue.

Baker & Bruemmer (1969) repeated these experiments and found quite a different composition of orange cloud material. According to them the inadequate analytical procedures applied by Scott et al. (1965) resulted in values too high for the pectin content and much too low for the protein content of the cloud. According to Baker & Bruemmer (1969) the composition of the cloud insolubles is 45% protein. Since the cloud insolubles constitute 75% of the total cloud, the protein content of the total cloud amounts to 34%. They found a pectin content of about 43% for the cloud insolubles instead of about 80% as given by Scott et al. (1965). This means that the pectin content of the total cloud amounts to about 32%.

Larsen (1969) prepared clouding agents from citrus peel by enzymic action and determined the sizes of the particles with a microscope and by means of filtration through filters with varying pore diameters. The particle sizes were found to range from 1 to 30 µm. The clouding agent consisted of protoplasma particles, cell wall fragments and hesperidin needles.

Mizrahi & Berk (1970) found particle sizes for orange cloud ranging from 0.05 µm to a few hundred µm. They stated that stable particles are smaller than 2 µm. Four types of particles could be distinguished: chromoplastids, pulpfragments, oildroplets and hesperidin needles. Heat treatment increased the cloud level, possibly due to an increase of the small particles at the cost of the bigger ones. Enhanced cloud stability after heat treatment is also recorded by Loeffler (1941) who suggested the increased cloudiness might be due to hydrolysis of protopectin to pectin or the release of pectin from enveloping cellulose. According to Mizrahi & Berk (1970) some extraction of pectin into the serum took place indeed, but this effect was too small to explain the stabilizing effect of heat. From a physico-chemical study the investigators concluded that for the particles to remain in suspension their

hydration was the most important factor.

Resch & Schara (1970) in an article about the anatomical elements of the orange fruit describe the various particles that are found in orange juice.

2.2.2 *Clarification and stabilization*

Attempts to elucidate the mechanism of clarification have only been scarcely reported in the literature after 1960. Primo Yúfera et al. (1961a) studied changes of the pectin of cloud particles. Cloud particles were separated from juice and suspended in water. During storage their low methoxy pectin content increased, whereas the insoluble pectin content decreased. This phenomenon was accompanied by an increased sedimentation rate. They also observed that cloud particles, centrifuged from juice after various storage times and then resuspended in water gave suspensions which were the less stable the longer the juice had been stored. Primo Yúfera et al. (1963) have also used the hydroxylamine-ferrichloride colouring method to show that the pectin in the cloud particles of a clarified juice was saponified. The conclusion of these authors is that the state of the pectin linked to the cloud particles and not the serum pectin is responsible for cloud stability.

Baker & Bruemmer (1969) have approached the problem by considering cloud behaviour of cloud particles separated from serum. They reported that cloud particles suspended in water made very stable suspensions, which disproves the old hypothesis of serum pectin stabilizing the cloud (Rouse & Atkins, 1955). Addition of pectin, calcium, sugar and citric acid to the resuspensions did not harm the stability; however, addition of KCl resulted in a rapid clarification although still slower than a suspension of cloud in original serum. Possibly KCl solubilizes PE. Sugar nullified the KCl-effect to a large extent. Experiments were carried out by recombining previously separated cloud and serum either without or following heat treatment of one, the other or both. With both fractions heated the recombination was stable, with both fractions unheated rapid clarification occurred. Heated cloud recombined with unheated serum was slightly more stable than when both fractions were unheated. Unheated cloud recombined with heated serum resulted in delayed clarification.

Treatment of the serum with tomato PE resulted in a virtually immediate clarification following resuspension of the cloud in the serum. Treatment of the serum with Klerzyme (PE and PG activities) was moderately effective in preventing cloud loss. The conclusion from these experiments is that while

serum pectin is not a cloud stabilizer it may act as a cloud destructing agent when saponified by PE, this PE being a serum soluble fraction. The PE adsorbed to cloud particles does not seem to play a role except when brought into solution by KCl addition. Baker & Bruemmer explain the contradictions of their findings to the results of Primo Yúfera et al. (1961a) by the small size of their cloud particles, obtained by centrifugation at 78 000 x *g* while the Spanish authors obviously worked with coarser pulp particles which had been obtained by centrifugation at 1000 to 2000 x *g*. For concentrates the mechanism postulated by Primo Yúfera et al. (1961a) would still apply. This points to two different mechanisms for cloud loss in juices and concentrate gelling. In the first instance serum pectin is saponified by serum PE and precipitates the cloud; in the second instance particle PE saponifies all pectin fractions. However, Pilnik & Rothschild (1960) had already shown previously that when underpasteurized juice after standing was separated by decantation in a pulpy and a supernatant fraction and these fractions were left standing again, the cloudy fractions were less cloud stable. There appears to be a problem of PE distribution the complexity whereof is demonstrated by the findings of these three groups.

The results obtained by suspending cloud particles into serum treated with Klerzyme (Baker & Bruemmer, 1969), indicated that removal of soluble pectin could delay or prevent cloud loss and motived these authors to treat freshly pressed orange juice directly with pectic enzyme preparations (Baker & Bruemmer, 1972a). One of seven commercial preparations investigated accelerated clarification, but the others stabilized the cloud to a larger or smaller extent. Stabilization of orange cloud is explained by depolymerization of the pectic substances to soluble low molecular weight pectates preventing the formation of high molecular weight insoluble pectates. The authors correlated the effectiveness of stabilization of the pectolytic preparation used with the ratio of depolymerizing activity on polygalacturonic acid and on citrus pectin respectively (PG_{PGA}/PG_{PEC}). Under optimal conditions cloud density in the enzyme stabilized juice was equivalent to that of fresh juice. It was found, however, that this cloud could be enhanced by treating fresh orange juice with 200 ppm Klerzyme combined with 100 ppm of the protease ficine during 50 min at 26.7°C, after which the juice was heated to 73.9°C (Baker & Bruemmer, 1971). An identical treatment of pasteurized, PE inactive, juice led to cloud loss. These findings were the basis of a patent (Baker & Bruemmer, 1973), which states that addition of a protease enhances the breakdown of calcium pectate by polygalacturonase.

The use of enzymes to achieve cloud stability found more widespread application. Gierschner & Baumann (1969) described the preparation of a stable cloudy apple juice by means of a commercial pectic enzyme preparation. This cloud stable apple juice could not be clarified within five days by pectic enzymes. With protease, however, complete clarification occurred within 24 h. The existence is suggested of a highly hydrated complex of pectin, protein and possibly polyphenols as a result of the enzyme activities during the preparation of the juice. The preparation of a cloud stable apricot nectar by means of pectic enzymes has also been described (Weiss & Sämann, 1972). Biggs & Pollard (1970) discussed the effect of various enzyme preparations on the cloud stability of freshly extracted orange juice. Their observation time of 48 h seems, however, too short to enable valid conclusions to be drawn since in this time even their PE active control samples did not clarify. All literature data on enzyme treatments of fruit juices with commercial enzyme preparations are difficult to interpret because these preparations had not been purified and their activities were ill-defined.

Baker & Bruemmer (1972b) furthermore studied the interaction between orange juice cloud and a floc developing in ultracentrifugally prepared orange serum upon standing. This floc was shown to consist of pectate and hesperidin. The floc was formed within 6 days, corresponding to the period in which most of the cloud in the juice was lost. The authors suggest that floc formation in juice keeps pace with floc formation in serum, but this is by no means certain. The floc clarified a suspension of orange juice cloud in aged deflocced serum. Addition of soluble citrus pectin to aged floc-containing serum before resuspension of cloud seemed to inhibit the coacervation of floc and cloud. In aqueous resuspensions the presence of calcium or potassium salts was found to be necessary for coacervation. The calcium required for clarification was not bound by the floc, but was effective only when cloud and floc were combined.

Reinert (1973) was able to produce orange juices that were cloud stable during 2.5 years. After pressing the juice was homogenized in a Waring blendor and kept for 24 h to 72 h. The juice was subsequently treated for 1 h with cation exchanger at $70^{o}C$ in order to extract pectic substances from the pulp particles. Before heat inactivation the juice was centrifuged for 10 min at 2400 x *g* and 100 to 1000 mg Ca per litre juice were added aseptically. After 2.5 years no clarification, gelation or sedimentation had occurred. Cloud was uniformly distributed over the whole sample. According to Reinert this cloud stabilization is achieved by increased pectin content and binding of soluble pectin to cloud particles by the action of the native PE and the added Ca-ions.

It should be noted that Reinert applied a technology in preparing orange juice strongly different from those commonly used and it is an open question whether his products can be considered as orange juice.

2.3 PROCESSING TECHNIQUES

Increasing sales of frozen concentrate in Europe where cold storage facilities are less universally available than in the USA and the increasing use of frozen concentrates as industrial raw material for the production of single-strength juices, squashes and beverages caused concentrate producers to look for techniques to produce more stable products of the same organoleptic qualities. All of these techniques are based on the PE hypothesis, i.e. they all try to achieve more stability by diminishing PE activity. They involve: Screening of cut back juice (PE is primarily bound to pulp particles), light pasteurization of cut back juice, increasing solids content of frozen concentrate by using freeze concentrated juice for cut back (Pilnik, 1969) or, more recently, the use of aroma recovery in place of cut back for pasteurized or unpasteurized concentrates with a high solids content (Wolford et al., 1969). Israeli research-workers (Peleg & Mannheim, 1970a, 1970b) suggested to concentrate serum separately from the pulp, achieving thus a high Brix (PE inhibition), organoleptically desirable unpasteurized concentrate. It is interesting to note that the possibility of inhibiting PE is only mentioned in a patent (Kew & Veldhuis, 1961) according to which grape leaf extract is used.

A completely different approach was made by Baker & Bruemmer (1973) who patented the application of pectin depolymerases and proteases to obtain cloud stability.

2.4 CLARIFICATION MECHANISM OF APPLE AND GRAPE JUICE

Although this study is concerned with the clarification mechanism of orange juice, it is useful to consider our knowledge about similar behaviour of some other juices. While clarification of apple and grape juices is a desirable process, which since the early thirties is achieved by adding pectic enzymes, the same phenomenon in citrus juices mostly is regarded as highly objectionable.

Some ten years ago the clarification mechanism of apple juice was studied by Japanese research-workers. Yamasaki et al. (1964) studied resuspensions of ultracentrifugal precipitates of apple juice in aqueous media. These precip-

itates were found to contain 36% protein and at least four sugars: arabinose, galactose, glucose and galacturonic acid. By electrophoresis technique the particles were shown to be negatively charged at pH 3.5. Their experiments led Yamasaki et al. to propose a mechanism of flocculation of the suspended particles: The cloud particles are a protein-carbohydrate complex, coated by negatively charged pectin as a protective colloid. If this pectin is partially degraded by enzymes the positive charge of the protein complex is exposed and flocculation occurs due to electrostatic attraction between particles with positive and negative charges. No flocculation occurs above pH 4.75, this pH value probably being above the isoelectric point of the protein.

Endo (1965a, 1965b, 1965c, 1965d) demonstrated that clarification of apple juice could be accomplished by the joint action of a purified endo PG and a purified PE, both produced by *Coniothyrium diplodiella*, while a combination of exo PG and PE had no effect on the clarification. Hydrolysis of the pectic substances in apple juice seemed to be indispensable for clarification. Endo distinguishes three stages in the clarification process: (1) solubilization of insoluble pectin bound to the suspended particles; (2) decrease in viscosity by hydrolysis of the soluble pectin, and (3) flocculation of the suspended particles. Endo (1965b) found soluble apple pectin to have a DE of approximately 88%, while commercial citrus pectin had a DE of 64%. According to Endo (1965a) activity of cellulase, amylase and protease is not indispensable for the clarification of freshly pressed apple juice.

Yamasaki et al. (1967) confirmed the findings of Endo to the extent that they were able to clarify apple juice by the joint action of a purified endo PG of *Aspergillus satoi* and a purified PE of *Sclerotinia arachnidis*. Addition of only PE to apple juice gave a gel but no clarification, while PG was able to clarify apple juice only partially after prolonged incubation. This effect probably should be ascribed to a combined action of the PG added with the native apple PE. The electrostatic charge neutralization mechanism (Yamasaki et al., 1964) was supported by further experimental data.

Ishii & Yokotsuka (1971, 1972) purified two PLs from a culture of *Aspergillus sojae* and were able to clarify both cloudy apple juice and cloudy grape juice by either preparation. However, to clarify grape juice five times more enzyme was necessary than for apple juice. PL was not able to clarify orange juice completely. Later Ishii & Yokotsuka (1973) showed that PL from *Aspergillus japonicus* was able to clarify apple juice, while its effect upon grape juice varied depending on the grape variety and ripeness. A purified endo PG, also from *Aspergillus japonicus*, did not change the cloud level of apple juice,

while its effect upon grape juice varied like that of PL. Sometimes PG was more effective upon grape juice than PL, sometimes the reverse was true. Apple pectin is always highly esterified (Gee et al., 1959; Endo, 1965b; Ishii & Yokotsuka, 1973) while apple fruit has a low PE content (Pollard & Kieser, 1951). This may explain why PL is highly effective and PG ineffective on apple juice. Grape juice pectin may have a much lower DE as a result of the activity of native grape PE (Ishii & Yokotsuka, 1973) and this explains the varying effectiveness of PL and PG upon grape juice.

3 Pectic substances and pectic enzymes

3.1 PECTIC SUBSTANCES

The review on cloud research given in Chapter 2 shows clearly that an intelligent approach to this problem is not possible without a thorough knowledge of pectic substances and pectic enzymes. Therefore the large volume of knowledge on these subjects is condensed in this chapter.

Definitions. Pectic substances are found in the tissues of all higher plants. They are mainly deposited in the middle lamella and the primary cell wall, where they act as intercellular cement, and therefore parenchymous and meristematic tissues are particularly rich in petic substances. The American Chemical Society (Kertesz, 1951, p. 6-8) gives the following definitions:

Pectic substances. Pectic substances is a group designation for those complex, colloidal carbohydrate derivatives which occur in, or are prepared from, plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chain-like combination. The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases.
Protopectin. The term protopectin is applied to the water-insoluble parent pectic substance which occurs in plants and which, upon restricted hydrolysis yields pectinic acids.
Pectinic acids. The term pectinic acids is used for colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Pectinic acids, under suitable conditions, are capable of forming gels with sugar and acid or, if suitably low in methoxy content, with certain metallic ions. The salts of pectinic acids are either normal or acid pectinates.
Pectin. The general term pectin (or pectins) designates those water-soluble pectinic acids of varying methyl ester content and degrees of neutralization which are capable of forming gels with sugar and acid under suitable conditions.

Pectic acid. The term pectic acid is applied to pectic substances mostly composed of colloidal polygalacturonic acids and essentially free from methyl ester groups. The salts of pectic acid are either normal or acid pectates.
Structure. The backbone of pectic substances consists of 1,4 linked α-D-galactopyranosyluronic acid units. The galacturonic acid possesses the Cl conformation; consequently the hydroxyl groups at the C_1 and C_4 atoms are in axial position and the polymer chain has a screw axis with a tendency to coiling. On hydrolysis most pectin preparations, even after extensive purification, yield not only galacturonic acid but also neutral sugars. These are primarily D-galactose, L-arabinose and L-rhamnose; in some cases also D-xylose and L-fucose, their respective 2-methylethers and D-apiose (Rombouts, 1972). Many authors investigate the structure of pectic substances by analysis of the breakdown products formed upon partial hydrolysis. Mild acid hydrolysis results in a rapid release of arabinose and sometimes fucose. Acetolysis (i.e. breakdown of acetylated polysaccharides in glacial acetic acid-sulphuric acid) and acid hydrolysis show that rhamnose units are part of the main chain. Aldobiuronic acids (i.e. disaccharides, consisting of a uronic acid unit glycosidically linked to a neutral sugar unit) are obtained by acid hydrolysis and some pseudoaldobiuronic acids (disaccharides consisting of a neutral sugar unit glycosidically linked to a uronic acid unit) by enzymic hydrolysis. These pseudoaldobiuronic acids are breakdown products of the side chains covalently linked to the main chain (at C_3 of galacturonic acid monomers and at C_4 of rhamnose monomers). Thus it has become clear that pectic substances are not homopolysaccharides but heteropolysaccharides. A model pectin molecule might consist of a main chain of galacturonan, containing blocks of rhamnose rich regions, with mainly arabinose, galactose and xylose in the side chains (Rombouts, 1972; Pilnik & Voragen, 1970).

Pectic substances are characterized by:

1. Degree of polymerization (DP). Figures for molecular weight given in the literature (Kertesz, 1951) range from 30 000 to 300 000, consequently the DP varies between 160 and 1600.
2. Degree of esterification (DE). Each carboxyl group of the uronic acids can be methylated. Theoretically the DE varies between 0 and 100%. When a pure galacturonan is fully methylated its methoxy content is 16.32%. Methoxy groups can be distributed at random or blockwise.
3. Degree of esterification with acetic acid. Pectic substances of some plants (especially sugar-beet and pear) are partially esterified with acetic acid at C_2 and C_3.

4. Content of non-galacturonide materials and the distribution of these along the main chain.
In view of all the possible variations, it becomes probable that there are hardly two identical molecules in a pectin preparation.

The structure of pectic substances is thus only partially understood. The water-insoluble parent substance of the pectic substances in plant cell walls, the protopectin, is believed to consist of giant molecules of soluble pectin of high molecular weight and bound covalently to hemicelluloses and possibly to other cell wall substances such as cellulose (Keegstra et al., 1973). Furthermore it is admixed with these substances and thus enmeshed mechanically as well as chemically.

Pectic substances have been extensively reviewed by Kertesz (1951). More recent reviews have been given by Doesburg (1965), Neukom (1967), Pilnik & Voragen (1970), Pilnik & Zwiker (1970), Voragen & Pilnik (1970a) and Rombouts (1972). The chemistry of protopectin has been reviewed by Joslyn (1962).

Deesterification and Ca-sensitivity. For the enzymic attack on pectic substances (see Section *1.3.2*) the distribution of the methyl ester groups along the pectin molecule is very important. It has been known for a long time that this distribution is determined by the way of preparation of low methoxy pectin (Baker, 1948; Kertesz, 1951). Deesterification of pectin can be accomplished by the following methods: (1) by the use of acids, (2) by the use of alkali, and (3) by the use of pectin esterase.
1. Acid deesterification without depolymerization is performed at a high acidity (e.g. pH 0.3) and temperatures below 50°C. Simultaneously many of the hemicelluloses present are hydrolyzed, which does not occur during alkaline or enzymic deesterification. Acid deesterification is a very slow reaction compared with alkaline saponification which proceeds more than 100 times faster at pH 11 and at the same temperature.
2. Alkaline saponification is thus much faster than deesterification by acid; however, under alkaline circumstances degradation also takes place. Already at room temperature β-eliminative cleavage of glycosidic linkages is observed. At elevated temperatures β-elimination becomes dominant. Degradation can be prevented by saponification at low temperatures (ca. 0°C). Noteworthy is the fact that β-elimination occurs already at pH 5 and 80°C (softening of vegetables during cooking). Pectates are by far more resistant against β-elimination as this type of cleavage occurs at the glycosidic linkage adjacent to an esterified carboxyl group. Alkaline saponification can also be

accomplished by the use of ammonia. In this case, however, the saponification can be accompanied by amidation (Slavíčková, 1961). Amidated pectins require less Ca to form gels and tolerate more Ca before they precipitate than low methoxy pectins obtained by acid deesterification (Lockwood, 1972).

3. Enzymic deesterification by plant pectin esterase is rapid at pH ca. 7 and 30-40°C. It is known that pectin esterase attacks a methyl ester group adjacent to a free carboxyl group and proceeds along the pectin molecule removing the ester groups one by one (Solms & Deuel, 1955). Chemical deesterification by acid or alkali occurs at random. Thus, enzymic deesterification results in a blockwise distribution of the free carboxyl groups and this is the cause for an increased Ca-sensitivity (Kohn et al., 1968). According to Deuel et al. (1950) pectinic acids are not precipitated by calcium ions when their DE is above 50%. However, enzymically deesterified pectin can be precipitated by Ca-ions at a higher DE. This is easily demonstrated by mixing a high ester apple pectin (75% esterified) with citrus-PE. The reaction mixture contains 100 mg Ca^{2+} per g pectin. As soon as a Ca-pectinate gel is formed the reaction is stopped by adding acid/alcohol mixture thus precipitating the pectin. The DE of the pectin is between 68 and 70% (Pilnik, private communication).

Solubility of pectic substances and their extractibility from plant material. The various types of pectic substances have different solubility characteristics. Pectic acid is only soluble in water after partial neutralization. The water-solubility increases with higher DE and shorter chain length. Fully methylated pectin is not sensitive to di- or polyvalent cations but with decreasing DE sensitivity to these increase and precipitation can occur. Based upon these characteristics methods for fractional extraction were developed (McColloch, 1952; Dietz & Rouse, 1953; Rouse & Atkins, 1955). At first an alcohol-insoluble-solids (AIS) precipitate is prepared. The AIS are then extracted with (1) water (this gives the high methoxy pectins), (2) water containing Ca binding agents such as oxalate, polyphosphate or EDTA (this gives the low methoxy pectins and pectic acid) and (3) with hot acid (0.05 N HCl at 80-90°C) or cold alkali (0.05 N NaOH) (this fraction consists of the protopectin). Fractional extractions are used in trying to correlate pectic changes in fruit and vegetables with ripening, storage and processing. The same is done in cloud stability studies (Koen Mosse & Royo Iranzo, 1960; Primo Yúfera et al., 1961b; MacDowell, 1962). The method is open to several objections. Extraction procedures (extraction times and temperatures) must be adapted to the various plant materials in order to give the highest yields for each

product. The number of investigations about the rate and extent of extraction of pectins from plant tissue preparations is limited, although a considerable amount of data is available on the chemical composition of extracted pectins (Joslyn & Deuel, 1963). The various pectic substances have overlapping solubilities. E.g. low methoxy pectins may also be water-soluble depending on the cation composition of the fruit; on the other hand pectins demethylated by enzyme may be Ca-sensitive at a relatively high DE (McCready & McComb, 1952).

Sinclair (1961b) has given pectin contents of various parts of citrus fruit expressed as Ca-pectate of the alcohol insoluble solids and calculated on a dry weight basis (percentages between brackets) as follows: (1) in whole peel 39.6% (17.4%), (2) in albedo 36.5% (19.6%), (3) in the vesicles 29.1% (3.1%) and (4) in the pulp 35.0% (5.1%). Pectin contents of orange juice, expressed an anhydrogalacturonic acid, range between 28 and 210 mg/100 g (Royo Iranzo, 1972).

3.2 PECTIC ENZYMES

Pectic substances can be attacked by two main groups of pectic enzymes, the saponifying enzymes or pectin esterases and the pectin depolymerizing enzymes. The pectic enzyme literature has been reviewed recently by Pilnik & Voragen (1970), Voragen & Pilnik (1970a), Voragen & Pilnik (1970b), Rombouts (1972), Rombouts & Pilnik (1972) and Voragen (1972).

Saponifying enzymes. Pectin esterase (PE) or pectin pectyl-hydrolase, number 3.1.1.11 of the International Enzyme Commission, is found in many higher plants e.g. apple, citrus and tomato (Pilnik & Voragen, 1970), but also produced by some microorganisms e.g. *Fusarium oxysporum* f.sp. *vasinfectum* (Miller & Macmillan, 1971) and *Clostridium multifermentans* (Lee et al., 1970). The optimum pH for PE depends on its origin: tomato and orange PE have an optimum pH of 7.5, apple somewhat lower, viz. 6.6, fungal PE has an optimum value between 4 and 5 and bacterial PE between 7.5 and 8. Molecular weights, determined by ascending chromatography on Sephadex G-75, superfine, are for tomato PE 27 500 (Miller & Macmillan, 1971), for 4 tomato iso-enzymes (determined by analytical column gelfiltration on Sephadex G-100 with 0.15 M NaCl) from 24 300 to 35 500 (Pressey & Avants, 1972), for fungal PE 35 000 and for clostridial PE 400 000 (by Sephadex G-200, supposed to be an esterase-lyase complex, Miller & Macmillan, 1971).

PE is very specific in hydrolyzing the methyl ester groups of pectinic acids. The methyl ester of polymannuronic acid (alginate) is not split off, neither are the methyl esters of mono-, di- and trigalacturonic acids (McCready & Seegmiller, 1954). According to MacDonnell et al. (1950) the glycol and glycerol esters are not attacked, but the ethyl ester is hydrolyzed very slowly; which has also been described recently by Manabe (1973). As regards the mechanism of attack Schultz et al. (1945) were the first to postulate that orange PE begins its saponifying action on methyl ester groups next to a free carboxyl group and then continues to act along the molecule. Solms & Deuel (1955) showed that PE acts more quickly on alkali presaponified pectin than on enzymically presaponified pectin. The enzyme would act in both directions. They noticed that pectin was not fully saponified, as always a residual DE of 10-11% was found. They ascribed this phenomenon to irregularities in the pectin molecules (e.g. neutral sugars). Lee et al. (1970) investigated a pectin esterase-pectate lyase complex of *Clostridium multifermentans*. The lyase activity and the pectin esterase activity were demonstrated to act simultaneously beginning at the reducing end of the pectin molecule. For tomato PE (Lee & Macmillan, 1970) and fungal PE (Miller & Macmillan, 1971) it was demonstrated that the PE activity initiated for more than 50% near the reducing end of highly esterified pectin molecules.

PE activity is considerably influenced by the concentration of cations present. At pH 7.5 orange PE has maximum activity if 0.12 M monovalent cations are present or 0.02 to 0.05 M divalent cations. Larger concentrations inhibit PE activity particularly in the case of divalent cations. At pH 3.8 0.1 M divalent cations or 0.4 M monovalent cations are necessary for maximum activity which in the former case is only little more than half and in the latter case one-third of the maximum activity observed at pH 7.5. Optimum pH is at pH 7.5 in 0.15 M NaCl but shifted to more alkaline pH values when the salt concentration is decreased (MacDonnell at al., 1945).

PE from one source can consist of several isoenzymes as reported for banana by Hultin & Levine (1963), Hultin et al. (1966) and for tomato by Pressey & Avants (1972).

Inhibition of PE by sugar is reported by Chang et al. (1965) for papaya PE, by Hultin et al. (1966) for banana PE and by Lee (1969b) and Lee & Wiley (1970) for apple PE. About 40% of the apple PE activity is inhibited in the presence of 15% sucrose.

Lee (1969a) and Lee & Macmillan (1968) found polygalacturonic acid inhibition of tomato PE. With pectin N.F. (70% DE) tomato PE has a K_m of

4×10^{-3} M anhydrogalacturonate residues and is inhibited competitively by polygalacturonic acid with a K_i of 7×10^{-3} M anhydrogalacturonic acid residues. The same authors tentatively proposed tomato PE to be a lipoprotein. Lineweaver & Ballou (1945) found alfalfa PE slightly inhibited by sodium pectate at pH 8.5, but strongly inhibited at pH 5.7 in the absence of cations. No inhibition occurs at pH 5.7 if sufficient cations are present.

According to MacDonnell et al. (1945) orange PE was found to be associated with the solid particles. Neither the clear juice of the edible portion of the orange nor the pressed juice of the flavedo contained appreciable activity. On a wet-weight basis, the relative enzyme contents of the flavedo, albedo, and cell sacks were approximately 1.0, 0.8 and 0.5. Jansen et al. (1960) discussed the binding of PE to orange cell walls, the nature of which is an enzyme-substrate complex. Pectin esterase activity in citrus products has been reviewed by Joslyn & Pilnik (1961).

Depolymerizing enzymes. Pectin depolymerizing enzymes can be classified according to three criteria namely: (1) hydrolytic or transeliminative splitting of the glycosidic bonds (Fig. 1), (2) statistical or terminal mechanism of attack (endo- respectively exo-enzymes) and (3) preference for pectic acid or pectin as substrate. Thus theoretically eight groups are obtained. The scheme according to Neukom (1963) and Koller (1966) is shown in Table 1.

Enzymes belonging to the exo PMG and exo PL groups have never been des-

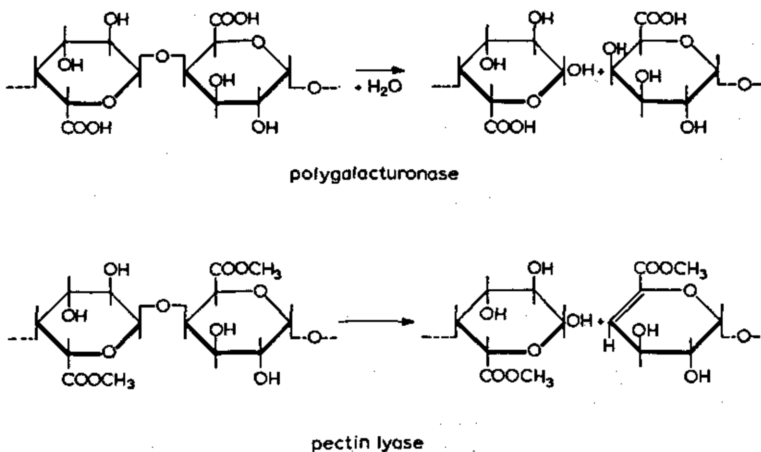


Fig. 1. Splitting mechanisms of the 1,4 glycosidic bond. Hydrolytic splitting of a pectic acid chain by polygalacturonase and transeliminative splitting of a pectin chain by pectin lyase.

Table 1. Classification of depolymerizing pectic enzymes (Neukom, 1963; Koller, 1966). (Numbers between brackets are based on the recommendations on enzyme nomenclature of the International Union of Biochemistry as assigned to the enzymes by Koller (1966).)

Pectic enzymes acting mainly on pectin		Pectic enzymes acting mainly on pectic acid	
polymethylgalacturonases (PMG)	pectin lyases[1] (PL)	polygalacturonases (PG)	pectate lyases[1] (PAL)
1. endo PMG (3.2.1.41)	3. endo PL (4.2.2.3)	5. endo PG (3.2.1.15)	7. endo PAL (4.2.2.1)
2. exo PMG	4. exo PL	6. exo PG (3.2.1.40)	8. exo PAL (4.2.2.2)

1. The term 'lyase' is preferred to 'transeliminase' by the International Union of Biochemistry (Florkin & Stotz, 1965).

cribed. Since the discovery of lyases by Albersheim et al. (1960) the existence of endo PMG has become disputable (Rombouts, 1972). The enzymes of Table 1 prefer high polymerized pectinic acids as substrate and degrade these to mixtures of oligomers. These oligomers can be further degraded by specific oligomerases (either hydrolytic or eliminative) which have the property of degrading their substrate at a rate which is inversely proportional to its chain length (Hasegawa & Nagel, 1967, 1968; Moran et al., 1968; Hatanaka & Ozawa, 1970). However, depolymerases which prefer high polymer substrate are also active on oligomers. A survey of the activity of pectate degrading enzymes on oligogalacturonides has been given by Voragen & Pilnik (1970b).

Recently it has become evident there are enzymes that can not easily be classed within the context of Table 1 because they are intermediates between PL and PAL. Specifically, enzymes produced by *Arthrobacter* and *Bacillus polymyxa* prefer 21 to 44% esterified pectinic acid. Pilnik et al. (1973) therefore proposed a new classification of pectin depolymerases based on activity

Table 2. Classification of pectin depolymerases - new proposal (Pilnik et al., 1973)

Hydrolases	Lyases	
polygalacturonases (PG)	LM pectin lyases (LMPL)	pectin lyase (PL)
1. endo PG	3. endo LMPL	5. endo PL
2. exo PG	4. exo LMPL	

upon glycol esters of pectin. Pectin lyases are glycol ester inactive, while 'low methoxy pectin lyases (LMPL)' are glycol ester active. The new proposal is shown in Table 2. Pilnik et al. (1974) obtained evidence from investigations with glycol esters of pectins, amidated pectins and PMR studies of breakdown products that PL is very specific for methoxy groups and LMPL for free carboxyl groups. Breakdown products from PL action will show a methylated unsaturated galacturonide unit at the non-reducing end and those from LMPL action will show oligomers with a non-methylated unsaturated galacturonide unit at the non-reducing end.

Endo-PGs are the most widely distributed and most frequently occurring pectin depolymerases in nature. They occur in fruits, stems and leaves of many higher plants (Pilnik & Voragen, 1970). It is the only pectin depolymerase known to be produced by yeasts (Phaff, 1966). The enzyme is produced constitutively by *Kluyveromyces fragilis* (Phaff, 1966), but most fungi produce it adaptively together with other pectic enzymes such as pectin esterase, exo polygalacturonase and pectin lyase. The preferred substrate is pectic acid but also pectin is attacked at a lower rate and to a lower hydrolysis limit. Hydrolysis limits for pectin preparations with different DEs, obtained by chemical saponification of completely esterified pectin, are found to decrease with increasing degree of esterification and to become zero at 75% esterification (Koller & Neukom, 1969). Several authors state that the carboxyl groups at both sides of the glycosidic bond to be split should be free (Ayers et al., 1969; Luh & Phaff, 1954; McCready & Seegmiller, 1954). Koller & Neukom (1969) deduced experimentally that two free carboxyl groups at a certain distance are required. Optimum pH values for PGs depend on the applied buffers; for oligouronides the optimum pH is often lower than for pectic acid. In most cases the optimum pH lies between pH 3.5 and 5.6. Optimum pH for the LMPL mentioned above lies between 8.0 and 9.8, for PL generally between 5.1 and 6.3, however, a new increased optimum pH may be found at pH 8.0 to 8.5 if calcium ions are present (Voragen et al., 1971). PL may still be active in orange juice whereas LMPL shows no activity below pH 7.0 and is therefore not further considered in this study.

According to MacDonnell et al. (1945) PG is absent in orange. Mannheim & Siv (1969) did not find PG or PG inhibitors in oranges, mandarins and lemons. However, some PG activity was found in grapefruit.

3.3 PECTIN ESTERASE IN ORANGE JUICE; ACTIVITY AND ACTION

Pectin esterase is the only pectic enzyme occurring in citrus fruit with the exception of polygalacturonase in some grapefruit varieties (Mannheim & Siv, 1969). Cloud loss in citrus juices or gelation in citrus concentrates is usually ascribed to PE activity (Joslyn & Pilnik, 1961). In the investigation of these problems methods to determine PE activity are required. When PE activity occurs the DE of pectinic acid decreases, one molecule of methanol is formed for each ester link split and the pH is lowered. All known PE assays are based on these phenomena, viz. the pH change, the increase of free carboxyl groups or decrease in methyl ester groups and the release of free methanol.

For a qualitative PE assay the sample to be investigated is mixed with a pectin solution, the pH adjusted and the pH drop followed either electrometrically or by means of indicators (Kertesz, 1951, p. 361). Besides the pH drop test the amount of alkali necessary to readjust the pH to the original value (titration test) can be taken as a criterium (Pilnik & Rothschild, 1960). Fractional extraction of pectic substances can give indication of PE presence but objections to this method have been discussed in Section 3.1.

Methyl ester groups of pectic substances can be converted by alkaline hydroxylamine into pectin hydroxamic acid, which forms a red coloured insoluble complex with ferric ions. When samples to be investigated for the presence of PE are brought upon filter paper discs and these placed on a pectin-agar gel plate for a certain time, these plates will show, after flooding with hydroxylamine, sodium hydroxide, hydrochloric acid and ferric chloride solutions, clear zones on a red background indicating which paper discs contained active PE. This method is sensitive to approximately 1 to 0.1 unit PE (µmol ester split/min, McComb & McCready, 1957, 1958). With this method, used histochemically, Gee et al. (1959) showed that pectin in fresh fruit (apple, pear, peach) reaches virtually full esterification at the onset of ripening, after which the DE decreases.

When diluted PE active fruit juice is mixed with a pectin solution (DE between 55 and 65%) and Ca-ions, the saponification of the pectin after some time will cause a Ca-pectinate gel to be formed. With this geltest Pilnik & Rothschild (1960) were able to detect within 4 days PE activity in a mixture of 99.5% pasteurized and 0.5% fresh juice.

For quantitative measurement of PE activity a titrimetric method based upon neutralization of released free carboxyl groups is recommended. The method

is described in detail by Kertesz (1951, p. 362), Vas et al. (1967) and Leuprecht & Schaller (1968). Stegeman (1970) determined the optimal conditions for tomato PE: 25 ml of a 1% ca. 65% esterified apple pectin solution (green ribbon, Obipektin, Bischofszell, Switzerland) with 0.1 M NaCl added are adjusted to pH 7.5 and N_2-gas is bubbled through the solution in order to expel carbon dioxide. After equilibration at 25°C, 0 to 10 ml of the enzyme solution to be assayed are added and the pH maintained at 7.5 by automatic titration with 0.01 N NaOH. Consumption of alkali is recorded and the activity derived from the cotangent of the line presented. Rombouts (1972) pointed out that no accurate measurements are obtained if the titrimetric method is applied in the acid range (pH≤4, necessary for e.g. fungal PE with a pH optimum near 4) because of the acid behaviour of pectins. He recommends to allow the mixture to react for a certain time and then to raise the pH of the solution quickly to near 6 with 0.1 N NaOH. Hereafter the reaction mixture is titrated to pH 7.0 with 0.01 N NaOH. The same is done in a blank determination with heat inactivated enzyme. From the titrigram the alkali consumed and the reaction time are taken and the activity calculated. The activity of PE is expressed as PMU (pectin methyl esterase units), calculated as milligrams of methoxy groups split off in 30 min, or as PEu (pectin esterase units) that is milli-equivalent ester saponified per min. Under the same conditions 1 PEu corresponds to 930 PMU. According to the Enzyme Commission of the International Union of Biochemistry one unit of enzyme is defined as that amount which will catalyse the transformation of 1 µmol of the substrate per min under standard conditions. Furthermore this commission suggested that these standard conditions be at 30°C and in an optimum chemical environment in respect to pH and substrate concentration. A pectin esterase unit calculated as 1 µmol ester saponified/min or 1 µmol methanol released/min under the conditions of Stegeman (1970) most closely approximates the definition of the Enzyme Commission.

All the methods discussed so far are not suitable to measure PE activity in situ. With the titrimetric method e.g. one determines PE activity under conditions and on a substrate that are quite different from those in a citrus juice.

As each enzymic split of a methyl ester group releases one molecule of methanol, quantitative determination of methanol offers the best perspective for in situ determination of PE. In determining methanol quantitatively one determines the exact action of PE while with the methods mentioned above only one activity is measured. However, the quantities of methanol being at issue

are very small. The mean value for pectin content in orange juice amounts to approximately 0.1% (w/v). Upon full deesterification, depending on the DE, about 0.01% (w/v) methanol as the maximum can be expected. This means that 0.001% and less methanol is to be determined.

Methanol can be determined either by the determination of methyliodide after reaction with HI (Zeisel method; Clark, 1932) or colorimetrically by the determination of formaldehyde after oxidation. Various colorimetric methods are practised in determining methanol: (1) with Schiff's reagent, (2) with chromotropic acid (Boos, 1948; Mathers, 1958 and Mathers & Pro, 1955), and (3) with pentane-2,4-dione (Wood & Siddiqui, 1971). In the Zeisel method ethers, esters, alcohols and thioethers will react with HI, e.g. the methoxygroup of the B-ring of hesperidin will also give methyliodide. The method can be made very specific for the various iodides by gas chromatography (Kratzl & Gruber, 1958; Vertalier & Martin, 1958). The colorimetric methods need an initial distillation step, are laborious and most of them lack sensitivity. The method of Wood & Siddiqui (1971) appears to be the most sensitive.

Gas chromatographic assays of polar compounds such as alcohols have never been very successful because of adsorption on the column materials which results in tailing peaks and poor separation of the low molecular compounds. Determination of alcohols as alcohol nitrites by head space technique is described by several authors (Gessner, 1970; Bartolome & Hoff, 1972; Litchman & Upton, 1972), however, quantitative assay by head space technique is known to be difficult. In the last 10 years porous polymers of ethyl vinyl benzene, styrene and styrene with divinyl benzene were developed. These polymers which are now available are in bead form with closely defined pore diameters. The porous polymers are used in gas chromatography without a liquid phase (gas solid chromatography, g.s.c). According to Cieplinsky & Spencer (1967) and Hollis & Hayes (1966) they can be applied for determination of trace amounts of water in organic solvents as well as for determination of trace organic chemicals in water. As an accurate assay of methanol in orange juice is of great help in studying cloud loss phenomena, much attention is given to this g.s.c. technique in this thesis.

4 The measuring of cloud changes

4.1 SEDIMENTATION RATE OF PARTICLES IN A FLUID

By a mechanistic approach to cloud problems an orange juice in the process of clarification could be considered as a sedimentation system. Stokes' sedimentation law describes the sedimentation of spherical particles in a fluid:

$$v_s = \frac{(\rho_s - \rho_f)\, g\, d^2}{18\eta} \tag{1}$$

where

v_s = sedimentation rate of the particle (m/s)
ρ_s = density of the particle (kg/m^3)
ρ_f = density of the continuous phase (kg/m^3)
g = acceleration due to gravity (m/s^2)
d = diameter of the particle (m)
η = viscosity of the continuous phase ($N \times s/m^2$)

An important parameter is the Reynolds number:

$$Re = \frac{\rho_f\, v_s\, d}{\eta} \tag{2}$$

The region of validity of Stokes' law is restricted to Reynolds numbers greater than approximately 10^{-4} and smaller than approximately 1. Under 10^{-4} particles do not show steady sedimentation because of the influence of the Brownian movement, above 1 laminar flow changes into turbulent flow. Another restraint is imposed by the relative volume of the dispersed phase: if the dispersed phase takes up 1% of the total volume of continuous phase sedimentation is hindered already because of interaction between the particles.

From Stokes' law it follows that the rate of sedimentation can be reduced by the following measures:

1. Diminishing the difference in density (a) by raising the density of the continuous phase, (b) by lowering the density of the particles.
2. As v_s is proportional to the square of the particle diameter, homogenizing

should be very effective.
3. Raising the viscosity of the continuous phase e.g. by adding thickening agents.

Application of Stokes' law to citrus juice does imply a considerable simplification of the natural system because,
1. Cloud particles are not all identical. As described in Section 2.2.1 cloud particles are composed of oil globules, particles of fruit tissue of different types, chromoplastids and hesperidin crystals. By an equilibrium zonal centrifugation technique it was found that for self pressed orange juice the density of most of the particles ranges from 1.15 to 1.40 x 10^3 kg/m^3.
2. Cloud particles in general are not spherical.
3. Cloud particles do not settle independently. They hinder each other e.g. by electrical and steric interaction.
4. Cloud particles may be changed by pectin esterase action. Instead of enhanced hydratation (a stabilizing factor) ionic bridges with e.g. Ca^{2+} can be formed as a consequence of such activity, which may result in condensed particles or coagulation.

As cloud destabilization is characterized by increasing changes in 'cloud density'[1], two principally different types of cloud stability measurement present themselves: (1) Measurement of the quantity of settled pulp. (2) Measurement of the turbidity of the supernatant.

4.2 THE QUANTITY OF SETTLED PULP

The pulp content of a juice or fruit-drink is a measure of the fruit content and of the turbid ingredients in the beverage. It adds to the eye appeal of the beverage and reminds the consumer of juice freshly expressed by hand. Determining the pulp content under well-defined conditions can give some information about the cloud stability of the juice or of the beverage.

According to an American standard (Hendrix & Jefferson) pulp content is defined as the portion of the suspended particles which settles when 50 ml juice in a graduated tube with a conical bottom are centrifuged for 10 min at approximately 360 x *g* The pulp content is expressed in % v/v. This method has a number of disadvantages as is apparent from a study of the published literature data.

1. This is an expression widely used in literature indicating the optical appearance.

Dupaigne (1960) enumerates some practical considerations. The stated centrifugal acceleration only applies to the bottom of the tube and decreases in the direction of the top of the tube. The acceleration profile in the tube therefore depends on the shape of the tube. Indeed, a larger diameter tube results in a more uniform distribution of the centrifugal field, but then the reading becomes more inaccurate. Cloud particles are deformable and elastic. In connection with this the sediment becomes much denser at a higher centrifugal acceleration or at a longer centrifuging time. Dupaigne also stated that the relation between pulp content and centrifuging time at a fixed centrifugal acceleration, as well as the relation between pulp content and centrifugal acceleration at a fixed centrifuging time, are both logarithmic functions. This means that small deviations in the number of revolutions or centrifuging time are sufficient to affect considerably the results of pulp content measurements. Besides, it is difficult to state a precise centrifuging time as the acceleration and braking of the centrifuge influence the result. Furthermore tachometers generally are not very accurate. As an improvement Dupaigne proposes to maintain a centrifuging time of 10 min but increasing the centrifugal acceleration to 4000 x g, thus diminishing the effect of deviations in rev/min or time. Schaller & Mihalovics (1959) demonstrated that the pulp volume is not a constant value. Because of its elasticity the pulp volume expands slowly after centrifugation. Rouse & Atkins (1955) also centrifuged off the pulp which they washed out several times and then dried and weighed. Dupaigne (1967) pointed out that there is no relation between dried total insoluble solids and the pulp content as determined by centrifugation. This is explained by the fact that the dry weight of the total insoluble solids does not depend on the shape or elasticity of the particles as does the centrifugal method.

After examining some other methods Dupaigne (1967) proposed a new method. An equal volume of acetone is added to the juice which leads to a partial coagulation of the pectin and thus favours sendimentation. The extra volume of sediment from soluble solids is negligible compared to the total pulp content. After centrifugation a more coherent sediment is obtained and values are more reproducible, especially if the quantity of pulp is determined by weighing after decantation. From a later paper of Dupaigne (1970) however, it appears that he has come back to the direct centrifugal method (10 min at 4000 x g).

Bielig & Klettner (1971) expressed the pulp content of tomato juice as the so-called ratio R = pulp/serum (g/g). In experiments with diluted concentrates these authors saw that this ratio is both dependent on the dilution of

the centrifuged juice (expressed in ^{o}Bx) and on the applied centrifugal acceleration. If the acceleration increases R decreases; if ^{o}Bx increases R increases too. At an optimal centrifugal acceleration it is possible to compose curves from the results of various juices giving the relation between ^{o}Bx and R. The authors were able to relate the relative position of these curves with the cloud stability of the juices examined. Besides the content of insoluble dry matter R also expresses the resultant of all forces that play a role between the particles.

Both, the described centrifugal methods and the determination of R are not suitable for routine measurements of cloud stability on small samples (e.g. 100 ml) during an extended period (several weeks). The first method is not very accurate and demands 50 ml sample for a single determination. The second method is too laborious and requires too much sample. It must also be realized that besides the content of fruit solids, the condition of the cloud (stable or coagulated) and the content and type of pectin affect the pulp volume considerably.

4.3 TURBIDITY IN THE SUPERNATANT PHASE

Turbidity measurement on the supernatant phase of centrifuged samples is a widely used method for determining cloud stability. A light beam is sent through a sample in a cuvette and attenuated by scattering by the cloud particles present. As a measure for the quantity of cloud particles in suspension one can determine either the scattered light or the transmitted light. The scattered light can be measured by a nephelometer as is often done in microbiology for bacterial suspensions but rarely in citrus industry and research. Usually the turbidity is determined by measuring the transmitted light beam. Different types of apparatus are suitable for this purpose. In colorimeters a small band of the polychromatic light is selected by a filter to determine extinction (E) or percentage of light transmission (%T). In spectrophotometers the same is done by means of nearly monochromatic light. Making use of the Trübungsmesser (Lange, Berlin) one determines an arbitrary value by means of polychromatic light: at first the cuvette is placed right before the photocell so that nearly all the light is caught and the meter is adjusted to zero. A second reading is made after the cuvette has been placed at a greater distance from the photocell, so that part of the scattered light is lost. This measurement is therefore not influenced by light absorption.

Most frequently use is made of a colorimeter. This is a cheaper appara-

tus than a spectrophotometer. The filters used range from 580 to 730 nm. Hendrix & Jefferson recommend a 650 nm filter, the Florida Citrus Experiment Station recommends 730 nm (cited by Hendrix & Jefferson). In practice a 650 or 660 nm filter is most widely used. Authors who use a spectrophotometer also work at 660 or 720 nm (Primo Yúfera et al., 1961a; Endo, 1964; Ishii & Yokotsuka, 1971). Making use of a relatively high wavelength is favourable as at the lower wavelengths (<600nm) attenuation of the light beam by absorption of the yellow colour of the juice is not negligible. Data obtained with colorimeters or spectrophotometers are presented as %T. Sometimes data are expressed as bentonite values (Peleg & Mannheim, 1970a, 1970b; Baker & Bruemmer, 1970, 1972; Mizrahi & Berk, 1970). In these cases the apparatus has been calibrated on a bentonite suspension according to Senn et al. (1955). To my knowledge only one author so far has expressed turbidity value as E (Lankveld, 1973). This is remarkable as on a theoretical base it is more logical to express turbidity by E than by %T (see below).

Kertesz (1950) describes a very simple apparatus (Turbidity Tester) for determining the Turbidity Number (TN). It is based on the principle how far (in cm) one can recognize a number looking through a suspension.

As regards the preparation of the supernatant many different combinations of centrifuging time and centrifugal acceleration are in use. Official recommendations are 10 min at approximately 360 x *g* (Procedure for Analysis of Citrus Juices, 1964; Hendrix & Jefferson).

It is useful to have a look at the theoretical background of light scattering. For spherical particles the following relation can be deduced:

$$E = \lg \frac{I_o}{I} = \frac{1}{4} \lg e \; \pi d^2 \, Q \, N \, l \qquad (3)$$

where

E = the measured extinction
I_o = intensity of incident light
I = intensity of transmitted light
d = diameter of the particle (m)
Q = the light scattering quotient
N = number of particles per unit volume (m^{-3})
l = optical path-length (m)

The extinction is seen to be proportional to the optical path-length (l), the concentration of the particles (N) and the projected area of the particle ($\frac{1}{4} \pi d^2$). The light scattering quotient (Q) is a function of ρ:

$$\rho = 2\pi d\ (n_d - n_c)/\lambda_o \qquad (4)$$

where

ρ = dimensionless parameter of particle size

d = diameter of the particle (m)

n_d = refractive index of the dispersed phase

n_c = refractive index of the continuous phase

λ_o = wavelength of the light in vacuum (m^{-1}) (practically the applied wavelength).

In the region of anomalous diffraction that we encounter in fruit juice suspension $Q = 2 - \frac{4}{\rho} \sin\rho + \frac{4}{\rho^2} (1 - \cos\rho)$ (Walstra, 1964).

In Fig. 2 (taken from Lankveld, 1970) this relation is shown for $n_d/n_c = 1.05$ both for Q and Q^* (the scattering quotient corrected for the forward scattering at an angle of acceptance = 1.5^o). The first maximum in the light scattering quotient occurs at about ρ=4 which corresponds to a particle diameter of about 7 µm at λ = 660 nm and n_d/n_c = 1.05. It is apparent that relatively small changes in particle size may change Q considerably. Applying turbidity measurements to cloud stability studies, one must recognize that the measured extinction does not only depend on the total scattering area ($\sim d^2 \times N$) but

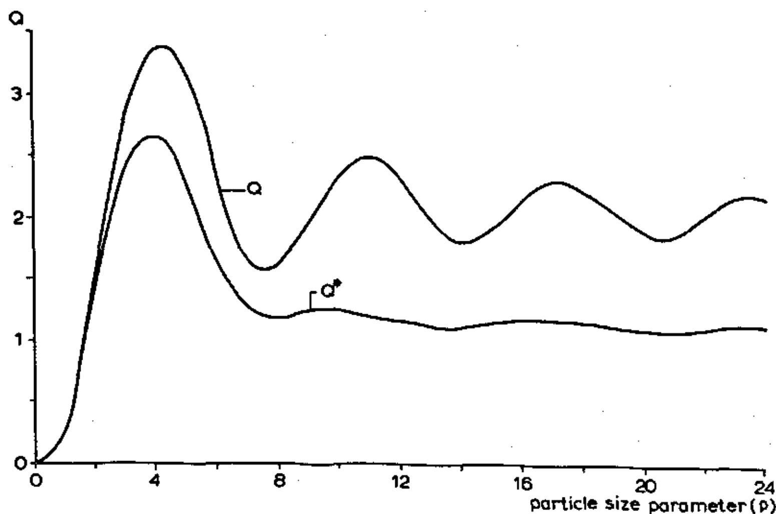


Fig. 2. Light scattering quotient Q, and the qotient Q^* corrected for forward scattering, as functions of the particle size parameter $\rho = 2\pi d(n_d - n_c)/\lambda_o$ for n_d/n_c = 1.05 (taken from Lankveld, 1973).

also on changes in Q (changes in refractive index (sugar content) and in particle size by flocculation or redispersion).

Although suspensions of fruit particles are very heterogeneous in size, shape and refractive index, Lankveld (1973) showed for lemon concentrate, which is normally more finely screened than orange concentrate, a good agreement with equation (3) (within 10%). No multiple scattering occurred and particles were shown mainly to be smaller than the size for which the first maximum in Q occurs. Lankveld was able to correlate the relative mean particle size of samples to the ratio of extinctions measured at 600 nm for a centrifuged and an uncentrifuged sample ($E_{600\ nm,\ centrifuged}/E_{600\ nm,\ uncentrifuged}$). The same could be done if an uncentrifuged sample was measured at two wavelengthes ($E_{600\ nm,\ uncentrifuged}/E_{430\ nm,\ uncentrifuged}$). A high value for $E_{600\ nm,\ centrifuged}/E_{600\ nm,\ uncentrifuged}$ as well as a low value for $E_{600\ nm,\ uncentrifuged}/E_{430\ nm,\ uncentrifuged}$ means a low mean particle size.

5 Materials and methods

5.1 MATERIALS

Orange juice. Oranges (varieties depending on the season) are bought commercially and after washing with tap water and drying stored in a cold room (ca. 5°C) for at least one night. The oranges are cut into halves and pressed by a household rosette juice extractor (Kenwood kitchen-aid). The juice is then finished either by squeezing through cheese-cloth or by screening with the aid of a Seibert vibrating sieve-machine (Ø 0.7 mm).

Orange concentrate. A pasteurized frozen, PE inactive, Valencia orange concentrate of ca. 65°Bx from Letaba (South-Africa) in 10 litre lacquered tin cans was supplied by The Coca-Cola Company. After extraction of the juice by FMC In Line extractors the juice had been finished through 0.20 mm perforation screens. Hold-up time between extraction and pasteurization was 35 min. The cans were kept in frozen storage at -20°C. When needed the contents of one can were distributed in 10 1-litre plastic screw bottles and these were likewise kept at -20°C.

Reconstitution of juice from the concentrate: to 200 g of concentrate 1738 mg $K_2S_2O_5$ (to make 1000 mg SO_2/litre) and ca. 550 ml distilled water are added. The pH is adjusted to 4.0 by the addition of 1 N KOH. In a 1-litre measuring cylindre the volume is then made up to 800 ml. Thus a juice of 10/8 x single-strength is obtained. Samples of 80 ml can now be added with reagents and/or distilled water to 100 ml and single-strength level.

Characteristics of the juice reconstituted from the concentrate: (it should be noted here that serum is the supernatant of juice centrifuged for 20 min at 48 000 x *g* and cloud is the sediment of juice obtained under the same conditions):

- concentration of total soluble solids (TSS):
  juice: 12.7°Bx by refractometer (20°C, uncorrected for citric acid)
  serum: 12.45°Bx by refractometer (20°C , uncorrected for citric acid)

- density:

    juice: 1.044 g/ml (22°C)

    serum: 1.049 g/ml (22°C)

- PE activity measured by gas chromatography (methanol formation) : none
- free methanol: 40 μg/100 ml (g.s.c.)
- pectin content:

    57.4 mg AGA/100 ml juice (AGA = anhydrogalacturonic acid)

    16.7 mg AGA/100 ml serum

    40.9 mg AGA/0.4625 g dried cloud (4 h at 105°C)

- total bound methanol content (obtained by alkaline saponification, g.s.c.):

    juice: 4930 μg/100 ml (4845 μg/100 ml colorimetrically)

    serum: 2000 μg/100 ml

    cloud: 2946 μg/0.4625 g dried cloud

- degree of esterification (DE), calculated from the above AGA and methanol data:

    juice pectin: 47.3%

    serum pectin: 66.0%

    cloud pectin: 39.6%

- protein content (6.25 x N):

    juice: 628 mg/100 g

    serum: 482.5 mg/100 g

    cloud: 151 mg/0.4625 g dried cloud

- cations (assays carried out by the Centraal Instituut Voedingsonderzoek at Zeist, the Netherlands). Na and K were determined by flame spectrophotometry, Mg and Ca by atomic absorption spectrometry.

The figures for depectinized serum are also given:

Cations	mg/100 ml juice	mg/100 ml depectinized serum
Na	1.0	3.0
K	195.6 (460)	462.0
Mg	12.6	12.8
Ca	9.9	10.3

The potassium value between brackets applies for juice with pH raised to 4.0, from which also the depectinized serum has been preparared.

Depectinized orange serum. To reconstituted orange juice 1 U citrus PE/ml juice is added. After 3 to 4 days of incubation at 30°C the flocculated

cloud is removed by centrifugation (15 min at 48 000 x *g*) and a clear serum obtained. The serum is inactivated by heat in a micro-wave oven. The viscosity of this serum could not be further reduced by PG action. The difference between this serum and the serum obtained by simple centrifugation is not only the absence of pectic substances, but also other factors can be removed e.g. hesperidin crystals.

Pectin preparations. Apple pectins (Obipektin AG, Bischofszell, Switzerland) of various degrees of esterification have been used: pink ribbon (ca. 26%), purple ribbon (ca. 35%), green ribbon (ca. 65%) and brown ribbon (ca. 75%).
Pectic acid: polygalacturonic acid (Nutritional Biochemical Corporation, Cleveland, Ohio, USA).

Orcat solution. An orcat solution is a solution of the cations Na, K, Mg and Ca in water with the same concentrations as in orange juice reconstituted from the concentrate mentioned above:

7613	mg KCl + 1738 mg $K_2S_2O_5$	- 4600 mg K/litre
25.4	mg NaCl	- 10 Na/litre
363	mg $CaCl_2.2H_2O$	- 99 mg Ca/litre
1054	mg $MgCl_2.6H_2O$	- 126 mg Mg/litre

Potassium metabisulfite. $K_2S_2O_5$, purum (Fluka AG, Buchs, Switzerland).

Citrus pectin esterase. Orange pulp is obtained by cutting and pressing (Hafico galenic press) 5 kg of whole oranges. The pulp is homogenized (Braun multimix) in 5 litre buffer, containing per litre: 42.5 g borax ($Na_2B_4O_7.10\ H_2O$), 27.5 g boric acid and 40.0 g sodium acetate (pH 8.2). The enzyme is extracted from the pulp after stirring for 2 h. The extract is filtered through cheese-cloth in the Hafico press. The enzyme is 'salted out' by adding 50 g ammonium sulphate per 100 ml filtrate (the pH should stay above 7, MacDonnell et al., 1945) and the solution stored overnight in the cold room. The precipitate which is collected by centrifuging for 15 min at 12 000 x *g* at 0^oC is dissolved in 400 ml distilled water and stored overnight in the cold room; it is then recentrifuged (15 min at 27 000 x *g* at 0^oC) to remove insoluble material. This enzyme solution is dialysed against running tap water for at least two days and can be concentrated by freeze-drying part of it and redissolving this fraction in the remainder of the solution. The en-

zyme solution is distributed in 10 ml portions in culture tubes and kept at -20°C. Three batches have been prepared: (1) from Sunkist oranges (California): 109.0 U/ml, pH 8.0; (2) from Ajui oranges (Argentinia): 248.3 U/ml, pH 7,5; and (3) from Jaffa oranges (Israel): 184.6 U/ml.

The Sunkist and Ajui orange PE preparations were shown to be protease active at pH 8.0 and pH 7.5 respectively. At pH 4.0 no proteolytic action occurred. The Jaffa preparation has not been investigated for protease activity. The Ajui preparation has also been investigated for some hemicellulase activities by G.F. Bom during graduate work in this department. He added the enzyme solution to sugar-derivatives of *p*-nitrophenol at pH 5.2. If enzymic hydrolysis occurs *p*-nitrophenol can be detected by measuring the extinction at 400 nm. In this way the presence of the following enzymes was demonstrated qualitatively in all glycosides available: α- and β-glucosidase, α- and β-xylosidase, α- and β-galactosidase and α-mannosidase.

Yeast polygalacturonase. An endo polygalacturonase is produced constitutively by *Kluyveromyces fragilis* (Jörgensen) v.d. Walt strain CBS 397 (Yeast Division, Centraal Bureau voor Schimmelcultures, Laboratorium voor Microbiologie, Delft, the Netherlands). No other pectin depolymerizing enzymes or PE are produced (Phaff, 1966). An aqueous solution (1080 ml) containing glucose (36 g) is distributed between 3 x 1-litre Erlenmeyer flasks with a large bottom surface. 'Yeast nitrogen base', a salts-vitamins mixture prepared by Difco, (8.04 g in 120 ml) is distributed in 3 x 100-ml Erlenmeyer flasks. All the flasks are sterilized for 15 min at 115°C. The vitamin solutions are aseptically admixed to the glucose solutions and these are then inoculated with the yeast. The flasks are incubated for 4 or 5 days at room temperature. The culture medium with the PG is then obtained by centrifuging for 15 min at 48 000 x *g* at 0°C. The centrifuged culture medium is dialysed against running tap water in the cold for 6 h and then twice for 3 h against distilled water in a 10 litre-container. The dialysate is then freeze-dried, redissolved in little distilled water and kept frozen at -20°C. The resulting PG stock solution still contains sugars and acid, but their presence was seen to favour enzyme stability. Two stock solutions have been prepared: (1) 105 U PG/ml; 6.7°Bx; pH 4.2 and (2) 156 U PG/ml; 8.6°Bx; pH 4.6.

Pectin lyase. Endo pectin lyase preparations were supplied by the Division of Biochemistry of the Agricultural University at Wageningen, the Netherlands. The enzyme is isolated from Ultrazym 20 (CIBA-GEIGY AG, Basel, Switzer-

land). Purification is carried out with adsorption and partition chromatography techniques. It has been demonstrated by van Houdenhoven (personal communication) that the commercial preparation possesses two iso-enzymes, called types I and II. By ion-exchange technique (DEAE Sephadex) the two types could be separated. All preparations used here contained type I: (I) 6.45 U/ml; (II) 179.6 U/ml and (III) 41.4 U/ml.

5.2 METHODS

pH measurements are made with an Electrofact pH-meter, type 52A.

Total soluble solids (TSS), expressed at ^{o}Bx, are measured by an Abbe refractometer (Carl Zeiss). Readings are not corrected for acid content.

Pectin content. Prepare saponified alcohol insoluble solids as follows:
- dialyse 100 g of the product to be assayed against running cold tap water for 24 h;
- empty the dyalysis tube and rinse the tube twice with 5 ml water;
- bring the sample to pH 11 with 10 N NaOH;
- maintain the sample at 30°C for 30 min with occasional stirring;
- neutralize to pH 7 with 2 N HCl and weigh to establish a dilution factor;
- bring 10.0 g of the sample into a 50 ml centrifuge tube and add 17.5 ml 96% ethanol of 70°C;
- place the tube into a waterbath of 85°C and stir occasionally for 10 min;
- lower the pH to 2.5 - 3 with 2 N HCl;
- centrifuge for 5 min at 48 000 x *g* (Sorvall RC2-B);
- remove the supernatant layer by decanting from the precipitated pectic material;
- wash the sediment with ethanol (70%, 20 ml) by stirring;
- adjust the pH to 2.5 - 3;
- recentrifuge the sample for 5 min at 48 000 x *g* and discard the supernatant;
- add one drop of 0.5 N NaOH to the sediment and dissolve itself to an end volume of 20 or 25 ml;
- homogenize pulpy samples e.g. with a vibrating mixer.

After dilution (2 to 10 times) the pectin content, expressed as AGA, is now determined with a carbazole reagent according to Bitter & Muir (1962). A stock solution of 100 µg AGA/ml is prepared by dissolving 120.5 mg D-galacturonic acid, previously dried in a desiccator over P_2O_5, into 1 litre dis-

tilled water saturated with benzoic acid. Dilutions from 5 to 50 μg AGA/ml are used to establish a standard curve; this is necessary for each series of experiments. The accuracy of the method is estimated at ca. 5%.

Measurement of turbidity. The sample is mixed thoroughly by inverting the bottle ten times. An aliquot (10 ml) is withdrawn and placed in a graduated conical 15 ml centrifuge tube. The tubes are centrifuged for 10 min at 360 x *g* in a Christ table centrifuge (type UJ1). About 3 ml of the supernatant are brought into a 10 mm glass cuvette and the extinction is measured at 660 nm ($E_{660\ nm}$) in a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. After the measurement this supernatant is returned into the centrifuge tube and then the contents are mixed by shaking. This juice is then admixed to the contents of the corresponding bottle.

Keeping of samples. Unless otherwise mentioned the samples are held in stoppered glass flasks (100 ml, Ø 52 mm o.d.) in an incubator at 30°C.

Separation of juice into cloud and serum. Separation into cloud and serum is achieved by centrifuging juice in a refrigerated superspeed Sorvall RC2-B centrifuge for 20 min at 48 000 x *g* at ca. 0°C.

Resuspension of cloud. Resuspension of cloud is accomplished by a vibrating mixer, type E1 (Chemap AG, Männedorf, Switzerland).

Determination of activity of citrus pectin esterase. The activity of citrus PE is determined titrimetrically according to Vas et al. (1967) by determining the amount of 0.01 N NaOH required to maintain a pH of 7.5 at 25°C by automatic titration (Combi Titrator 3 D, Metrohm AG, Herisau, Switzerland) when 0.02 ml enzyme or a few ml of orange juice are added to 1% green ribbon pectin solution in 25 ml 0.1 M NaCl. The activity obtained is expressed as μmol ester saponified per ml enzyme solution or orange juice.

PE activity can also be determined by the release of methanol. The enzyme is assayed in a 0.5% brown ribbon pectin solution in 1/3 McIlvaine buffer pH 4.0 or in orcat solution pH 4.0 at 30°C. During ca. 6 h occasionally 5 ml samples are drawn and analysed for methanol content. Methanol release is plotted against incubation time. PE activity is derived from the slope of the curve.

Viscosimetric assay of depolymerase activity. The activity of PG stock solutions is determined by following the decrease in viscosity of a 0.25% low methoxy pectin solution as a function of time: 8 ml substrate solution (0.375% pink ribbon pectin in 0.15 M acetate buffer pH 5.0) are pipetted into an Ubbelohde capillary viscosimeter kept at 30°C. 4 to 0 ml distilled water and 0 to 4 ml (diluted) enzyme solution are injected into the viscosimeter with a syringe. The contents are mixed immediately by bubbling air through. The reciprocal specific viscosity $t_o/(t-t_o)$ (where t_o= flow time of buffer solution; t = flow time of reaction mixture) is plotted against the reaction time in min choosing an amount of enzyme which reduces specific viscosity to half within about 20 min. From the slope of the curve the activity is determined:

$$\text{Activity (units)} = \text{tg}\ \alpha \cdot D \,/\, x\ (\text{min}^{-1} \cdot \text{ml}^{-1}) \qquad (5)$$

where

x = amount of enzyme solution added ($0 < x \leq 4$ ml);

D = dilution factor of the enzyme solution added in respect to stock solution.

The activities of PG and PL can also be determined on various pectins in depectinized orange serum. 300 mg of pectin are moistened with 1 ml ethanol and dissolved in 100 ml depectinized orange serum by heating under stirring to 95°C and rapid cooling to room temperature. The solution is then filtered by suction through a glass-fritted filter. 8 ml of this serum pectin solution are injected with a syringe into the Ubbelohde viscosimeter and hereafter 2 ml 0.3% pectin solution in water and 2 ml diluted enzyme solution in depectinized orange serum are injected into the same vicosimeter. Activity is determined as in the assay described above.

Spectrophotometric assay of the activity of pectin lyase. Lyase activity is determined by following the increase in extinction at 232 nm (absorbance of double bond between C_4 and C_5 conjugated with the esterified carboxyl group) of a high methoxy pectin solution. Into a 10 mm quartz cuvette are pipetted 1.0 ml 1.0% 93% esterified pectin solution (obtained by methylation of brown ribbon pectin), 1.0 ml 2/3 McIlvaine buffer pH 6.5, 0.4 ml distilled water and 0.1 ml (diluted) enzyme solution. Extinction ($E_{232\ nm}$) is measured at 30°C by a Zeiss PMQII spectrophotometer and recorded by a Servogor recorder. One unit of lyase activity is that amount of enzyme that causes an increase of 1 of the extinction at 232 nm per min under the above conditions.

Determination of proteolytic action. Assays of proteolytic activity have been carried out only qualitatively and comparatively. The assay is based on the capability to detach the silver layer from the celluloid of a slip of an exposed and developed black and white film by degrading the gelatin layer. These slips are dipped half into the diluted enzyme solution (pH adjusted) and each hour the slip is wiped with a tissue to determine whether the gelatin has been degraded. The slips can also be placed in orange juice samples to detect protease activity.

Electron microscopic investigation. Apparatus: Philips transmission electron microscope, type EM 300, voltage of acceleration: 60 kV. Samples of dialysed orange concentrate or dialysed orange juice were brought on a copper grid coated with foam Formvar. After drying in air for a few minutes the sample can be introduced directly into the apparatus by the vacuum lock.

Methanol determination. In order to remove interfering substances samples should be distilled before methanol can be assayed. 5 ml of the methanol containing sample are pipetted into a semi-micro Kjeldahl Markham (Quickfit) steam distillation apparatus and rinsed by 3 to 5 ml distilled water. After the apparatus has been heated by steam part of the steam is bubbled through the sample to obtain ca. 1 ml distillate per min. About 10 ml of distillate are collected and weighed to the second decimal. This distillate may be stored in a glass stoppered tube in the refrigerator. In any case care must be taken that no traces of methanol vapours from the outside can penetrate into the solution. The methanol content can be determined either by gas chromatography or by colorimetry.

Gas chromatographic determination. Hewlett-Packard 5750G gas chromatograph with double flame ionization detector. Stainless steel column, 10 ft (3.05 m), 3/16 inch o.d. (4.8 mm), packed with Chromosorb 101, 80-100 mesh (a styrene divinyl benzene polymer; Johns-Manville, New York), conditioned overnight at 200°C. Column temperature 102°C, detector temperature 200°C, injection block temperature 150°C. Carrier gas nitrogen, ca 30 ml/min, hydrogen ca. 20 ml/min, air ca. 500 ml/min. Methanol standard solutions containing 0.79 to 79 µg methanol/ml distilled water are prepared from a 1.0% (v/v) methanol A.R. (Merck) solution. Aliquots of 10 µl are injected with a Hamilton 702 N (25 µl) syringe, in which the sample is on both sides enclosed by ca. 2 µl air and ca. 2 µl distilled water. The methanol peak appears after

ca. 4 min and if no interfering compounds are present (ethanol, acetone) about 10 injections per hour can be made. One Chromosorb 101 filling lasts for ca. 1 month.

Calculation. Each run contains a standard of a concentration which gives a comparable peak area (height multiplied by width at halp height) (Nederlandse Norm, 1970). The methanol content of the sample is then calculated according to the formula

$$G_m = \frac{O_m\ G_s \times 1000}{O_s\ y\ z} \qquad (6)$$

where

G_m = µg methanol/ml sample
O_m = peak area sample
G_s = µg methanol in standard
O_s = peak area standard
x = quantity of distillate
y = quantity of sample distilled
z = quantity of distillate injected in µl

A more simple way of calculation consists in making a standard curve and reading the factor $O_m \times G_s/O_s$ directly from this curve. The first method is used when only a few samples have to be assayed, the latter is preferred when a considerable number has to be assayed.

Colorimetric determination of methanol. Methanol is oxidized to formaldehyde which is determined colorimetrically. The method of Wood & Siddiqui (1971) is used with the following slight modifications: Before the addition of $KMnO_4$ the acidified samples are cooled in an ice-water bath for 15 min. Instead of 0.2 ml 0.5 M $NaAsO_2$ in 0.12 N H_2SO_4 and 0.6 ml distilled water 0.8 ml 0.5 M $NaAsO_2$ in 0.12 N HCl are added. Before the addition of pentane-2,4-dione the tubes are placed for 1 h in a waterbath of 30°C rather than standing at room temperature. After the heating at 60°C the tubes are again placed in the waterbath of 30°C and after exactly 15 min the colour is measured spectrophotometrically at 412 nm. Each sample is determined in triplicate. In each run a control is included. As the time schedule for each run allows for a maximum capacity of 18 tubes, only 5 samples per run can be determined.

Experiments carried out to determine the best method for turbidity measurement

Calibration of turbidity meters. The Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer, used throughout the present study, has been calibrated with a suspension of bentonite with known sieve-analysis (BDH), according to Senn et al. (1955). In Fig. 3 the calibration curve is plotted as $\%T_{660\ nm}$ against g/litre bentonite, in Fig. 4 as $E_{660\ nm}$ against g/litre bentonite.

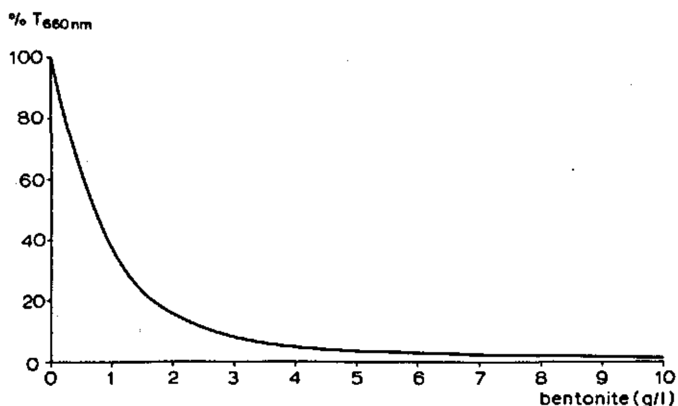


Fig. 3. Calibration of Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer with bentonite suspension. Turbidity expressed as % light transmission.

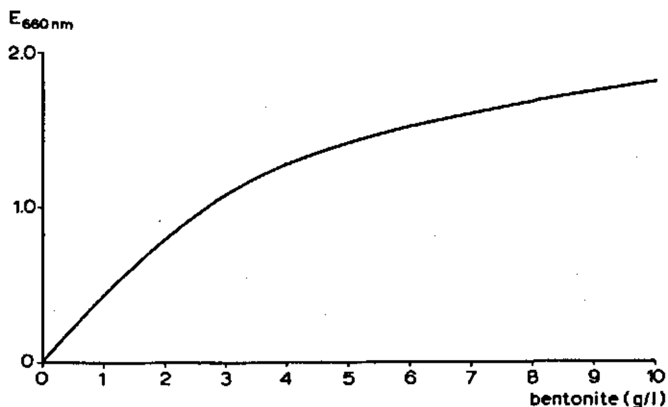


Fig. 4. Calibration of Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer with bentonite suspension. Turbidity expressed as extinction.

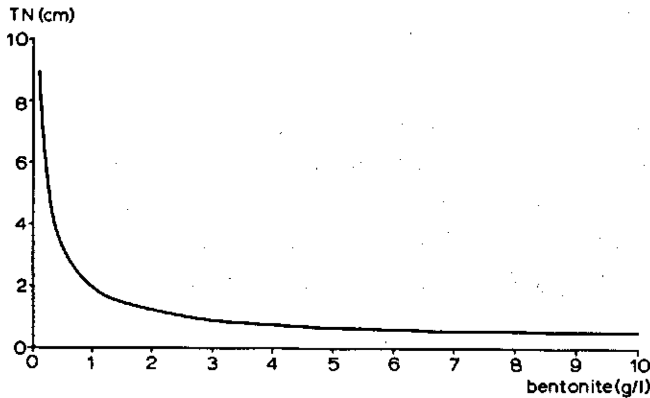


Fig. 5. Calibration of Kertesz turbidity tester with bentonite suspension. Turbidity expressed as cm transparent.

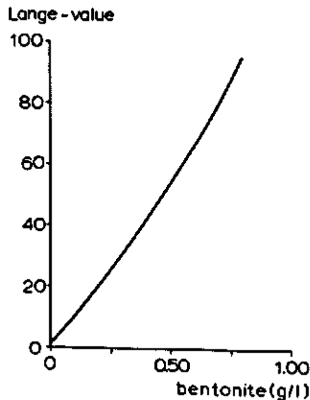


Fig. 6. Calibration of Lange Trübungsmesser with bentonite suspension.

A Kertesz turbidity tester (Kertesz, 1950; Fig. 5) and the Trübungsmesser (Fig. 6) were calibrated with the same bentonite suspension.

It is demonstrated that Lambert-Beer's law is only obeyed in the range 0 to 1.5 g bentonite/litre (Fig. 4). From the %T-plot it becomes evident that transmission values vary considerably with small changes in bentonite concentration at the low concentration and do not vary much with large changes in bentonite concentration at the higher concentrations. This is even more pronounced for the Kertesz turbidity tester. The Trübungsmesser is extremely sensitive to low concentrations of bentonite. The Trübungsmesser and the Kertesz turbidity tester are not very practical for our purpose because of the large volume of sample that is needed or the necessity for dilution.

The preference (cf. Section 4.3) for extinction measurement on theoretical grounds is confirmed by the bentonite calibration curves. The extinction measurement is thus the method of choice for determining turbidity values.

Comparison of different turbidity measurement methods. Several turbidity measurement methods have been compared with a visual judgment of cloud stability in order to evaluate the different methods. Samples of orange juice reconstituted from concentrate were brought to various levels of cloud stability by adding enzymes:

Sample	Added enzyme
A	- (control)
B	1.0 U yeast PG/ml juice
C	0.75 U citrus PE/ml juice
D	0.50 U citrus PE/ml juice
E	0.25 U citrus PE/ml juice
F	1.0 U yeast PG + 1.0 U citrus PE/ml juice
G	0.036 U PL-II/ml juice
H	0.036 U PL-II + 1.0 U citrus PE/ml juice

For each sample 500 ml portions were prepared and divided into 5 100 ml glass stoppered jars. The bottles were kept at 30°C. The various methods used were:
1. The bottle is turned ten times and a 10 ml sample is transferred into a conical centrifuge tube and centrifuged during 10 min at 360 x *g*. Thereupon the extinction at 660 nm of the supernatant is measured in a 1 cm cuvette in a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. The measured supernatant is returned into the centrifuge tube. This is shaken and the contents returned into the corresponding bottle. The $E_{660\ nm}$-values are plotted against incubation time (Fig. 7);
2. The bottle is turned ten times and a 10 ml sample is centrifuged for 10 min at 360 x *g*. 1 ml supernatant is diluted with 14 ml distilled water, mixed by a Whirlmix and measured in a white light Nephelometer (Unigalvo Type 20, Evans Electroselenium Ltd.). The 9 ml left are discarded. The measured values are plotted against incubation time (Fig. 8);
3. The bottle is turned ten times and 1 ml of sample is pipetted into a 1 cm cuvette. After this 2 ml tap water are added, the sample is mixed and the extinction measured both at 720 nm and 550 nm in the same spectrophotometer as described sub 1. The measured values are multiplied by 3. Another 10 ml sample is centrifuged during 10 min at 360 x *g* and the supernatant measured at

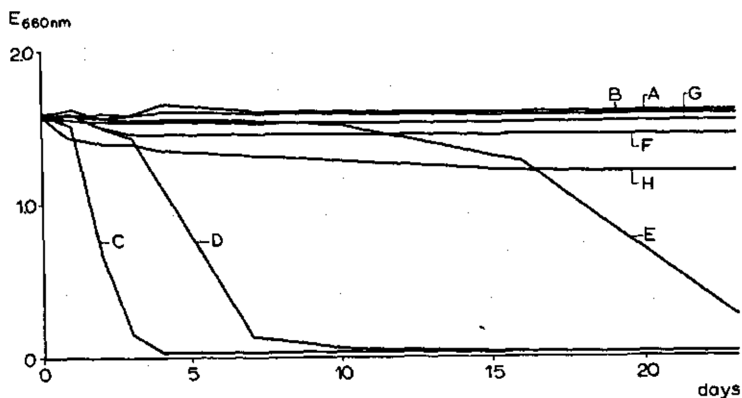


Fig. 7. Cloud stability of 8 different samples of orange juice (see text), as measured with a Hitachi Perkin-Elmer spectrophotometer.

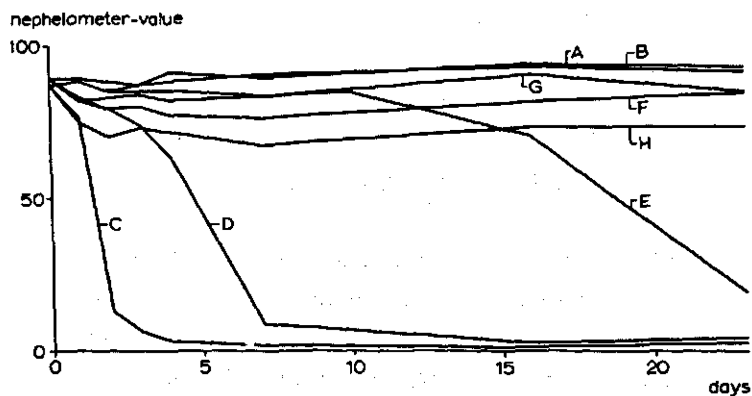


Fig. 8. Cloud stability of 8 different samples of orange juice (see text), as measured with an EEL nephelometer.

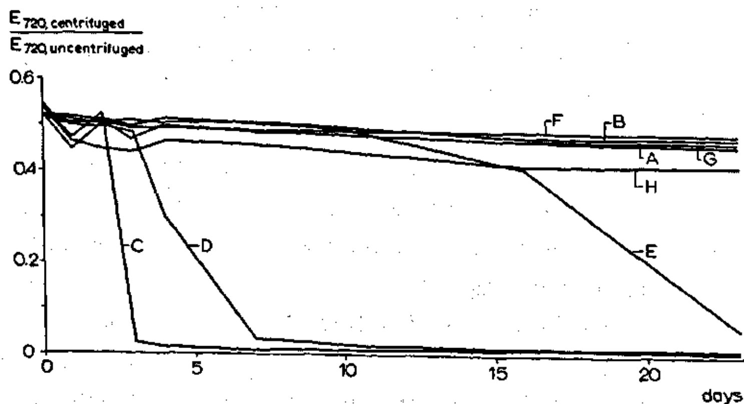


Fig. 9. Cloud stability of 8 different samples of orange juice (see text), expressed as the ratio of extinctions at 720 nm of centrifuged and uncentrifuged samples.

720 nm. Two plots are made: (1) $E_{720\ nm,\ centrifuged}/E_{720\ nm,\ uncentrifuged}$ against the incubation time (Fig. 9), and (2) $E_{720\ nm,\ uncentrifuged}/E_{550\ nm,\ uncentrifuged}$ against incubation time (plot not shown);

4. The bottles are not displaced during the incubation time. When layers are observed in the sample, 5 ml of the upper layer are pipetted into a 100 ml flask and made up with tap water. After this the contents are transferred into a 100 ml cuvette and measured in the Trübungsmesser (Lange, Berlin).

Table 3. Visual judgment of relative turbidity after 4, 11 and 46 days. The more turbid the sample is, the more + signs it gets.

Sample no.	4 days	11 days	46 days
A	++++++++	+++++++	++++++
B	++++++	++++++	+++++
C	+ (clear)	+	+
D	++	+	+
E	+++++++	+++++	+
F	++++	+++	++
G	+++++	++++	++++
H	+++	++	+++

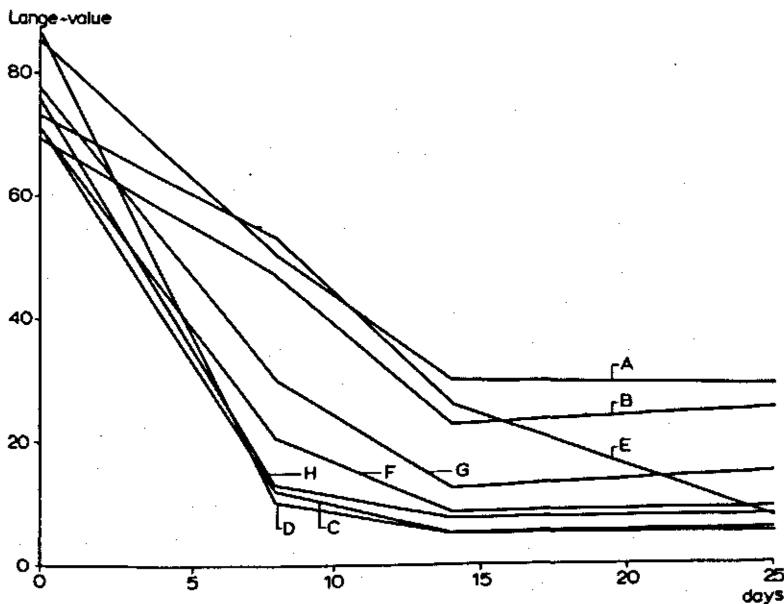


Fig. 10. Cloud stability of 8 different samples of orange juice (see text). 5 ml of supernatant layer was diluted to 100 ml and measured in a Lange Trübungsmesser.

The measured values are plotted against incubation time (Fig. 10);
5. The visual method: the bottles are kept untouched during the incubation time. From time to time the cloudiness of the supernatant is judged and degree of turbidity indicated by one or more + signs (Table 3).

Comparison of the different methods shows that the results of the methods 1 and 2 are essentially the same. For my purpose the spectrophotometer is preferable to the nephelometer because no dilution of sample is required and the measured juice can be returned to the bottle afterwards. If these advantages do not matter, the nephelometer can be used as a relatively cheap apparatus without objections.

Method 3 gives the same curves as the previous methods. The extinction values of the uncentrifuged samples are constant so that actually the curves of $E_{720\ nm,\ centrifuged}$ divided by a certain factor are obtained. The following drawbacks of the method were noted. Uncentrifuged orange juice could not be measured without dilution because of its high turbidity. The diluted samples had to be measured after standardized time intervals because of rapid settlement of the particles. Because of this drawback and because two measurements and a (simple) arithmetical operation are required, the method is less suitable if compared to the others mentioned above. Upon plotting the $E_{720\ nm,\ uncentrifuged}/E_{550\ nm,\ uncentrifuged}$-values one straight line at about 0.74 was obtained, identical for all the samples. This can be explained as follows. In the uncentrifuged samples the mean particle size is so high that Q is not altered by changes in the particle size (ρ) due to coagulation (see Fig. 2). As the plot does not give relevant information, it is omitted.

The curves of method 4 differ considerably from the previous methods. It is seen that even the blank A drops to Lange-value less than 30. Besides A only B is evidently a stable juice, as, according to the other methods, A, B, F, G and H, to a somewhat lesser extent, were stable. Some drawbacks of method 4 are: a 5-ml supernatant sample can only be taken if a sufficient thick supernatant layer has been formed and this layer is well-separated from the sediment. This leads to difficulties, especially in the case of self-pressed juices. In the taking of samples only from the supernatant, the composition of the remaining juice is altered. Continued drawing of samples from the same bottle is thus possible only to a very limited extent. Also care must be taken not to disturb the bottles when drawing the sample. After a certain period even stable juices (like A) show low values, so that figures for stable and unstable juices do not differ much. All in all this method is not

suitable for serial determinations.

A comparison of methods 1 to 4 with the visual method (5) does demonstrate that method 4 correlates best. The main reason for this is, of course, that in both cases samples are left standing so that indeed method 4 seems very suitable for shelf life studies. However, when the mechanism of cloud loss is studied and consequently frequent turbidity measurements together with other assays (e.g. methanol determination) are required, method 4 is not suitable and a centrifugation technique offers more possibility. It is evident that statements about cloud stability are closely related to the measuring method applied. A juice with a high extinction value after centrifugation might lose cloud on standing, whereas samples holding their cloud upon standing may lose it by centrifugation (cf. Section 6.2.5, Fig. 28b).
Being aware of these limitations I chose method 1 for the present work because it is a generally used method in the citrus industry and in research, not considering the fact most authors express turbidity in %T. It has the additional advantage to be a very convenient method.

5.3 DISCUSSION

The majority of experiments has been carried out with orange juice reconstituted from concentrate as the uniformity of the starting material allowed better correlation of the experimental results. As many experiments involved added enzymes the holding temperature was raised to 30°C and the pH to 4.0 in order to optimize the requirements of the enzymes and to accelerate the various phenomena. Optimum pH for yeast PG is 4.4, for Ultrazym 20 PL about 6.0 and for citrus PE about 7.5.

In Table 4 the cation contents of reconstituted juice and depectinized

Table 4. Cation contents (in mg/100 ml) of reconstituted juice and depectinized serum compared with literature data for single-strength juice.

	Rec. juice	Dep. serum[2]	Single-strength juice[1]			
			a	b	c	d
K	195.6	462.0	150 -215	186 -375	114 -193	98 -200
Na	1.0	3.0	0.4- 2.4	0.25- 0.9	0.4- 2.0	0.2- 2.5
Ca	9.9	10.3	8 - 24	1.5 - 5.5	5.4- 15.6	9.3- 21.5
Mg	12.6	12.8	3 - 19	4.9 - 15.0	7.3- 15.3	5.3- 15.1

1. a: Koch & Hess (1971), b: Attaway & Carter (1971), c: Benk (1966, 1968, 1970), d. Primo Yúfera & Royo Iranzo (1965) and Royo Iranzo (1971).
2. Depectinized serum has been prepared from juice which had been brought to pH 4.0 by addition of KOH (see Section 5.1).

serum are compared with literature data for single-strength juice. There is a wide variation in the literature values. Possibly this variation is due to the variability of the natural products as well as to different methods of analysis used. The K- and Na-values seem normal, while the Mg-value is slightly high and the Ca-value somewhat low compared with literature data. The high K-value of the depectinized serum is explained by the addition of KOH to bring pH to 4.0. It is surprising that Ca- and Mg-values have not been lowered by the removal of the flocculated cloud by centrifugation.

In Table 5 the pectin content is compared with literature data. The pectin content of the reconstituted juice appears to be normal.

The DEs of the pectin fractions of the reconstituted juice appear to be rather low. As is stated in Section 3.1 pectin with a DE < 50% becomes sensitive to Ca and an enzymically deesterified pectin might precipitate at a still higher DE. Particularly the cloud pectin should be Ca-sensitive, yet the reconstituted juice is cloud stable. Perhaps the cloud pectin is well surrounded by the particles and relatively inaccessible to Ca-ions or binding with Ca-ions does not affect cloud stability. Deesterification of the dissolved pectin (serum pectin) could then be of greater importance.

The methanol determination has proven difficult. Initially distillation was carried out in a Quickfit micro-distillation apparatus, but it was realised that an additional Vigreux column was necessary to prevent boiling over of juice, possibly as aerosol. Although the 5 ml round bottom flask with 2 ml of sample was heated by a micro-burner, the contents were easily burnt which resulted in too high methanol levels in the distillate. An advantage was, however, a twofold concentration of methanol while methanol in the method described in Section 5.2 is diluted twice.

As the distillate of fresh orange juice does not only contain methanol but also acetaldehyde and ethanol, the gas chromatographic method should separate all these peaks well from each other and from the water ghost peak.

Table 5. Pectin content of reconstituted juice compared with literature data.

	mg AGA/100 ml	mg AGA/100 g
reconstituted juice	57.4	55.0
Koch & Hess (1971)	28.0-83.0	
Benk (1966, 1968, 1970)	60.0-210.0	
Attaway & Carter (1971)	soft squeeze:	min. 46.7, av. 56.9, max. 80.0
	hard squeeze:	min. 87.9, av. 120, max. 216

With Porapak Q (an ethyl divinyl benzene polymer, Waters Associated Inc., Framingham, Mass., USA) methanol and acetaldehyde had the same retention times. As Norman (1970) was able to separate orange volatiles with Chromosorb 101 by head space technique, we also tried Chromosorb 101. It was seen that the same conditions could not be applied as water gave two interfering ghost peaks when the column temperature was 135°C. To find optimal conditions we varied column length, column diameter, column material, temperatures of the column, injection port and detector, gas flows, applied auxiliary gas etcetera. It was demonstrated that good peak separation was influenced by the conditioning of the Chromosorb. Overnight conditioning at 270°C resulted in good separation from the water peak, but methanol and acetaldehyde were still not separated well; overnight conditioning at 170°C facilitated separation of methanol from acetaldehyde but not of water from methanol. An acceptable compromise was found in conditioning overnight at 200°C. Also other column packing materials and g.l.c. techniques have been tried. All these preliminary experiments are described by de Boer (1972) and Faddegon (1973). Eventually optimal conditions were found and the method worked well with good reproducibility and good recovery (Krop et al., 1974). Most of the methanol determinations have been made by this method; however, during the period of this investigation it was not always possible to reproduce these optimal conditions and in these cases it was decided to employ a colorimetric method for methanol determination. This occurred after dismantling and cleaning the detector. Thus the cause for these phenomena should possibly be sought in the geometry of the detector. This was confirmed by Bal (Chrompack Nederland N.V., Vlissingen, the Netherlands) who stated that the signal from water differed for various apparatus; e.g. water in a Varian 600 gives a much smaller signal than in a Becker gas chromatograph. The influence of water upon the sensitivity of the flame ionization detector is described by Lucero (1972). Bal tested several other columns (Carbowax liquid phases on teflon in stainless steel, glass and teflon columns) for me, but without success. The g.s.c. method described proved successful under very precise conditions, but is not suitable for serial assays. Although laborious the colorimetric method worked satisfactorily.

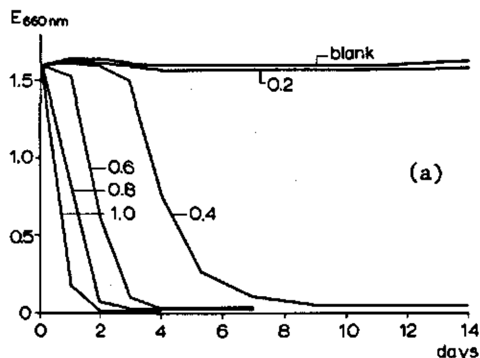
6 Experiments and results

6.1 INFLUENCE OF EXPERIMENTAL FACTORS ON CLOUD STABILITY

6.1.1 Cloud change assay methods

Influence of repeated measurements upon juice turbidity and methanol release. Turbidity was measured as described in Section 5.2. After the extinction has been measured the 10 ml juice is shaken and returned to the corresponding bottle. This procedure makes it possible to carry out many measurements on a relatively small sample. Since this procedure may influence the turbidity of the sample, the following experiment was carried out. Juice was reconstituted from the concentrate and samples were prepared in duplicate with 6 PE levels. The 100 ml portions were filled into 100 ml plastic screw bottles. These bottles were attached to a plexiglass disc rotating at 5.4 rev/min in a waterbath kept at 30 ^{o}C, thus ensuring continuous mixing. Of the duplicate samples one was measured by the method described in Section 5.2 (here called method 1), whereas the measured juice of the other one was discarded (method 2). The cloud stability of both series is shown in Figs. 11a and 11b, while methanol release is shown in Figs. 12a and 12b. It is shown that the extinction curves for both series are identical. The methanol curves for the two highest PE levels in method 1 are found to be somewhat lower, the other curves somewhat higher compared to method 2. Thus it can be concluded that no significant differences appear and therefore method 1 (readdition of measured juice) is applicable without objections.

Influence of the frequency of turbidity measurements upon the turbidity. A sample of reconstituted juice with 0.2 U PE/ml was kept according to the method described in Section 5.2. During the first 20 days of incubation the turbidity was measured frequently, then no measurements occurred during the next 20 days, and thereafter again frequent measurements were carried out. From Fig. 13 it is shown that clarification occurred only after the 40th day of incubation. It is suggested here that the frequency of measurements possibly



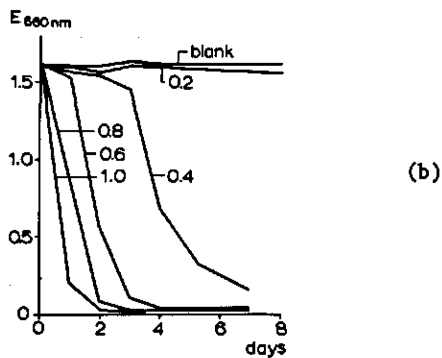
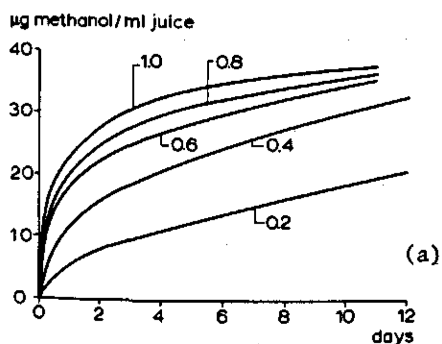


Fig. 11. Clarification of reconstituted orange juices adjusted to various PE levels and mixed continuously. After extinction measurements the measured juices were (a) returned to the corresponding bottles or (b) discarded. The figures for the curves indicate PE level as U/ml.



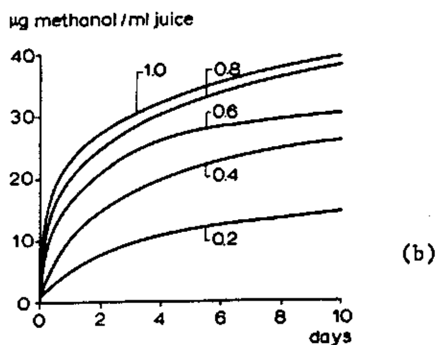


Fig. 12. Methanol release in orange juice samples of (a) Fig. 11a and (b) Fig. 11b.

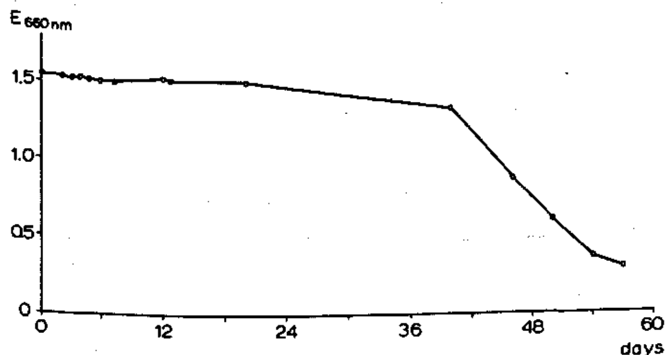


Fig. 13. Clarification of reconstituted orange juice adjusted to 0.2 U PE/ml. The sample was left standing at 30°C.

Table 6. Extinction values of samples with different frequency of measurement.

Incubation time (h)	$E_{660\ nm}$ of samples with different frequency of measurement[1]					
	a	b	c	d	e	f
0	1.600	1.600	1.590	1.600	1.600	1.600
1	1.655	1.648				
2	1.332	1.317	1.288			
3	0.655	0.643		0.609		
4	0.275	0.271	0.226		0.198	
5	0.134	0.144				
6	0.093	0.098	0.090	0.098		
7	0.064	0.078				
8	0.057	0.057	0.057	0.055	0.059	0.058

1. Code: frequency of measurement a: each hour; half between two measurements the sample is inverted ten times, b: each hour, c: each 2 h, d: each 3 h, e: each 4 h, f: after 8 h

influences the rate of cloud loss. Therefore a less time consuming experiment was set up as follows. Juice was reconstituted from the concentrate and brought to 30°C. Under stirring an amount of PE was added to give 3 U PE/ml juice. This PE activated juice was distributed in 6 100 ml-stoppered glass flasks and kept according to the method described in Section 5.2. The extinction values are tabulated in Table 6. It is shown that less frequent measurement leads to more rapid clarification but differences are very small and not significant. Possibly effects of a different frequency in measurements appear only when low PE levels are applied. This, however, has not been investigated further.

Influence of juice keeping method. The results of the experiment described in the former section as method 1 were compared with a similar experiment in which the bottles did not rotate but were left standing according to the method described in Section 5.2. In Fig. 14 the extinction values of both experiments have been plotted against incubation time. In Fig. 15 methanol release has been plotted as the percentage saponified methoxy groups as a function of incubation time and in Fig. 16 extinction values have been plotted against percentage saponified methoxy groups. From the figures we see that saponification occurs more rapidly in rotating bottles and the pectin appears to be saponified to a somewhat greater extent (76% against 70%).

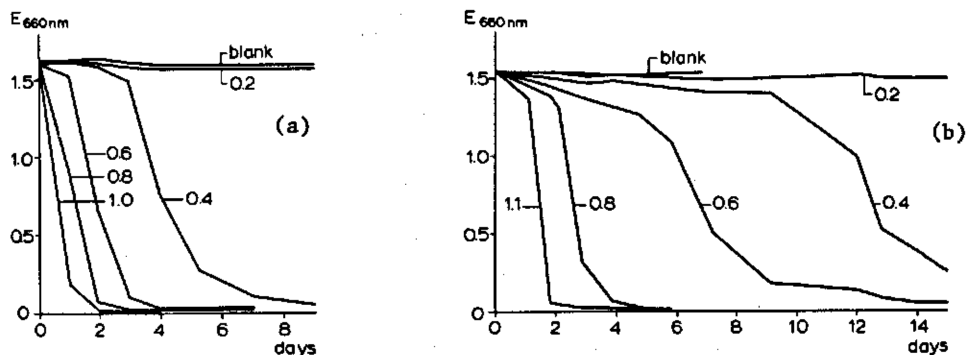


Fig. 14. Clarification of reconstituted orange juices adjusted to (a) various PE levels and mixed continuously and (b) to various PE levels; samples were left standing at 30°C. The figures for the curves indicate PE level as U/ml.

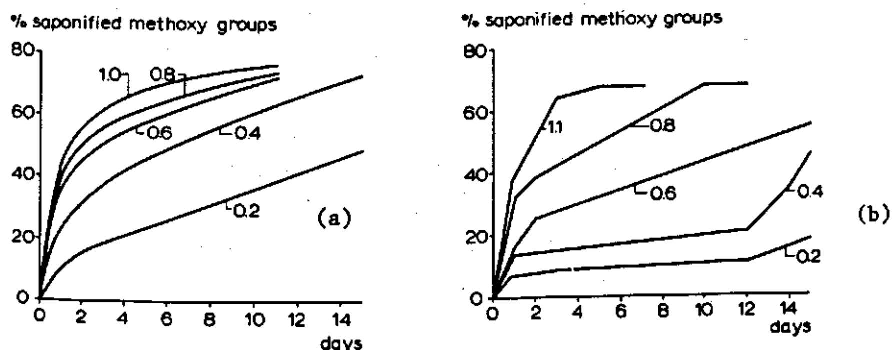


Fig. 15. Methanol release in orange juice samples of (a) Fig. 14a, and (b) Fig. 14b, expressed as % saponified methoxy groups of total saponifiable galacturonic acid methyl ester.

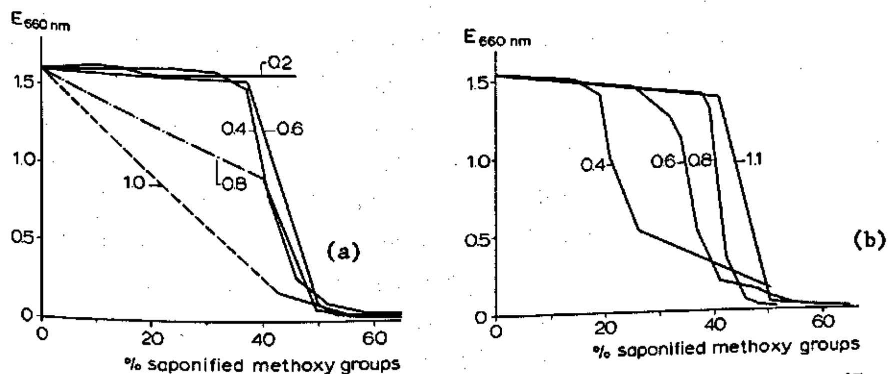


Fig. 16. Clarification as a function of pectin deesterification (% saponified methoxy groups) of orange juice samples of (a) Fig. 14a and (b) Fig. 14b.

Similarly clarification is more rapid if the juice is mixed continuously; the samples with the two highest PE levels clarified so rapidly that their curves in Fig. 16a could not be drawn completely. Remarkably the sample with 0.2 U PE/ml juice when mixed continuously did not clarify even after saponification of 46% of the methoxy groups. The continuously mixed samples seem to clarify at a distinct percentage of saponified methoxy groups. However, in resting samples this value is related to the PE level (Fig. 16b).

Other variations in keeping methods were compared in preliminary experiments: Standing was done in 100 ml stoppered glass flasks (52 mm o.d.), but also in 100 ml stoppered graduated cylinders (29.5 mm o.d.). Besides standing methods 100 ml salve pots (47 mm o.d.) attached to a rotating plexiglass disc (5.4 rev/min) thermostated at 30°C and 100 ml Erlenmeyer flasks in a shaking incubator (Gallenkamp) at 30°C were applied. All experiments were carried out with self prepared juices of different varieties of oranges. Samples in the graduated cylinders often tended to retain their cloud somewhat better than in stoppered glass flasks, possibly by wall influence. Both 'moving' methods tended to a slight increase in turbidity as compared with the standing method, possibly as a result of the vigorous movements which might reduce particle size. Samples of some varieties coagulated in the rotating disc method. Therefore the standing method in glass flasks was considered to be the most appropriate method.

6.1.2 Juice making-up methods

Influence of heat treatment. Addition of 1.0 U citrus PE to the juice reconstituted from concentrate brings about clarification in 2 days (Fig. 17). If the reconstituted juice was heated at 90°C for 5 min in a boiling waterbath before PE addition complete clarification occurred within 24 h (Fig. 17) and also methanol was released more rapidly (Fig. 18). So it is clear that heat treated juices are more susceptible to PE than untreated juices. It was thought that the heat treatment might cause extraction of pectin from cloud particles into the serum which would result in new substrate for PE. Pectin analyses showed this to occur to a limited extent only. In one experiment 16.2 mg AGA/100 g serum was found before heat treatment of diluted concentrate (5 min 90°C) and 18.3 mg AGA/100 g serum after the treatment. These figures correspond to 29.4 respectively 33.2% of the total pectin content of the juice. A more important effect of the heat treatment might be a reduction in particle size or a better accessibility of the enzyme to its sub-

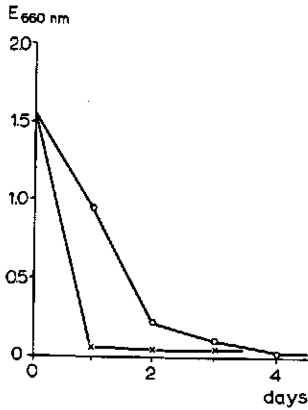


Fig. 17. Clarification of reconstituted orange juice with 1 U PE/ml added, as influenced by an extra heat treatment. o : no extra heat treatment; x : sample heated for 5 min at 90°C.

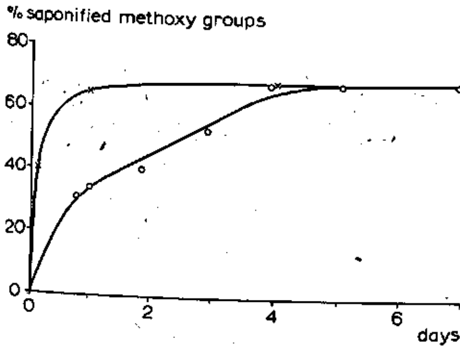


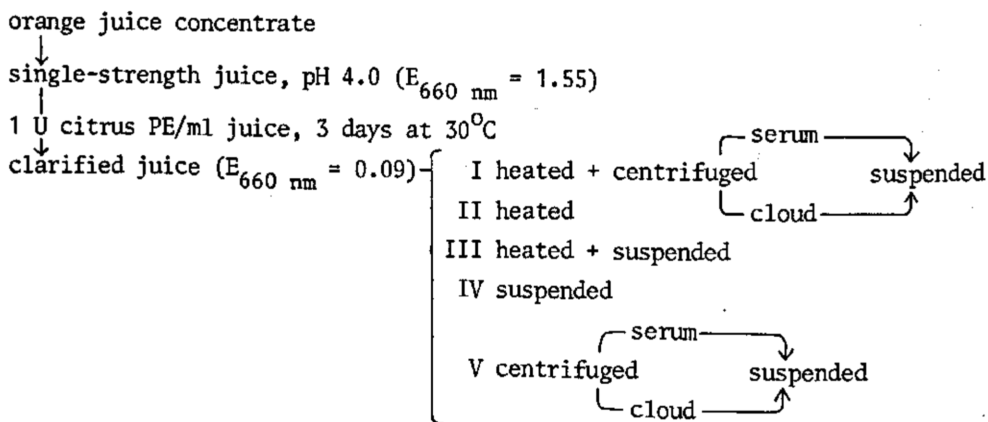
Fig. 18. Methanol release in orange juice samples of Fig. 17.

strate. This, however, has not been investigated.

Influence of homogenization of juice. It is most likely that the mechanical handling of juice in the processing industry (pumping, mixing) influences cloud stability. Therefore juice prepared from Switie oranges (Surinam) was homogenized in a Braun multimix at full speed for 60 s. Afterwards the juice had to be deaerated by evacuation. However, no differences in the extent or rate of clarification between homogenized and unhomogenized samples could be observed.

Influence of resuspension with and without heat treatment. In preliminary experiments involving resuspension of cloud in various media in order to check the results of Baker & Bruemmer (1969) I was confronted with problems of reproducibility. The problems might be caused by factors inherent to the oranges (variety, origin, growing circumstances, maturity, etc., Biggs & Pol-

lard, 1970) or to the methods used. As regards the latter, I suspected mechanical factors, although homogenizing of juice in a Braun multimix had no influence (see above). This was investigated according to the following scheme:



heated = juice heated up to 90°C in a boiling waterbath, holding time 5 min, then cooled to room temperature
centrifuged = juice centrifuged during 20 min at 48 000 x *g* at 0°C
suspended = cloud suspended in serum by vibrating mixer during 30 min

After preparation of the samples according to the scheme, cloud stability was monitored for two weeks. Fig. 19 shows that a combination of all the treatments enhances the cloud level nearly to the level of single-strength juice which, furthermore is maintained (I). Heat treatment alone does not enhance the initial cloud level, a slow increase, however, is observed afterwards (II, cf. also III with IV). The suspension treatment enhances the initial cloud level by about 0.5 E (III, IV, V). The centrifugation step has no effect if the clarified juice is centrifuged directly (cf. V with IV), but it

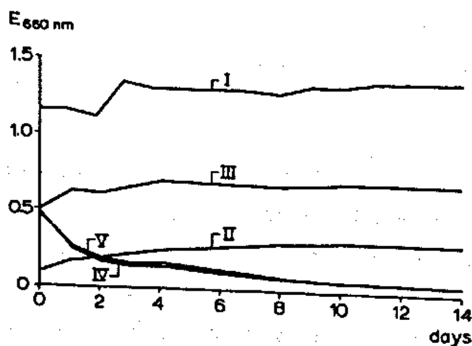


Fig. 19. Cloud stability of clarified orange juice samples after different treatments. (For explanation roman numbers see text.)

does have effect if the juice has been heated previously (cf. I with V). It has become clear now, that the treatments mentioned have a pronounced effect upon cloud level and cloud stability. These results do indeed explain irreprocucibility in resuspension experiments.

6.2 INFLUENCE OF INTRINSIC FACTORS ON CLOUD STABILITY

6.2.1 Clarification behaviour of unpasteurized orange juices of different varieties.

Juice was prepared from 4 varieties of oranges: Navel (Spain), Salustiana (Spain), Switie (Surinam) and Jaffa (Israel). The samples were kept in stoppered glass flasks at room temperature. Cloud stability was measured several times during the first day (Fig. 20a) and occasionally in the next two weeks (Fig. 20b). From Fig. 20a it is seen that the juices are rather clear immediately after pressing but the turbidity increases rapidly to reach its maximum value 1 to 3 h after pressing. Then it decreases rapidly during the first day of incubation and at a slower rate in the following days (Fig. 20b). Differences in clarification behaviour between varieties are very small except for Jaffa juice which does not clarify completely. This phenomenon has also been observed in other experiments with Jaffa juice. PE activities have been determined titrimetrically but no correlation has been found between these PE activity values and the clarification behaviour: Navel 1.53 U/ml, Switie 1.72 U/ml, Salustiana 2.93 U/ml, Jaffa 2.55 U/ml.

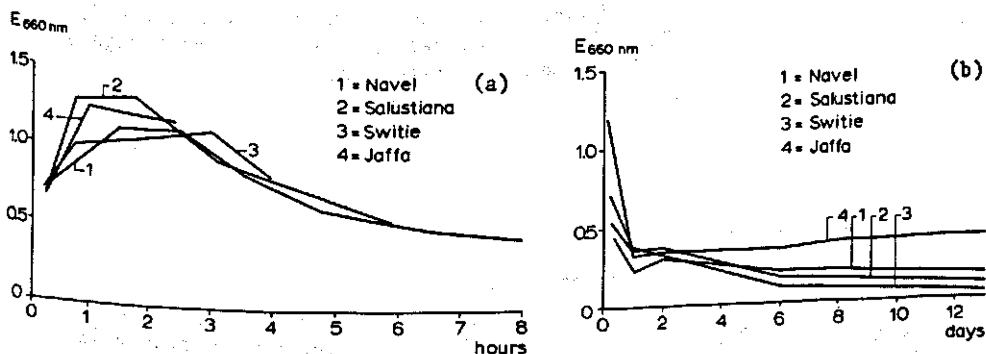


Fig. 20. (a) Changes in cloud level of orange juices during the first hours after pressing. (b) Clarification of orange juice samples of Fig. 20a. Initial extinction values were measured after 2.5 h (Jaffa), 4 h (Switie), 6 h (Salustiana) and 8 h (Navel).

6.2.2 *Clarification behaviour of mixtures of pasteurized and unpasteurized juices*

From a 1:1 mixture of Navel (Spain) and Switie (Surinam) juice samples were prepared with different ratios of pasteurized and unpasteurized juice. The cloud stability of the samples was measured for 2 weeks and the results are shown in Fig. 21. It is seen that a smaller amount of unpasteurized (PE active) juice does delay clarification. After two weeks only samples with more than 50% unpasteurized juice were clarified. In a similar experiment with pure Switie juice it was shown that a mixture with only 15% unpasteurized juice was still clarified after 8 weeks (Fig. 22).

6.2.3 *Relation between clarification and pectin esterase action*

In view of the foregoing experiments and in view of the PE hypothesis in Section 2.1 varying amounts of PE were added to reconstituted orange juice. Cloud stability (Fig. 23) and methanol formation (Fig. 24) have been monitored

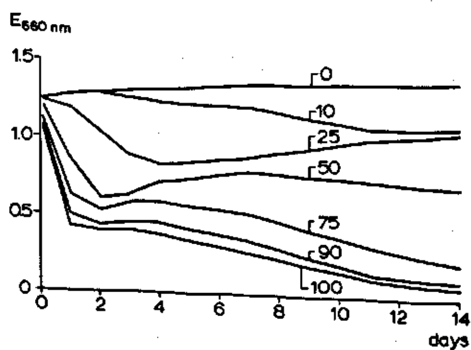


Fig. 21. Clarification of 1:1 mixtures of self pressed Navel and Switie orange juices. Part of each mixture was inactivated by heat. The figures for the curves indicate the percentage unpasteurized juice.

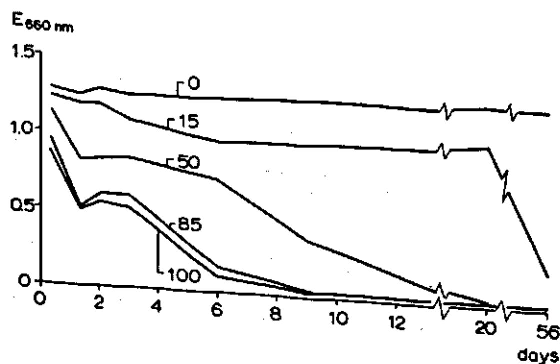


Fig. 22. Clarification of self pressed Switie orange juice. Part of the juice samples was inactivated by heat. The figures for the curves indicate the percentage unpasteurized juice.

for a 2 weeks period. Methanol is released rapidly during the first 24 h, but then the rate decreases which might be ascribed to product inhibition (Lee, 1969; Lee & Macmillan, 1968). A sudden increase in the rate of methanol release might be ascribed to the inhomogeneity of the system, as in a similar experiment, in which the samples were mixed continuously, methanol release was very even (Fig. 15a). In Fig. 25 the turbidity (calculated as extinction) is

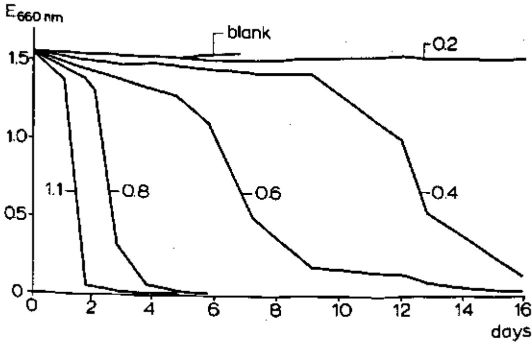


Fig. 23. Clarification of reconstituted orange juices adjusted to various PE levels. The figures for the curves indicate PE level as U/ml.

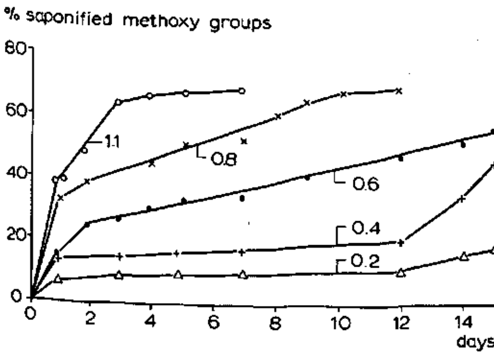


Fig. 24. Methanol release in orange juice samples of Fig. 23, expressed as % saponified methoxy groups of total saponifiable galacturonic acid methyl ester.

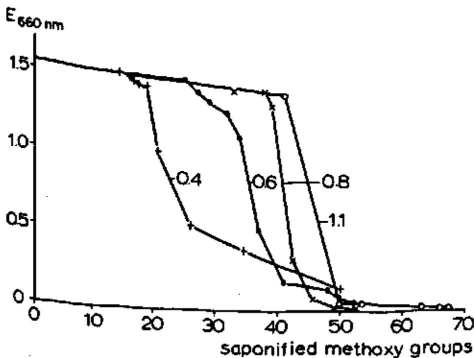


Fig. 25. Clarification as a function of pectin deesterification (% saponified methoxy groups) of orange juice samples of Fig. 23.

plotted against the percentage of saponified methoxy groups. It is interesting to note that clarification takes place at a decreasing percentage of saponified methoxy groups with decreasing PE activity of the juices (although it takes longer). As PE is known to follow along the pectin molecule (Solms & Deuel, 1955; Lee & Macmillan, 1970) one explanation could be that at a high PE concentration a certain methanol content may mean just a few saponified groups in each of many pectin molecules whereas the same methanol content at a low PE concentration might indicate a few but almost completely saponified pectin molecules. These would then coagulate with calcium and destabilize the cloud. A juice with an extinction of 0.22 (corresponds with 60% transmission) can be considered as clarified. Fig. 25 shows that for all activities 40-50% of the methoxy groups must be deesterified to attain this extinction value.

6.2.4 *Confirmation of pectin esterase inhibition by pectic acid*

The slowing down of methanol release described in the foregoing section can be explained by an end product inhibition. A model experiment was therefore carried out: Methanol release in a 0.5% brown ribbon pectin solution in McIlvaine buffer pH 4.0 by 2 U citrus PE/ml both with and without 10 mg Ca/100 ml added is shown in Fig. 26. A similar experiment was done with 0.1% (w/v) pectic acid added (without Ca). In Fig. 26 it is shown that citrus PE is rather strongly inhibited by pectic acid and therefore the supposition that product inhibition might occur is confirmed.

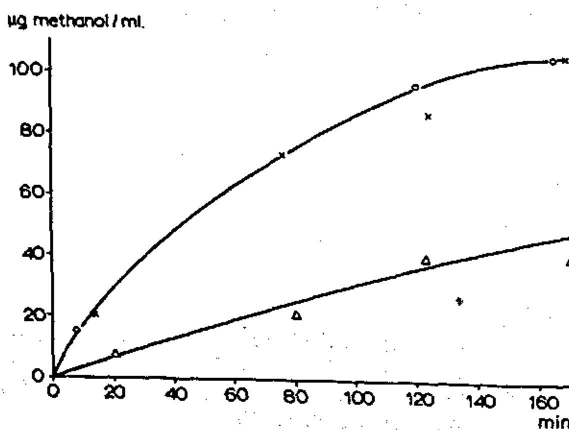


Fig. 26. PE action under different circumstances. o : methanol release of 2 U citrus PE/ml 0.5% (w/v) brown ribbon pectin solution in McIlvaine buffer pH 4.0; x : idem, 10 mg Ca/100 ml solution added (as $CaCl_2.2H_2O$); Δ: idem, 0.1% (w/v) pectic acid added.

6.2.5 *Effect of addition of pectic acid and bivalent cations on cloud loss* (Krop & Pilnik, 1974a)

An insight into the role of calcium ions in clarification phenomena was obtained by precipitating them as calcium oxalate. For this purpose 15.3 mmol ammonium oxalate per litre orange juice were added during the reconstitution of the orange juice. This quantity corresponds to twice the molar concentration of the bivalent cations Ca and Mg. The juice was then activated by adding 1 U citrus PE/ml juice. Turbidity measurements and methanol determinations were performed for a period of 2 weeks and Fig. 27a shows that the juice with ammonium oxalate (curve B) remains cloud stable in spite of normal PE action as indicated by methanol release (Fig. 27b).

Since pectic acid or a pectin fraction with a low content of methoxy groups can be assumed to be the end result of PE action, I investigated also the effect of pectic acid addition to orange juice. This was done by pipetting increasing amounts of a pectic acid solution to orange juice and to orange juice containing added ammonium oxalate as described before. Pectic acid was brought into solution by partially neutralizing with KOH to pH 4. The pH values of orange juices varied from 3.99 to 4.12. Centrifugal turbidity measurements were made within 12 min after the addition of pectic acid. Samples were also left standing undisturbed at room temperature for 3 days and the height of the sediment measured and expressed as percentage of the total sample height. Curves A in Fig. 28a and 28b show that pectic acid does indeed clarify orange juice. It is also seen that as the quantity of pectic acid is

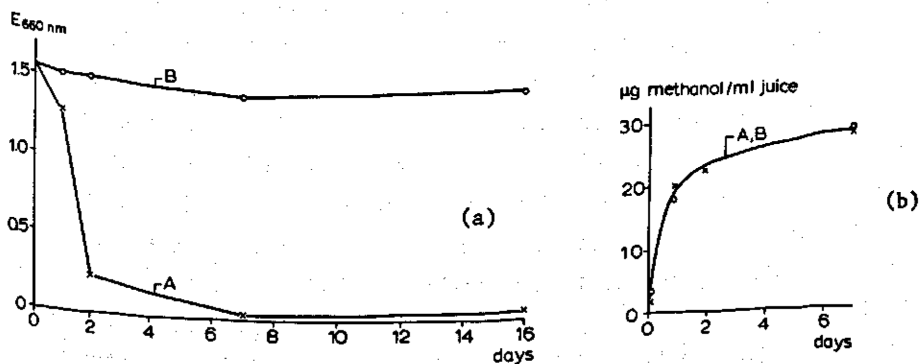


Fig. 27.(a) Cloud stability of reconstituted orange juice with 1 U PE/ml. Curve A: without addition of ammonium oxalate; Curve B: with addition of 15.3 mmol ammonium exalate per litre. (b) Methanol release in orange juice samples of Fig. 27a.

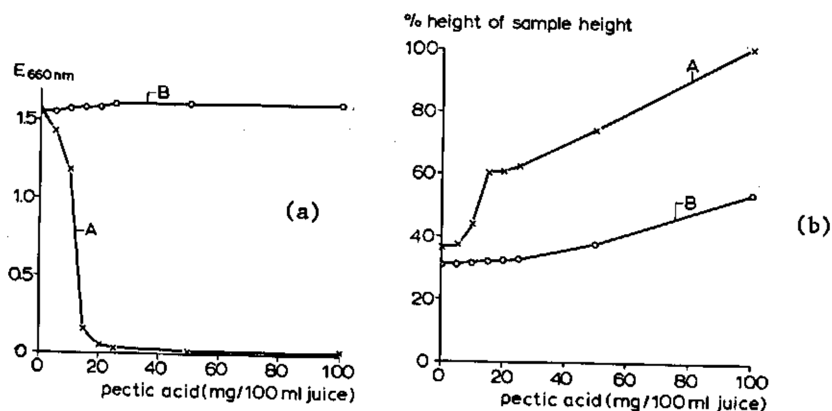


Fig. 28.(a) Cloud stability of PE inactive, reconstituted orange juice with various additions of pectic acid expressed as extinction of supernatant after centrifugation. Curve A: regular orange juice; Curve B: orange juice containing 15.3 mmol ammonium oxalate per litre. (b) Sediment of orange juice samples of Fig. 28a expressed as % height of sample height after letting stand undisturbed for 3 days.

increased sediment volume increases eventually reaching 100%. The sediment may be considered as a Ca-pectate gel including most of the cloud particles up to 15 mg pectic acid/100 ml, and all of them - leaving a clear supernatant - above this concentration. At the same time the gel volume increases up to 100%. At this extreme point the juice appears to be 'cloud stable' but the stability of this gel towards syneresis upon standing or towards flocculation on technological treatments (pasteurization, filling, transport) remains to be seen. It has already been shown that the cloud collapses upon heating. Again clarification is prevented by removing calcium ions (curves B).

From these experiments and from those described in Section 6.2.3 it is obvious that PE clarifies orange juice only if the Ca-ions can coagulate pectic acid molecules or pectin molecules with large saponified sections. Fig. 28a indicates that an addition of 5 to 10 mg pectic acid per 100 ml juice is critical for cloud loss. The pectin content of the orange juice has been determined to be 57.4 mg AGA per 100 ml with a DE of 47.3% (Section 5.1). From Fig. 25 it is seen that about 20% of the methoxy groups present (corresponding to 5.4 mg AGA) must be saponified by PE before clarification starts. The figures of both experiments therefore agree with each other.

6.2.6 The respective role of cloud pectin and serum pectin in cloud loss

Cloud from clarified Salustiana juice was suspended by means of a vibrating mixer into the following media: (1) distilled water, (2) distilled water + 0.03% citrus pectin (Sigma, grade II; St. Louis, Miss., USA) + 0.4% KCl and (3) serum of Salustiana juice. Cloud stability of these suspensions is shown in Fig. 29. In all cases the turbidity values remained at the initial levels. It is seen that the suspension in water (1) has the highest turbidity and the suspensions in water + pectin + KCl(2) and in serum (3) have equal turbidity. The difference between a suspension in water and a suspension in serum can partially be explained by the difference in refractive indices of the continuous phases. Depending on the particle size a greater difference between refractive index of the particles and the continuous phase ($n_d - n_c$) leads to a greater light scattering quotient (Q) and consequently to a higher extinction value (about 1.5 x; Section 4.3). However, in this way the difference in turbidity of the suspensions in water and in water + pectin + KCl can not be explained. Probably a difference in electrical charge of the particles play a role. Cloud particles of flocculated juice may be assumed to have a higher negative charge than those of a cloud stable juice. By the addition of citrus pectin (DE approximately 70%) and KCl the negative charge has been lowered by adsorption of the pectin upon the particles and by the decay of the electrical double layer due to the salt. Consequently the particles in this medium are less stabilized by electrical repulsion factors and more easily sedimented by centrifugation.

Cloud of reconstituted juice was suspended by means of a vibrating mixer in distilled water with added $K_2S_2O_5$ giving 0.0156 N K, and in the same medium with extra KCl added to give 0.150 N K. The same suspensions were prepared

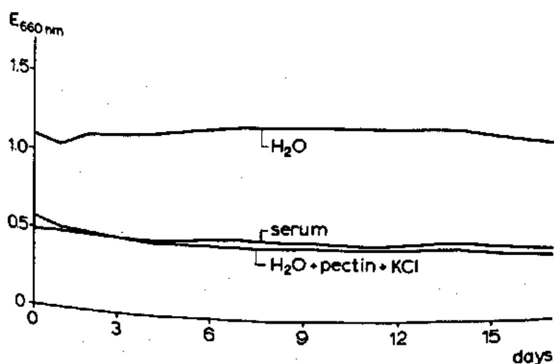


Fig. 29. Cloud stability of suspensions of cloud from clarified Salustiana orange juice in different media: distilled water, serum of Salustiana orange juice and distilled water + 0.03% citrus pectin + 0.4% KCl.

with the addition of 1 U citrus PE/ml solution. Fig. 30a shows the cloud stability of the suspensions and Fig. 30b the methanol release in the suspensions with PE added. None of the suspensions clarify (Fig. 30a) although PE is active (Fig. 30b). The salt has in this case (cloud from stable juice) no effect on the turbidity (Fig. 30a), but it heightens the PE action (Fig. 30b).

The same cloud suspension in orcat solution does, however, show clarification (Fig. 31a) and a more rapid PE action (60% of the methoxy groups saponified within 30 h instead of 14 days, Fig. 31b).

If PE is added to serum, the serum pectin is saponified relatively quickly and to a greater extent than the pulp pectin. Apparently, it is an easily attacked substrate for PE (Fig. 31b).

By means of these experiments and some others I tried to find out the role of serum pectin and cloud pectin in cloud loss phenomena. These roles could not be elucidated completely but it has been demonstrated that both serum pectin and cloud pectin are saponified by PE. Most probably both pectin fractions are important in cloud loss phenomena. A certain ionic strength is necessary for PE action and clarification, as determined by centrifugal turbidity measurements, occurs only if bivalent cations are present. In experiments not described here, I found an exchange between PE adsorbed on cloud particles and serum PE. Quantitative data could not be obtained, but it seemed that the greater part is adsorbed to the particles.

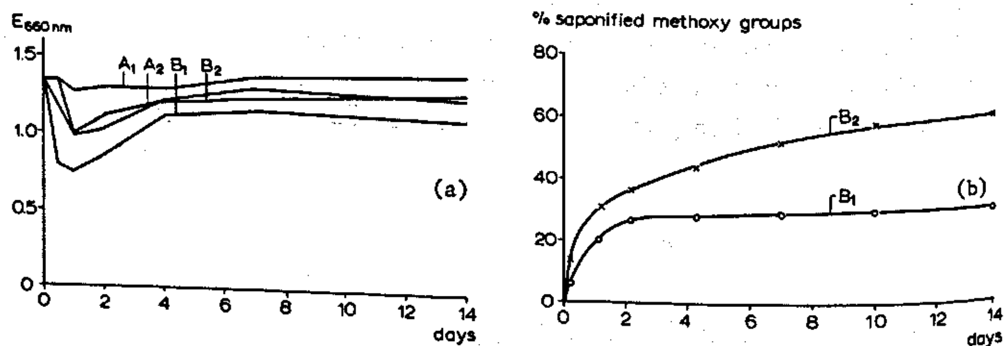
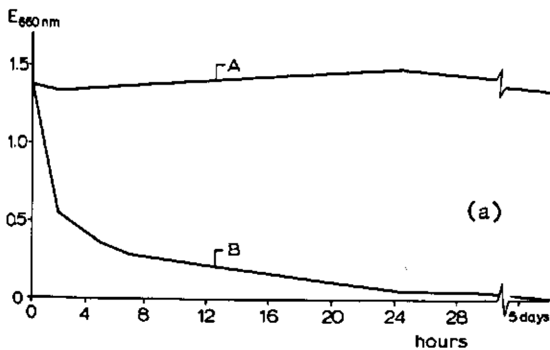


Fig. 30a. Cloud stability of suspensions of cloud from reconstituted orange juice in aqueous media.
A_1 : distilled water, 0.0156 N K from $K_2S_2O_5$ addition, no PE added;
B_1 : distilled water, 0.0156 N K from $K_2S_2O_5$ addition with 1 U PE/ml;
A_2 : distilled water, 0.150 N K from $K_2S_2O_5$ and KCl additions, no PE added;
B_2 : distilled water, 0.150 N K from $K_2S_2O_5$ and KCl additions with 1 U PE/ml.
Fig. 30b. Methanol release in PE active suspension samples of Fig. 30a.



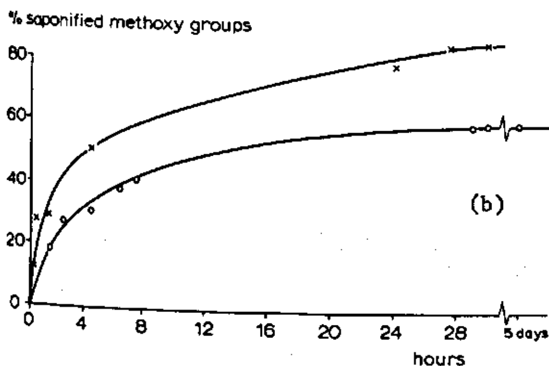


Fig. 31a. Cloud stability of a suspension of cloud from reconstituted orange juice in orcat solution. Curve A: no PE added; Curve B: with 1 U PE/ml added.

Fig. 31b. o : methanol release in PE active suspension of Fig. 31a (curve B); x : methanol release in PE activated (1 U/ml) serum of reconstituted orange juice.

6.2.7 The possible role of hesperidin in cloud loss as seen by electron microscopic investigation

If one lookes to electron micrographs of juice reconstituted from concentrate one sees a great variety of particles dispersed all over the preparation (Figs. 32a-32d). The quantity of needle-like crystals (thickness 0.03-0.25 µm, length 0.7-7 µm, cf. Mizrahi & Berk, 1970) is striking. Most likely, these crystals are hesperidin. Micrographs of the same juice clarified by PE show that all cloud particles are caught in clusters (15-20 µm), the space between the clusters being optically empty (Figs 33a-33b). The hesperidin crystals are included in the clusters too, so that the question arises

Fig. 32a. Electron micrograph of dialysed reconstituted orange juice (Photo: TFDL, Wageningen).

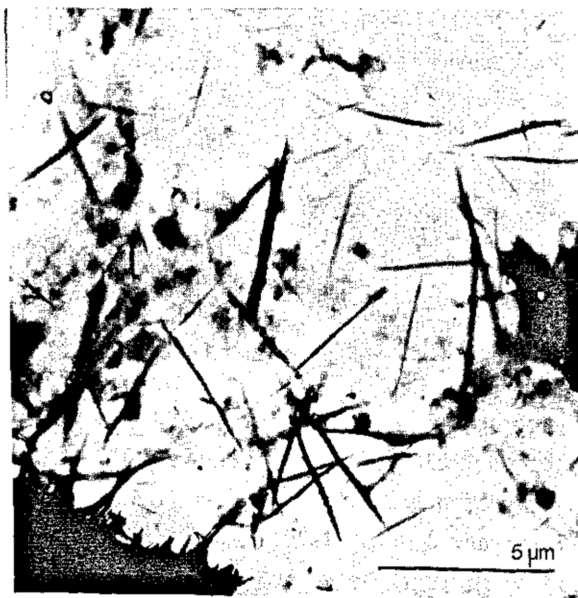


Fig. 32b. Electron micrograph of dialysed reconstituted orange juice (Photo: TFDL, Wageningen).

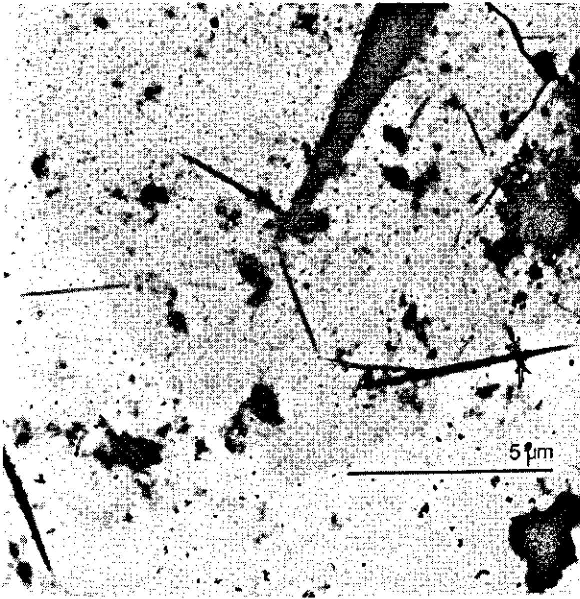


Fig. 32c. Electron micrograph of dialysed orange concentrate (Photo: TFDL, Wageningen).

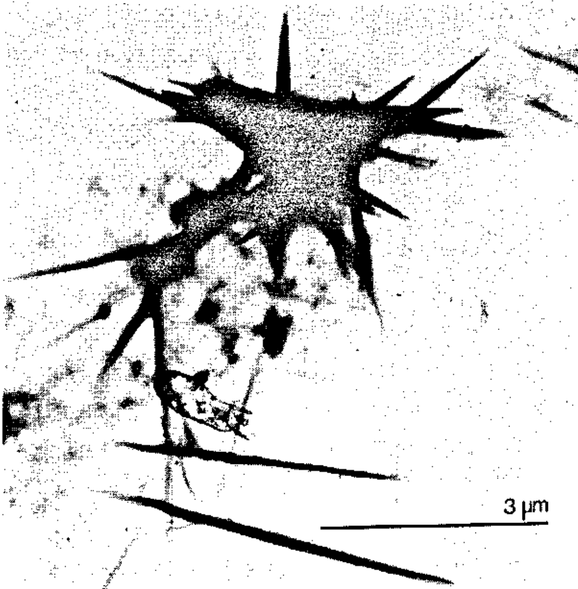


Fig. 32d. Electron micrograph of dialysed orange concentrate. Crystals shown are probably hesperidin (Photo: TFDL, Wageningen).

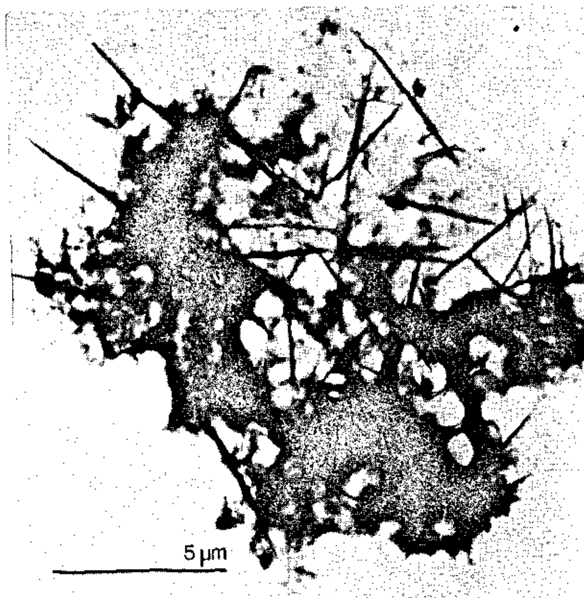


Fig. 33a. Electron micrograph of dialysed reconstituted orange juice clarified by citrus PE (Photo: TFDL, Wageningen).

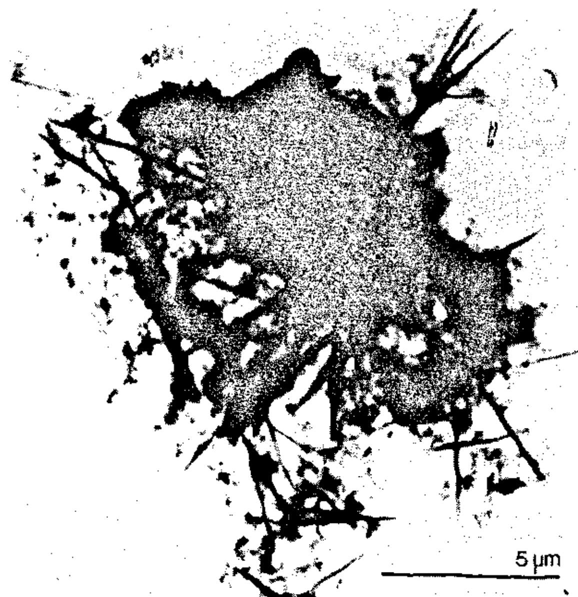


Fig. 33b. Electron micrograph of dialysed reconstituted orange juice clarified by citrus PE (Photo: TFDL, Wageningen).

whether they contribute to the clarification process and if so, to what extent. Stokes' law is given by the expression:

$$v_s = \frac{(\rho_s - \rho_f)\, g \; d^2}{18\, \eta} \qquad (1)$$

If clarification occurs all factors except g can change: The viscosity is affected by the degradation of pectin. The formation of clusters probably increases $(\rho_s - \rho_f)$. The greatest change occurs in respect to d, as all the particles with dimensions varying from less than 1 μm to approximately 20 μm coagulate to clusters of 15-20 μm. Moreover, d is raised to the square in the formula.

To check up the role of hesperidin in clarification the density of it (Merck A.G., Darmstadt, Germany; melting point after recrystallization from formamide (Pritchett & Merchant, 1946) 259-261°C) has been determined with a pyknometer and was seen to be ca. 1.45. According to Weast (1971-1972) the melting point of pure hesperidin is 261-263°C. The density of crystals isolated from serum of Sunkist oranges was 1.23 only, probably because of impurities (melting point 251-252°C). Purification is difficult as we wanted to maintain the original form of the crystals and therefore could not recrystallize. The density of hesperidin crystals is much higher than the density of juice (ca. 1.05), thus the contribution of hesperidin to cloud loss might not be negligible.

6.3 INFLUENCE OF PECTIN AND PECTATE DEPOLYMERASES ON THE CLOUD STABILITY OF ORANGE JUICE

6.3.1 *Cloud stabilization by a yeast polygalacturonase* (Krop & Pilnik, 1974b)

In Section 6.2 the ability of PE to clarify orange juice could be correlated to the saponifying action it exerts on pectin by quantitative determination of methanol formed. In this section it is shown that by hydrolyzing the deesterified pectin by a yeast PG this harmful effect of PE can be cancelled.

Activity and stability of polygalacturonase solutions. The PG activity of stock solution I was 105 U PG/ml (dialysate 1.79 U/ml). I noticed that activity of PG solutions in water or McIlvaine buffer of pH 7 was lost rapidly. Therefore PG was diluted with one tenth of normal strength McIlvaine buffer

pH 5.0 or pH 4.0. An addition of Ca to the buffer increased the stability. At a 0.001 M Ca concentration about one third of the activity is lost after 4 h incubation at 30°C. This loss is only about 10% (with a higher initial activity) if the Ca concentration is increased tenfold (Fig. 34). If, however, PG was diluted in depectinized serum no loss in activity could be detected even after incubation for 6 h at 30°C and the initial activity was even higher (82.6 ± 4.1%). This activity was even maintained after a storage period of 26 days at 4°C followed by 11 days at 30°C. Even after further incubation for 188 days at 30°C the activity was still 25% of the original.

Activity of polygalacturonase on pectins of different degree of esterification in depectinized serum. Fig. 35 shows PG activities on green ribbon and purple ribbon pectins. The activity curve obtained with green ribbon pectin is linear only during a short time because the enzyme can only split glycosidic bonds next to a free carboxyl group, with a second free carboxyl group at a certain distance (Koller & Neukom, 1969). The low esterified purple ribbon pectin gives a linear activity curve.

The activities of PG measured under different circumstances are summarized in Table 7.

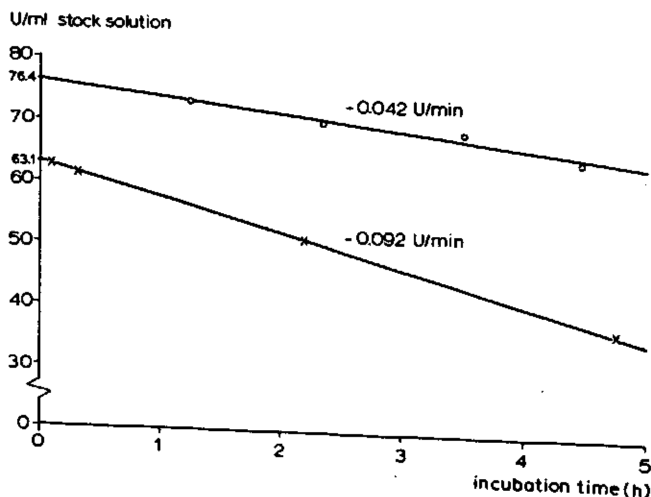


Fig. 34. Loss of PG activity at 30°C. Substrate: 10 ml orange serum with 0.3% purple ribbon pectin added. Enzyme solution: 2 ml 0.04% PG solution in 0.1 McIlvaine buffer pH 4.0 with 0.001 M Ca (x) or 0.01 M Ca (o) added. The enzyme solutions were incubated at 30°C.

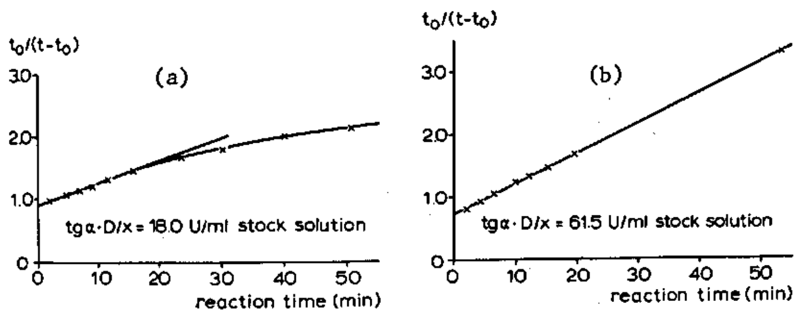


Fig. 35a. Activity of PG on green (a) or purple (b) ribbon pectin in orange serum at 30 °C. Substrate: 10 ml orange serum with 0.3% green (a) or purple (b) ribbon pectin added. Enzyme solution: 2 ml 0.1% PG solution in 0.1 McIlvaine buffer pH 4.0. x = ml of enzyme solution added; D = dilution factor of the enzyme solution added in respect to stock solution.

Table 7. The activity of yeast PG measured under different circumstances.

Substrate	Enzyme dissolved in	Initial activity (U/ml stock solution)	
0.25% pink ribbon pectin in acetate buffer pH 5.0	0.1 McIlvaine pH 5.0	PG-I	105
		PG-II	155
0.25% green ribbon pectin in depectinized serum pH 4.0	0.1 McIlvaine pH 4.0	PG-I	18.9 ± 9%
	depectinized serum pH 4.0	PG-II	44.5 ± 10%
0.25% purple ribbon pectin	0.1 McIlvaine pH 4.0 + 0.001 M Ca	PG-I	63.1 by extrapolation
in depectinized serum pH 4.0	0.1 McIlvaine pH 4.0 + 0.01 M Ca	PG-I	76.4 by extrapolation
	depectinized serum pH 4.0	PG-I	82.6 ± 4.1%
	depectinized serum pH 4.0	PG-II	134.3 ± 2.2%

Cloud stabilization by polygalacturonase. Fig. 36 shows that additions of PG to reconstituted orange juice in concentrations from 0.18 x 10^{-3} to 0.18 U PG/ml juice had no influence on the cloud stability during 15 days. After this period 1 U PE per ml juice was added to all samples. Fig. 36 shows that all PG concentrations, except the lowest one, stabilize the cloud for at least another 24 days although methanol analyses showed that in all samples 55% of the pectin methoxy groups present were saponified already 2 days after the PE addition.

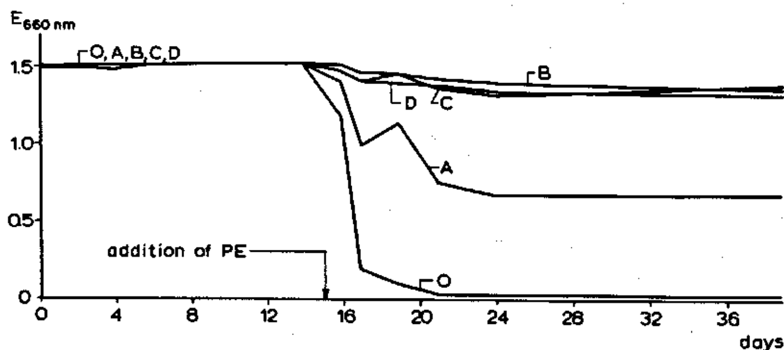


Fig. 36. Cloud stability of reconstituted orange juice with addition of 1 U PE/ml juice after a preincubation period of 15 days with various PG concentrations.

sample	U PG/ml juice
O	-
A	0.18×10^{-3}
B	0.18×10^{-2}
C	0.18×10^{-1}
D	0.18

In another experiment, carried out in duplicate, PG stock solution was added to reconstituted juice to give 0.1, 1.0 and 10 U PG/ml juice. After 19 h half of the samples were inactivated by heating them to 90°C in a boiling waterbath and immediate cooling. To all samples, PG active and PG inactivated, 1 U PE/ml juice was then added. Fig. 37a shows the cloud stability and Fig. 37b the corresponding methanol release by PE. It is seen again that the cloud is stabilized by PG if this enzyme is actively present during PE action. A pretreatment only with PG has no effect (Fig. 37a, curve D); clarification and methanol release are more rapid after the heat treatment (cf. Section 6.1.2).

In industrial practice PG would have to be added to PE active juice. In further experiments therefore 1 U PG and 1 U PE/ml juice were added at the same time to samples of reconstituted juice. In a control experiment inactivated PG was added instead of active PG. From Fig. 38a it is seen that preincubation with PG is not necessary to obtain cloud stabilization and the analysis of liberated methanol shown in Fig. 38b demonstrates clearly that PG does not affect the PE activity.

Establishment of the minimum ratio PG/PE required for cloud stabilization
To determine which ratio of PG/PE should be considered as a minimum for cloud stabilization, experiments were carried out with 3 PE levels and 8 PG/PE ra-

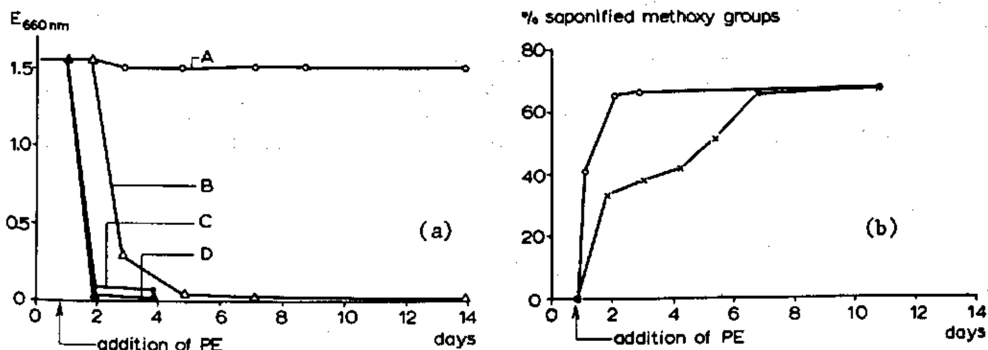


Fig. 37.(a) Cloud stability of reconstituted orange juice with addition of 1 U PE/ml juice after a preincubation period of 19 h with various PG concentrations (0.1, 1.0 and 10 U PG/ml juice).
Before addition of PE the PG was inactivated by heat in half of the samples.
A : PG, PE added without heat inactivation of PG;
B : blank without PG, PE added without heat treatment;
C : blank without PG, PE added after heat treatment;
D : PG, PE added after heat inactivation of PG.

Fig. 37.(b) Methanol release in orange juice samples of Fig. 37a. x : A and B; o : C and D.

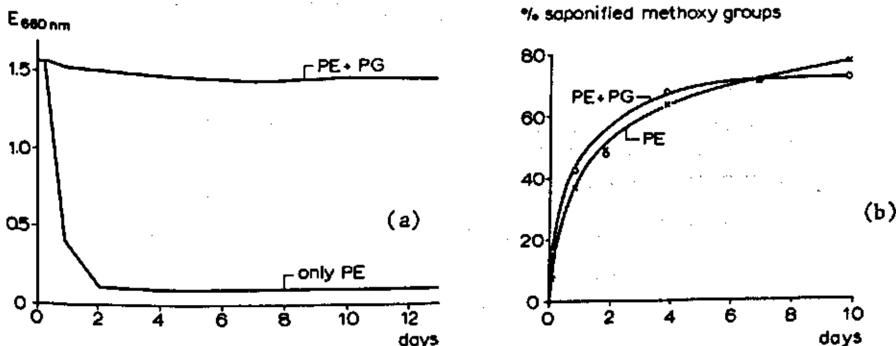


Fig. 38.(a) Cloud stabilization by PG (1 U/ml) added to reconstituted orange juice at the same time as PE (1 U/ml) and (b) Methanol release in orange juice samples of Fig. 38a.

tios according to Table 8. The Figs 39a, 39b and 39c show the cloud stability of the various samples. It is seen that all ratios enhance cloud stability; however, the lowest ratio, being 1×10^{-4}, does not suffice at the higher PE levels. Fig. 40 shows the turbidity after 14 days as a function of PE level. It is clearly seen that cloud stabilization is better at lower PE levels. There is also a tendency for higher ratios to stabilize better. Par-

Table 8. PG/PE ratios and absolute citrus PE levels to establish the minimum stabilizing ratio.

	Sample number for the various PE levels		
Ratio PG/PE	A: 0.5 U PE/ml	B: 1.0 U PE/ml	C: 2.0 U PE/ml
1.0	1	1	1
1.0×10^{-1}	2	2	2
1.0×10^{-2}	3	3	3
1.0×10^{-3}	4	4	4
5.0×10^{-4}	5	5	5
2.5×10^{-4}	6	6	6
1.0×10^{-4}	7	7	7
-	8	8	8

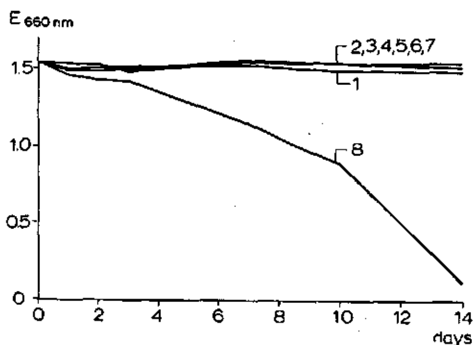


Fig. 39. Cloud stability of reconstituted orange juice samples with both PG and PE added to different PG/PE ratios. PE level (a) 0.5, (b) 1.0 and (c) 2.0 U/ml. The figures for the curves indicate sample numbers and have been explained in Table 8.

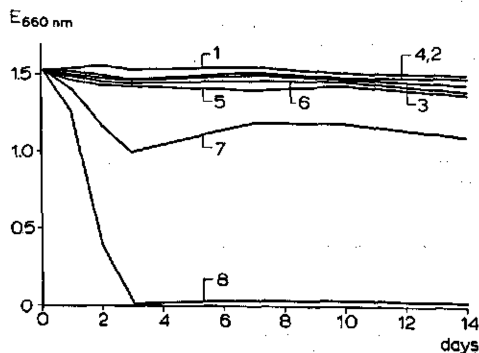


Fig. 39b.

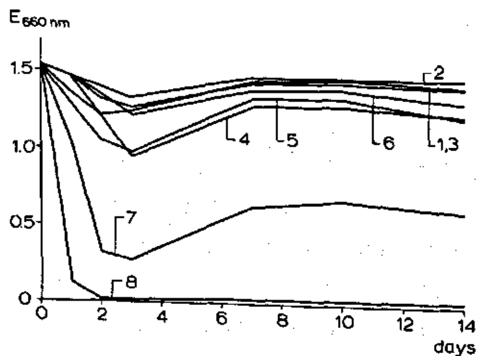


Fig. 39c.

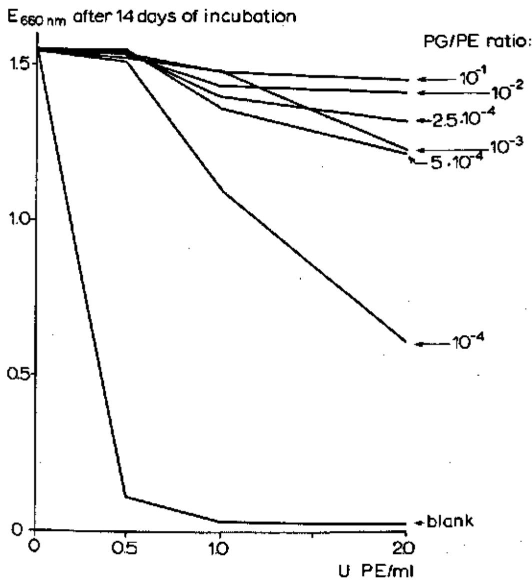


Fig. 40. Turbidity (as extinction after centrifugation) of orange juice samples of Fig. 39 after 14 days of incubation as a function of PE level.

ticularly the curves of Fig. 39c show a minimum after about 3 days. Possibly here the PE action is greater than the PG action and particles start to coagulate. Particle diameter could become so great that the light scattering quotient Q decreases and consequently the extinction value decreases too. Afterwards a reduction in particle size might occur by PG action with a resulting increase of Q.

Influence of calcium salt addition to reconstituted juice upon the stabilization by polygalacturonase. It is conceivable that there is a competition between the reaction of free carboxyl groups produced by PE action with Ca and with PG. Possibly the Ca in orange juice is present as a Ca-citrate complex and consequently the Ca-ions are released into the juice only slowly, so that PG can hydrolyze the low methoxy pectin before insoluble Ca-pectate has been formed. Therefore extra Ca was added to reconstituted juice to investigate if a surplus of Ca might inhibit stabilization by PG. The native Ca content of the juice is 0.25 mmol/100 ml; additions of $CaCl_2 \cdot 2H_2O$ were made to give 10.0 mmol Ca/100 ml.

From Fig. 41 it is seen that yeast PG stabilizes the cloud in spite of the surplus of Ca (curve D). Therefore it can be concluded that either PG competes more successfully or PG is also able to attack Ca-pectate. Further it is seen that reconstituted juice with added Ca is very stable (curve B). If this juice is activated by PE, however, clarification occurs very rapidly (curve C).

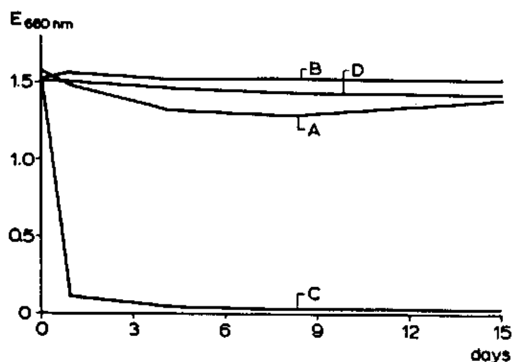


Fig. 41. Cloud stabilization by PG of reconstituted orange juice with extra Ca added.
A : orange juice containing 0.25 mmol Ca/100 ml, 1 U PG/ml and 1 U PE/ml; B : orange juice containing 10.0 mmol Ca/100 ml; C : orange juice containing 10.0 mmol Ca/100 ml and 1 U PE/ml; D : orange juice containing 10.0 mmol Ca/100 ml, 1 U PG/ml and 1 U PE/ml.

6.3.2 Cloud stabilization by a pectin lyase

In this section it is shown that the harmful effect of PE upon cloud stability can also be cancelled by degrading the juice pectin before and/or during PE action by a pectin lyase purified from the commercial preparation Ultrazym 20 (CIBA-GEIGY AG, Basel, Switzerland).

Pectin esterase activity of pectin lyase preparations. In spite of purification the preparations still have some PE activity which must be well described in order to be able to judge its influence. Firstly the pH-spectrum of the PE activity exhibited by the commercial preparation Ultrazym 20 was established. An enzyme solution was prepared by stirring 3 g of Ultrazym 20 in 150 ml distilled water during 1 h. The suspension was then filtered through a paper filter and a D3 glass fritted filter. The PE activity was measured titrimetrically with the Combi titrator at pHs ranging from 3.5 to 7.0 in the following way: Into the reaction vessel 0.625% green ribbon pectin solution in 20 ml 0.125 M NaCl were pipetted. After equilibration at 30°C the pH was brought to the desired value with 0.1 N NaOH from a microburette. Then 5 ml enzyme solution were added and the desired pH was kept constant titrimetrically while the amount of 0.01 N NaOH against time was recorded. When approximately 1% of the pectin had been saponified, the pH of the solution was raised quickly to about 6 with 0.1 N NaOH from the microburette. Finally the reaction mixture was titrated to pH 7.0 with 0.01 N NaOH from the titrator. A control determination was carried out with heat inactivated enzyme (5 min at 100°C). All assays were carried out in duplicate. The activity is found by subtracting the amount of NaOH used in the blank determination from the total amount of NaOH, and dividing this value by the reaction time at the respec-

tive pH. The pH-spectrum is shown in Fig. 42, optimum pH is at about 4.25, at pH values of 6.0 and higher the PE is completely inactive.

The methanol release of the 2% (w/v) Ultrazym 20 solution added to a 0.5% (w/v) brown ribbon pectin solution in 1/3 McIlvaine buffer pH 4.0 is shown in Fig. 43. Fig. 44 shows the methanol release by the purified PL preparations I, II and III. There is an initial linear rate of methanol format-

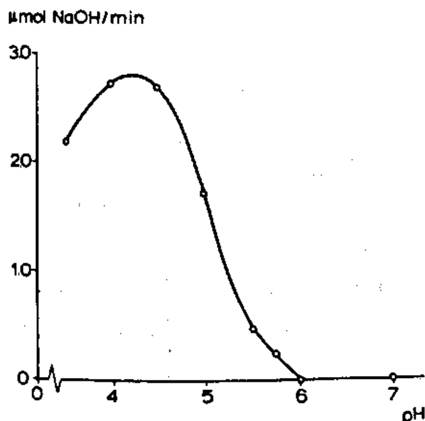


Fig. 42. pH spectrum of the PE activity in Ultrazym 20.

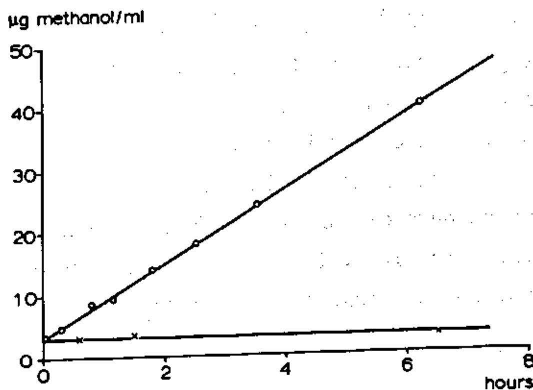


Fig. 43. Methanol release in a 0.5% (w/v) brown ribbon pectin solution in 1/3 McIlvaine buffer pH 4.0, containing crude Ultrazym 20 to give 2.7 x 10^{-3} U PL/ml (0); x: blank without added enzyme.

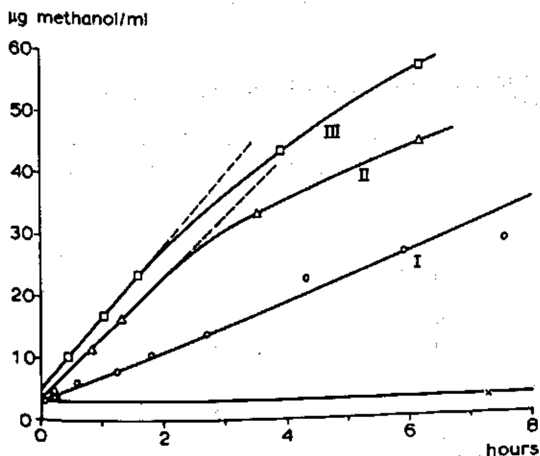


Fig. 44. Methanol release in 0.5% (w/v) brown ribbon pectin solutions in 1/3 McIlvaine buffer pH 4.0, containing purified U20 PL preparations.
I : containing 42.7 x 10^{-3} U PL-I/ml;
II : containing 59.8 x 10^{-3} U PL-II/ml;
III : containing 41.4 x 10^{-3} U PL-III/ml.
x : blank without added enzyme.

ion, however, after a few hours, depending on the amount of PE, methanol release decreases. From the slope of the initial velocity the PE activity can be deduced. The PL activity is divided by this value and the quotient compared with that for the crude enzyme. Thus the purification factor can be calculated (Table 9).

Cloud stabilization by pectin lyase. In preliminary experiments involving preincubation for 21 h of reconstituted juice with PL-I followed by PE addition cloud stabilization was observed at all PL levels applied (12.9×10^{-3}, 32.2×10^{-3} and 64.5×10^{-3} U/ml), in spite of normal enzymic saponification in the PE activated samples as indicated by methanol determinations. An experiment was then carried out with various PL/PE ratios at different citrus PE levels and without preincubation. In the PL/PE ratios given the PE activities of the PL preparations are not included; thus PE means here citrus PE. In Table 10 the ratios applied and the preparations used are given. The Figs 45a, 45b and 45c show the cloud stability of the various samples. It is seen that at the lowest PE level all ratios and pre-

Table 9. PL and PE activities of liquid PL preparations.

	U PL/ml	x[1]	$\frac{\mu mol\ methanol}{min \times (150 + x) ml}$	$\frac{U\ PL}{(150 + x) ml}$	PL/PE	Purification factor
2% crude U20	0.41	1.0	0.47	0.41	0.88	-
PL-I	6.45	1.0	0.31	6.45	20.50	23.2
PL-II	179.6	0.05	0.77	8.98	11.74	13.3
PL-III	41.4	0.15	0.91	6.21	6.83	7.7

1. x: the amount of PL preparation added to 150 ml pectin solution

Table 10. PL preparations, PL/PE ratios and absolute citrus PE levels used for establishing the best stabilizing ratio.

	Ratio PL/PE	A: 0.5 U PE/ml	B: 1.0 U PE/ml	C: 2.0 U PE/ml
-	-	1	1	1
PL - I	3.6×10^{-3}	2	2	2
PL - I	36×10^{-3}	3	3	3
PL - II	3.6×10^{-3}	4	4	4
PL - II	36×10^{-3}	5	5	5
PL - II	360×10^{-3}	6	6	6
PL - III	36×10^{-3}	7	7	7
PL - III	360×10^{-3}	8	8	8

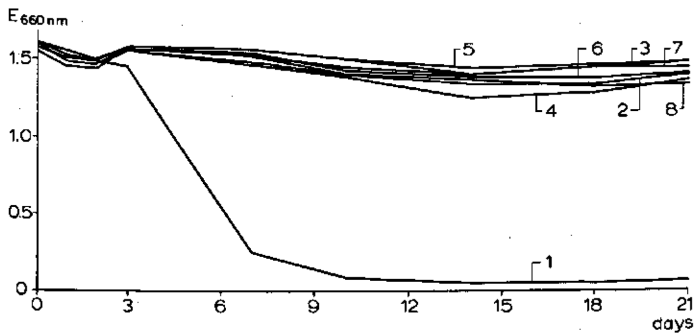


Fig. 45. Cloud stability of reconstituted orange juice samples with both PL and PE added to different PL/PE ratios. PE level (a) 0.5, (b) 1.0 and (c) 2.0 U/ml. The figures for the curves indicate sample numbers and have been explained in Table 10.

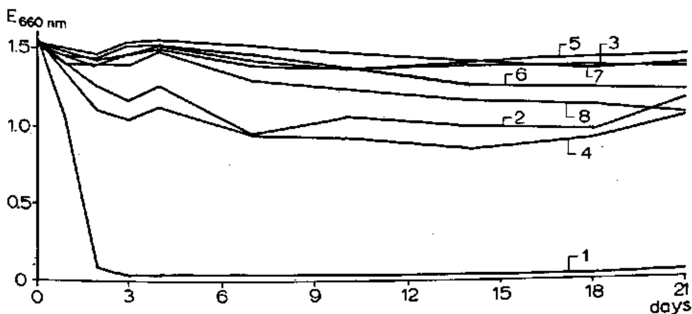


Fig. 45b.

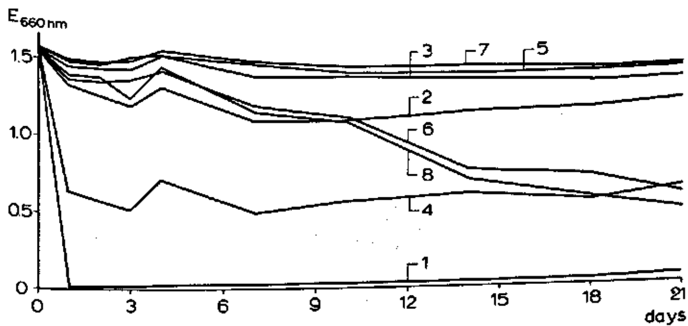
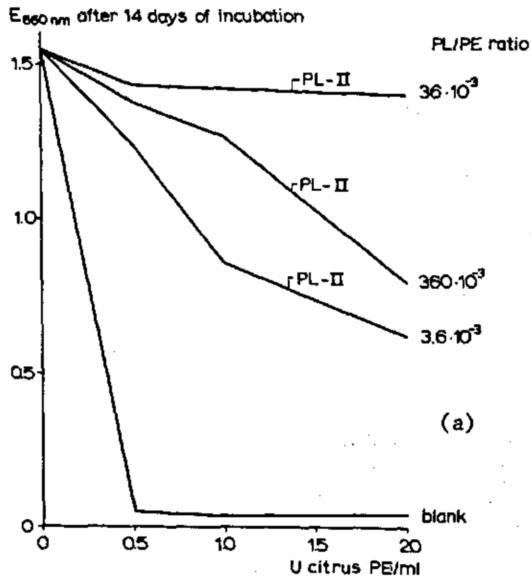


Fig. 45c.

parations stabilize the cloud well. At the higher PE levels cloud stabilization occurs but in some cases the stability is only moderate. Figs 46a and 46b show the turbidity after 14 days as a function of PE level. Two facts are striking: (1) cloud stabilization does not appear to be dependent on the purification of the PL preparation as all three preparations applied can give



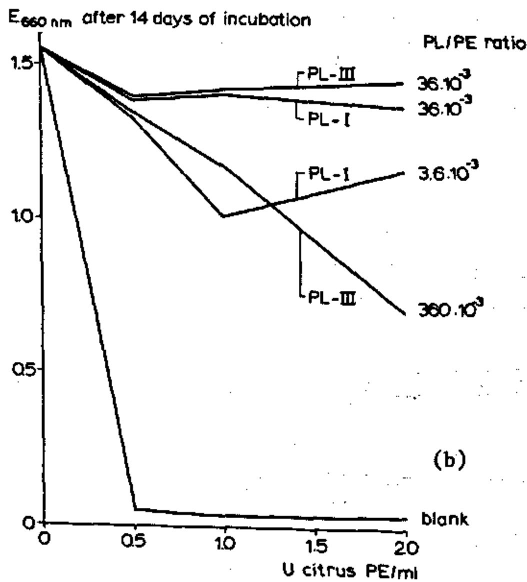


Fig. 46. Turbidity (as extinction after centrifugation) of orange juice samples of Fig. 45 after 14 days of incubation as a function of PE level. (a) Curves for PL-II are given. (b) Curves for PL-I and PL-III are given.

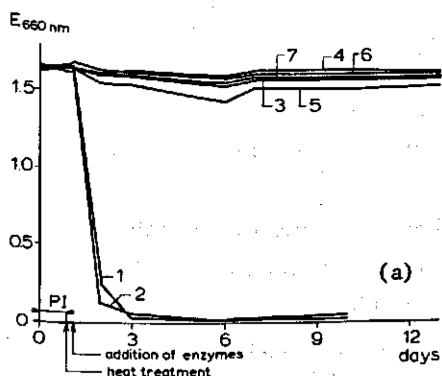
good cloud stability, and (2) best stabilization is not achieved by the highest PL/PE ratio, but a ratio of approximately 36×10^{-3} seems to be better than both lower and higher ratios.

The influence of preincubation of the juice with PL upon the cloud stability has been investigated in an experiment carried out according to Table 11. Fig. 47a shows the cloud stability and Fig. 47b the methanol release of the samples. In this experiment it is seen again that clarification and methanol release proceed more rapidly after an extra heat treatment (cf. sample 2 with

Table 11. Design of experiment to investigate influence of preincubation with PL upon cloud stability.

	22 h preincubation with PL-II[1]	Heat treatment after preincubation (5 min at 90°C)	Addition of PE[2] and/or PL-II[1] after preincubation and heat treatment
1	no	no	PE
2	no	yes	PE
3	yes	no	-
4	yes	yes	PL-II
5	yes	no	PE
6	yes	yes	PE
7	yes	yes	PL-II + PE

1. Each addition of PL-II amounts to 36×10^{-3} U PL/ml juice
2. Each addition of citrus PE amounts to 1.0 U PE/ml juice



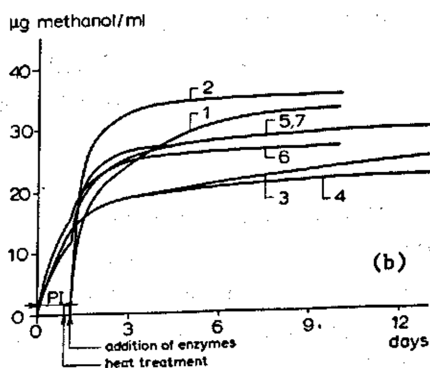


Fig. 47.(a) Influence of preincubation of reconstituted orange juice with PL upon cloud stability. The figures for the curves indicate sample numbers and have been explained in Table 11. (b) Methanol release in orange juice samples of Fig. 47a. PI = preincubation.

sample 1; cf. also the Figs 17 and 18). From the methanol release during the preincubation of the samples 3 to 7 and during the whole incubation of the samples 3 and 4 it is clearly demonstrated that the own PE activity of the PL-II preparation is not negligible. However, if PE is added after the preincubation methanol release is suddenly increased. It is clearly shown that after two weeks in all samples at least 45% of the methoxy groups of the pectin have been saponified. In spite of this, all samples preincubated with PL-II showed a stable cloud (Fig. 47a). The most striking result is that of sample 6, in which the juice preincubated with PL-II and submitted to citrus PE activity only after inactivation of this PL activity does show cloud stability. This means that, contrary to results obtained with PG, preincubation with PL suffices for cloud stabilization. Apparently PL is able to degrade the juice pectin to such an extent that saponification of the breakdown products afterwards does not yield Ca-precipitable pectates which could clarify the juice. This theory is supported by the known mechanism of action of PL (Voragen, 1972).

6.3.3 *Comparison of polygalacturonase and pectin lyase as pectin depolymerases and as cloud stabilizers*

In order to be able to compare the activities of PG and PL, their depolymerizing activities on green ribbon pectin have been determined viscosimetrically as described in Section 5.2. Green ribbon pectin (DE = ca. 65%) which is attacked both by PG and PL was used as substrate. The activities were determined in quadruplicate and the average depolymerase values (DU = depolymerase unit) were:

- PG-II: 44.5 $\pm$ 10.0% DU/ml stock solution;
- PL-II: 37.7 $\pm$ 3.2% DU/ml stock solution.

The activity of PG is linear with time only during about 10 min; however, the activity of PL is still linear after 40 min.

At a level of 1 U citrus PE/ml juice cloud is stabilized if the PG/PE ratio is 2.5×10^{-4} or higher or the PL/PE ratio is about 36×10^{-3}. These figures correspond with 71.7×10^{-6} DU of PG/ml juice and with 75.6×10^{-4} DU of PL/ml juice respectively. So it seems that PG is approximately 100 times more effective than PL. Probably this higher effectiveness of PG can be explained by two facts: (1) the average DE of the juice pectin is lower than that of green ribbon pectin, so that the juice pectin is a better substrate for PG and a less suitable substrate for PL than green ribbon pectin, and (2)

when PE action occurs the juice pectin is saponified, thus continuously becoming a better substrate for PG and a less suitable one for PL.

6.4 ACTIVITY OF PECTIC ENZYMES AND CLARIFICATION IN CONCENTRATED SYSTEMS

6.4.1 Inhibition of citrus pectin esterase, yeast polygalacturonase and U20 pectin lyase by sugars and concentrated depectinized serum

The possible inhibition by sugars and concentrated depectinized serum of the various pectic enzymes used in this study was investigated in order to determine what their activities might be in a 40°Bx orange concentrate if added to prevent gelification. 40°Bx was chosen as the usual degree of concentration of commercial frozen concentrates. The enzymes PG and PL were tested viscosimetrically on purple ribbon pectin and brown ribbon pectin respectively. Citrus PE was assayed titrimetrically on green ribbon pectin. To investigate inhibition by sugars a mixture of sucrose, fructose and glucose (2:1:1 by weight (Sinclair, 1961a)) was added to make a 0.25% pectin solution of about 36°Bx. A concentrated depectinized serum of 50°Bx was added to make a similar 0.25% pectin solution of about 36°Bx. The inhibition of PE by concentrated depectinized serum could not be measured as the pH of the

Table 12. Inhibition of pectic enzymes by sugars (sucrose : fructose : glucose = 2:1:1) and concentrated depectinized orange juice serum.

	Substrate	Activity (U/ml stock solution)				
		Not inhibited (10.7°Bx)	Sugars (36°Bx)	Inhibition	Conc. dep. serum (36°Bx)	Inhibition
PG-II[1]	0.25% purple ribbon pectin in depectinized serum pH 4.0	134.3	83.1	38.1%	31.9	76.2%
PL-II[1]	0.25% brown ribbon pectin in depectinized serum pH 4.0	33.0	30.0	9.1%	17.8	46.1%
Citrus PE[2]	0.1 M NaCl, 1.0% green ribbon pectin pH 7.5	184.6	44.85	75.7%	•	•

1. Viscosimetrically assayed
2. Titrimetrically assayed, no serum or sugars present

serum was 4.0 and the amount of alkali required to bring to pH 7.5 would cause a salt effect to dominate the serum effect. In Table 12 the activities are shown and it is seen that the pectin depolymerizing enzymes are inhibited as well by sugars only as by the concentrated depectinized serum. Inhibition by the latter takes place to a greater extent and in both cases PG is more strongly inhibited than PL. The greater inhibition effect of the concentrated depectinized serum might be ascribed to the high content of cations, particularly potassium. Citrus PE is relatively strongly inhibited by sugars. From the literature (Joslyn & Pilnik, 1961) it is known that gelation and clarification processes find optimal conditions at 40°Bx concentration. An activating effect of the cations of concentrated orange juice may therefore be assumed.

6.4.2 Preliminary experiments on cloud stability of juice stored as 40° Brix concentrate

The cloud stabilizing property of PG in single-strength juice suggested that this enzyme could also prevent clarification effects in PE active 40°Bx concentrates. 40°Bx concentrates were prepared from the 65°Bx concentrate by mixing with self pressed, PE inactivated orange juice or with distilled water and kept at the natural pH value of 3.3 and at pH 4.0, after PG, PE and a combination of these had been added. After dilution to single-strength juice turbidity was measured. If PE had been added singly the reconstituted juice showed complete clarification. If PE had been combined with PG the reconstituted juice was not clear but turbidity was poor ($E_{660\ nm}$ = 0.40). It was seen that PG, if added singly, affected the cloud stability negatively particularly at pH 4.0. This is not the case in single-strength juice and no explanation is offered for this phenomenon. This negative effect is quite unexpected and, moreover, offers little hope for successful application of PG in concentrates.

6.5 A model experiment with pectic enzymes in a calcium pectinate solution

A model experiment was designed to demonstrate the effects of yeast PG and U20 PL upon the Ca reactivity produced in pectins by PE. The model system consisted of pectin solutions containing 0.5 g brown ribbon pectin/100 ml solution and 0.5 g green ribbon pectin/100 ml solution respectively. In addition 50 mg Ca/100 ml solution and 0.5 g citric acid/100 ml solution were

added; pH was adjusted to 4.0. Table 13 summarizes the experiment and the results obtained. If we compare gelation in this experiment with cloud loss in the experiments described in the Sections 6.3.1 and 6.3.2 the correlation is striking. Brown ribbon and green ribbon pectin solutions are gellified by citrus PE; this is, however, totally prevented by PG and to a lesser extent by PL. After preincubation with PG and PL respectively no gelation occurs at all.

Table 13. Influence of PG and PL upon gelification of pectin solutions in a model experiment.

Pectin solution	Enzymes added	Gel formation[6] after incubation overnight
A. Without preincubation		
brown ribbon	PE[1]	+++
green ribbon	PE[2]	+++
brown ribbon	PE + PG[3]	-
green ribbon	PE + PG[3]	-
brown ribbin	PE + PL[4]	+
green ribbon	PE + PL[5]	+
B. Addition of PE after preincubation during 3 h with PG or PL		
brown ribbon	PG PE	-
green ribbon	PG PE	-
brown ribbon	PL PE	-
green ribbon	PL PE	-
C. Preincubation during 3 h with PG or PL. Addition of PE after inactivation of PG or PL		
brown ribbon	PG PE	+++
green ribbon	PG PE	-
brown ribbon	PL PE	-
green ribbon	PL PE	-

1. To brown ribbon pectin solutions 3.69 U PE/ml were added in each experiment
2. To green ribbon pectin solutions 1.85 U PE/ml were added in each experiment
3. Always 1.55 U PG/ml were added
4. To brown ribbon pectin solutions 126 x 10^{-3} U PL/ml were added in each experiment
5. To green ribbon pectin solutions 269 x 10^{-3} U PL/ml were added in each experiment
6. +++ gel, ++ weak gel, + slightly gelled, - no gelation

In the case of preincubation with both depolymerases and their inactivation before PE addition PL sufficiently degrades both pectins to prevent gelling. The same is true for PG and green ribbon pectin whereas the more highly esterified brown ribbon pectin is not broken down by PG and therefore still gels by PE action. The average DE determined in orange juice pectin is 47.3% and it is therefore a better substrate for PG than green ribbon pectin. It appears therefore surprising that preincubation of juice with PG and subsequent pasteurization cannot prevent clarification by PE. It must be realized, however, that this is an average DE only and more highly esterified pectin fractions exist which are comparable to brown ribbon pectin.

6.6 *Effect of commercial protease preparations on cloud loss* (Krop & Pilnik, 1974c)

According to Lankveld (1973) clarification of citrus juice by PE must be ascribed among other things to the increased electrostatic interaction between the positively charged protein and negatively charged pectin molecules. Lankveld suggests that the cloud stabilizing property of pectinase (Baker & Bruemmer, 1972) may be due to the prevention of this interaction which will also prevent bridge formation between the dispersed cloud particles. From this point of view protease should stabilize the cloud by a similar mechanism as the pectin depolymerizing enzymes.

Assay of the proteolytic activities. 4 commercial preparations, which are all active in the acid range, have been used in this study:

1. Fungal protease (Takamine, Elkhart, Indiana, USA),
2. Bakterien Proteinase N (Röhm & Haas Pharma Gmbh, Darmstadt, Germany),
3. Papain AS-400 (Wallerstein Company, Deerfield, Illinois, USA),
4. Corolase L-10 (Röhm & Haas, Pharma Gmbh, Darmstadt, Germany).

Corolase L-10 is a liquid preperation, while the other ones are powdered. 200 mg of the powders were dissolved in 10 ml distilled water and afterwards centrifuged (3 min at 1200 x *g*). Corolase was diluted 1:10. These protease solutions were diluted and brought to two pHs: 4.0 (the pH of juice) and 7.5 (the pH of citrus PE stock solution). Filmslips were dipped into all these solutions and protease activity was checked according to Section 5.2. The citrus PE stock solution was tested in a similar manner. The results are shown in Table 14. The protease activity was also determined by putting the filmslips into the protease activated juice samples to have the conditions

Table 14. Proteolytic activity of enzyme solutions of different concentration at pH 7.5 and pH 4.0 expressed as number of hours for positive protease reaction

	Undiluted	1:1	1:100	1:200	1:500	1:1000	1:2000	1:5000
pH 7.5								
Fungal protease	.	.	5	5	18	18	18	18
Bakterien Proteinase N	.	.	1	1	2	3	5	6
Papain AS-400	.	.	2	4	6	.	.	.
Corolase L-10	.	.	4	6	18	.	.	.
Citrus PE	2	5	.	.	.	.	.	.
pH 4.0								
Fungal protease	.	.	4	18	18	18	18	18
Bakterien Proteinase N	.	.	4	6	6	27	27	.
Papain AS-400	.	.	2	5	27	.	.	.
Corolase L-10	.	.	2	6	27	.	.	.
Citrus PE	–	–	.	.	.	.	.	.

– : negative
. : no observation

of the juice. Juice samples contained per 100 ml juice 4 ml of the protease solution prepared as described above (corresponding to 80 mg protease powder preparation), however, the juice with Corolase L-10 contained 0.1 ml of this liquid preparation/100 ml juice. Each hour the filmslips were checked and from Table 15 it can be seen that all protease preparations tested are active on gelatin under juice conditions.

The effect of proteolytic activity on cloud stability. 80 mg of the various commercial preparations were added in aqueous solution to 100 ml orange juice as described above. From Corolase L-10 as a liquid preparation again 0.1 ml per 100 ml juice was added. The enzymes were added to juice:

Table 15. Proteolytic activity under juice conditions expressed as number of hours for positive protease reaction.

Juice activated with protease	Number of hours
Fungal protease	5
Bakterien Proteinase N	6
Papain AS-400	2
Corolase L-10	4
Blank	–[1]

1. After 3 days still negative

A. singly,

B. combined with 1 U citrus PE/ml,

C. combined with 1 U citrus PE/ml plus 1 U yeast PG/ml,

D. combined with 1 U yeast PG/ml.

The results of A and B are represented in Fig. 48a and the results of C and D in Fig. 48b. Fig. 49 shows methanol release of A and B. Curves A in

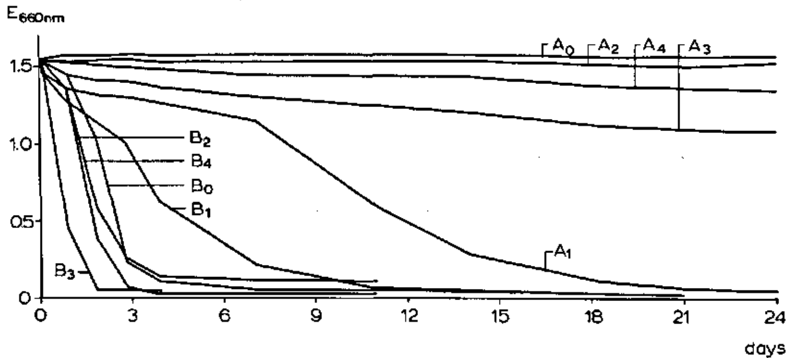


Fig. 48. Cloud stability of reconstituted orange juice samples.
A : protease added (80 mg/100 ml);
B : protease (80 mg/100 ml) and citrus PE (1 U/ml) added;
C : protease (80 mg/100 ml) and yeast PG (1 U/ml) and citrus PE (1 U/ml) added;
D : protease (80 mg/100 ml) and yeast PG (1 U/ml) added;
0 : no protease;
1 : fungal protease;
2 : Bakterien proteinase N;
3 : papain AS-400;
4 : Corolase L-10.

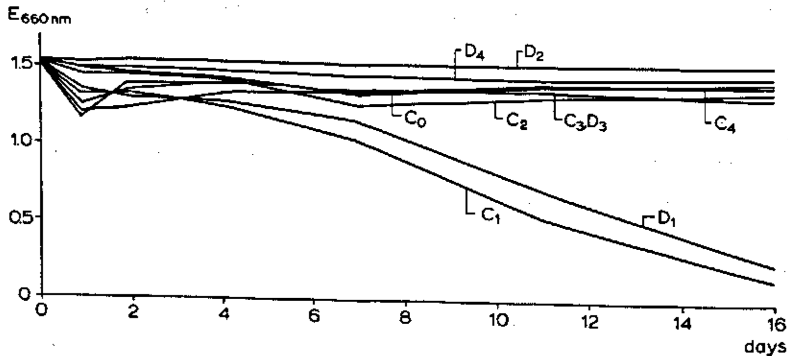


Fig. 48b.

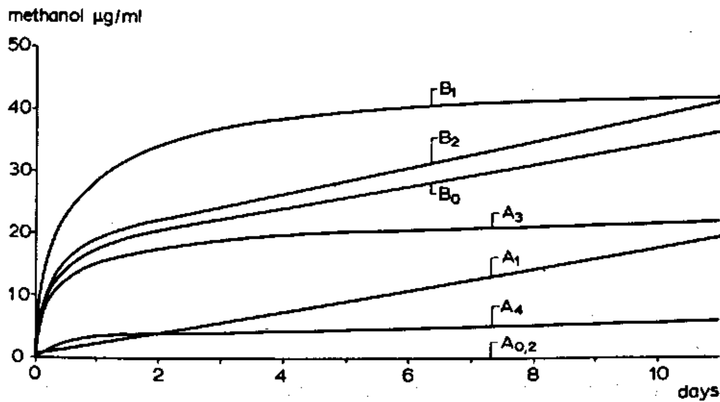


Fig. 49. Methanol release in orange juice samples of Fig. 48a.

Fig. 48a show that fungal protease clarifies the juice whereas the action of other enzymes results in a smaller cloud loss. If the juice is PE active (curves B) all the samples with protease do clarify. From the methanol release (Fig. 49) it is seen that fungal protease has a pronounced PE activity. Remarkable is also the PE activity of papain which, however, causes only slight clarification. Fig. 48b shows that the cloud stabilizing property of yeast PG is retained also in the presence of protease; only in the case of fungal protease clarification occurs.

It may be concluded that the commercial protease preparations tested affect the cloud stability to a greater or smaller extent, only in partial correlation to the PE activity of the enzyme. Bakterien Proteinase N contains no PE and affects cloud stability scarcely. Corolase L-10 has only a slight PE activity and provoked somewhat more cloud loss. On the other hand papain clarified the juice only slightly in spite of its rather high PE activity whereas fungal protease caused a total cloud loss, though its PE activity was less than that of papain. Conceivably the PEs of the various preparations have a different mechanism of action. I was unable to confirm literature data (Biggs & Pollard, 1970; Lankveld, 1973) concerning positive cloud stabilising properties of proteases (curves B, Fig. 48a). Although neither by those authors nor in my own investigation has it been shown conclusively that the enzyme preparations tested attack the juice protein the experiments described above present a basis for this assumption.

7 Discussion and conclusions

In Section 6.1 investigations of various experimental factors which might influence the cloud stability of orange juice have been described. It was shown that neither repeated measurements nor the frequency of measurement, at least if PE level was relatively high, affected the cloud loss process. However, the way samples are stored can have an effect. It was clearly shown that continuous mixing of samples increased considerably both the rate of clarification and the rate of methanol release. An additional effect was the more even course of the curves for methanol release (cf. Figs 15a and 15b). This can be explained by the heterogeneity of the orange juice samples being masked by the continuous mixing. This mixing results in a distinct percentage of saponified methoxy groups at which clarification takes place (Fig. 16a). This value is the same as for the 1.1 U PE/ml level of the samples of the standing method, while in this case samples with less PE clarify at a lower percentage saponified methoxy groups (Fig. 16b). Possibly in the mixed samples PE is redistributed continuously between particles and serum and consequently between particle pectin and serum pectin. In this way PE might saponify less effectively, by deesterifying many pectin molecules to a small extent rather than a few molecules to a great extent.

Both mechanical and thermal factors were shown to affect cloud stability. Heat treatment of juice accelerates clarification and methanol formation. Although a pectin extraction from the particles is restricted, other effects might be more important, e.g. an enhanced accessibility of the enzyme to its substrate by reduction of particle size. These effects, however, have not been investigated. Reconstituted orange juice activated with PE, clarified more rapidly than fresh orange juice. This result may be explained by the heat treatment of the concentrating process as well as by the addition of KOH in reconstituting the juice. Addition of KOH to adjust pH to 4.0 optimizes PE requirements with regard to both pH and cation content (cf. Section 3.2).

A combination of thermal and mechanical factors considerably increased the cloud level of clarified juice (Fig. 19). Moreover these treated samples

sometimes showed good stability. Possibly the treatments produced cloud particles which are differently stabilized compared with cloud stable juice that has not been clarified by PE. However, this was not investigated further. However these experiments did show that resuspension experiments (Baker & Bruemmer, 1969) are not really suitable for determining which factors are decisive for cloud stability of orange juice.

In Section 6.2 it was shown that immediately after pressing the supernatant of a centrifuged orange juice is rather clear. Maximum turbidity is reached between 1 and 3 h after pressing. Possibly this is connected with reactions of the polymers present. After pressing it takes some time before equilibrium has been attained. Polymers are adsorbed on particles and are also able to react with each other. These processes could influence turbidity. The reason why Jaffa orange juice often does not clarify completely is not evident. Perhaps this is connected with a different composition of the cloud. However, it was not within the scope of this study to investigate this phenomenon. It was established that the rate of clarification can not be correlated with PE activities which have been determined titrimetrically. Obviously, other factors are important too, e.g. cloud composition and pH. Possibly different orange PE iso-enzymes do exist as has been described for banana (Hultin & Levine, 1963) and for tomato (Pressey & Avants, 1972). However, for orange no literature data on this subject are known.

From the experiments with mixtures of pasteurized and unpasteurized orange juices (Figs 21 and 22) and from the experiments with PE additions to reconstituted juice (Figs 23-25), it has become obvious that ordinarily orange juice clarification depends on the PE present.

In fact clarification is correlated strongly with PE action, as I have proved by determining methanol formation. After an initial rapid methanol formation, always a slow-down takes place, which can be explained by the product inhibition of PE occurring as is demonstrated in Fig. 26. An accidental, sudden increase in methanol formation can be ascribed to the heterogeneity of the system as has been pointed out when continuous mixing was compared with the standing method.

From the investigations on the roles of bivalent cations (Fig. 27) and pectic acid (Fig. 28), it has become clear that a mere saponification of juice pectin is not sufficient for cloud loss. Bivalent cations are indispensable for clarification (Fig. 27a) as was also demonstrated when pectic acid was added to cloud stable reconstituted juice (Fig. 28a). Suspensions of

cloud in aqueous media with PE added did not clarify (Fig. 30a), while a cloud suspension in orcat solution containing bivalent cations, did (Fig. 31a). In Section 2.2 it was suggested, based on literature data, that serum pectin and cloud pectin might play different roles in cloud loss phenomena. In this study, however, it was not possible to differentiate sharply between these pectin fractions. As is shown in Fig. 31b serum pectin is saponified rapidly upon PE addition, but also cloud pectin is attacked by PE when the cloud has been suspended in aqueous media (Fig. 30b) or in orcat solution (Fig. 31b). Curve B in Fig. 31b shows that a cloud suspension is able to clarify without serum pectin present. On the other hand Baker & Bruemmer (1972b) described the clarification of a cloud suspension by the addition of a floc developed in the serum. Possibly the distinction between serum pectin and cloud pectin is not very sharp. Cloud pectin could be partially adsorbed on the particles with loops extending from the particle into the serum. These pectin loops could then be considered as 'serum pectin'.

From the results of the described experiments and from the electron micrographs of stable and clarified orange juice the clarification mechanism can be described as follows. If orange juice contains active PE, this PE saponifies the juice pectin, both soluble serum pectin and pectin adsorbed to the cloud particles. When a certain limit of saponification has been passed, the partially deesterified pectin becomes sensitive to calcium ions and, if these are present, insoluble calcium pectate will settle down. The insoluble calcium pectate forms three-dimensional structures by intermolecular interactions. These structures enclose all cloud particles, thus leaving a crystal clear supernatant.

To prevent clarification various measures can be taken. (1) Inactivation or inhibition of citrus PE. As no inhibitors are known, which inhibit PE for 100%, in practice PE is always inactivated by heat. (2) Removal of Ca from the juice, as is demonstrated in Section 6.2.5 (Figs 27 and 28), prevents clarification though PE action is normal. (3) Prevention of the formation of high molecular calcium pectate by enzymic splitting of the juice pectin. In principle this can be achieved in two ways: breakdown of the pectin before PE can exert its saponifying action and breakdown of the pectate after the action of PE. Both possibilities have been investigated and were described in Section 6.3.

In Section 6.3.1, PG proved to be a suitable agent to hydrolyse pectate after PE action. If reconstituted orange juice had been preincubated with PG and repasteurized before PE addition, no positive effect was obtained. How-

ever, if PG and PE were allowed to act simultaneously on the juice pectin, cloud stabilization was achieved. Even a very high calcium content of the juice did not cancel the positive effect of PG (Fig. 41). It was shown that the ratio of PG to PE enzyme activity should be a minimum. Baker & Bruemmer (1972a) correlated the effectiveness of stabilization by commercial pectinase preparations with the ratio of depolymerizing activity on polygalacturonic acid to that on citrus pectin (PG_{PGA}/PG_{PEC}). These authors recognized that the PG_{PEC} activity does not come from one enzyme activity. They ascribed this activity to a combined activity of PG and PMG. Indeed PG can degrade citrus pectin (DE approximately 70%) to a restricted extent (cf. Fig. 35a) and its action will be increased considerably by PE, which is mostly present in commercial preparations. Based upon recent literature data (Rombouts, 1972), it is disputable whether PMG does exist and therefore it is obvious that their PMG activity in reality is a PL activity. Because of data from Ishii & Yokotsuka (1971), Baker & Bruemmer omitted to assay their commercial preparations for lyase activity. However, commercial preparations with high lyase activity really do exist, e.g. Ultrazym 20 (CIBA - GEIGY A.G., Basel, Switzerland), Pektolase FL32 (Grindstedvaerket, Aarhus, Denmark) and Pektosin C (Kikkoman Shoyu Co. Ltd., Chiba-Ken, Japan). The correlation found by Baker & Bruemmer, as mentioned above, is shown in the Figs 50a and 50b. These curves suggest that a further increase of the PG_{PGA}/PG_{PEC} ratio will render higher cloud levels. However, I have taken the figures of Baker & Bruemmer (1972a) given

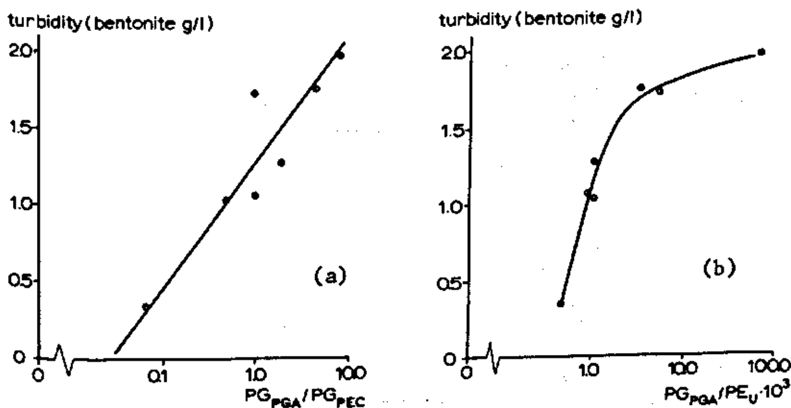


Fig. 50. Relation between turbidity and (a) PG_{PGA}/PG_{PEC} ratio on a logarithmic scale according to Baker & Bruemmer (1972a) or (b) PG_{PGA}/PE_{u} x 10^3 ratio on a logarithmic scale, data taken from Baker & Bruemmer (1972a, Table IV).

in Table IV of their publication and calculated another ratio, viz. $PG_{PGA}/PE_u \times 10^3$. I plotted the turbidity values given in Table I of their publication against this ratio and found a much better correlation. It also shows clearly that a ratio $PG_{PGA}/PE_u \times 10^3$ above 3 does not help much in increasing cloud level further (Figs. 50b and 51b). Although Baker & Bruemmer's (1972a) approach to cloud stability problems was different from mine, their findings about cloud stabilization by PG agree with mine.

However if we assume that the PG_{PEC} activity of Baker & Bruemmer, at least partially, is a PL activity, our findings do not agree. According to them a higher PG_{PEC} activity would result in a less stable cloud, whereas I on the contrary was able to stabilize the cloud with PL. Even preincubation of reconstituted orange juice with PL and inactivation of this activity before addition of citrus PE was effective in preventing cloud loss (Fig. 47a), probably because the juice pectin can be broken down to a greater extent by PL than by PG. PG needs at least two free carboxyl groups at a certain distance from each other (Koller & Neukom, 1969), while PL can split the glycosidic bond adjacent to an esterified galacturonic acid residue in the pectin chain. Yet it was found, based on depolymerase units, that PG is more effective than PL in stabilizing orange cloud in PE active juice. This fact can be explained by the action of the citrus PE that changes the juice pectin into a more suitable substrate for PG and a less suitable substrate for PL.

The results of the model experiment with PG and PL showed a good correlation between gelation of pectin solutions and clarification of orange

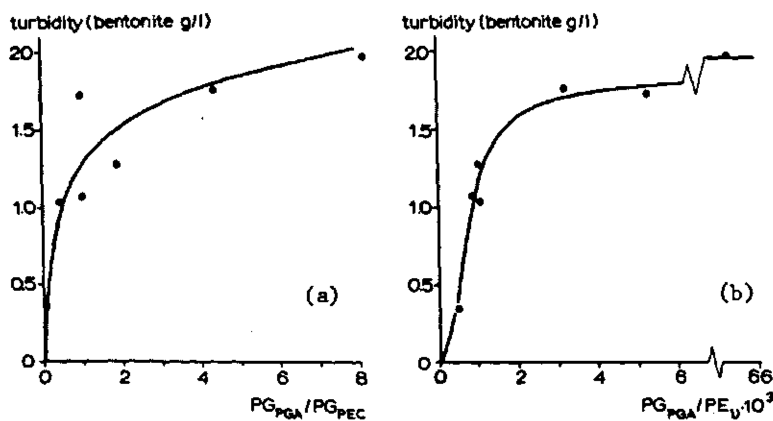


Fig. 51. (a) As Fig. 50a, on a non-logarithmic scale. (b) As Fig. 50b, on a non-logarithmic scale.

juice. The experiment with proteases (Section 6.6) disproved the supposition of Lankveld (1973) that clarification is mainly due to electrostatic interaction between low methoxy pectin and proteins. The results of the present experimental study stress the importance of the role of the trio PE-pectin-calcium ions in cloud loss of orange juice. Though this study shows that cloud may be stabilized by pectic enzymes, a comment of page 49 must be repeated, viz. statements about cloud stability are closely related to the measuring method applied. From Fig. 10 and Table 3 it is shown that cloud stabilization by PG and PL (curves F and H) is less sensational when determined by a method that approximates visual judgment than when the spectrophotometric turbidity measurements after centrifugation are applied. The reverse may also occur. In Fig. 28b an orange juice sample with pectic acid added shows a sediment volume of 100% due to internal structure of the precipitation of calcium pectate. Visually this sample appears to be 'cloud stable'. However, upon treatments like pasteurization, this structure collapses and the flocculated character of this juice becomes evident. Likewise, I suspect extremely cloud stable orange juices, as described by Reinert (1973), to be flocculated systems with a high internal structure masking the flocculated character.

As is stated on page 49 one has to choose a method for determining 'cloud stability' out of the available ones to progress in the investigation of the mechanism of cloud loss. It is hoped that this study is a contribution to the elucidation of this difficult and complex phenomenon.

Summary

The purpose of this study was to gain a better insight into the factors which cause cloud loss in turbid fruit juices. Orange juice was chosen as the material of investigation.

Chapters 2 and 3 are literature surveys. Chapter 2 reviews the older literature on cloud loss problems of orange juice, cloud composition and recent developments of the research in this field. Also the clarification mechanism of apple juice and grape juice have been described.

Chapter 3 pays attention to the structure and properties of pectic substances. As pectin esterase is a native enzyme of the orange fruit and pectin depolymerizing enzymes were applied in the experiments, the second part of this chapter reviews pectic enzymes.

Methods to demonstrate and determine PE activity have been described in Section 3.3. Since it was often more important to be informed about the actual action of PE than about the number of PE enzyme units present, a distinction was made between the terms 'action' and 'activity'. The action of PE can be determined by assaying quantitatively the methanol that is formed as a result of this action. Some methods to determine methanol therefore have been described.

Chapter 4 concerns the measurement of cloud stability. Initially the sedimentation of particles in a fluid was reviewed. Literature date on the measurement of settled pulp have been discussed as a possible method for determining cloud stability. It was concluded that this method is not suitable for this purpose. Next theoretical backgrounds of turbidity measurements were considered. This led to the conclusion that turbidity should be expressed as extinction rather than as transmission.

Chapter 5 gives the materials used and the methods applied in this study. In the conclusion of Section 5.2, various methods for the measurement of turbidity were compared in connection with some experiments. For the experimental part of this study the centrifugal extinction measurement method was chosen. In Section 5.3 the composition of the orange juice concentrate applied was discussed, as well as the gas chromatographic methanol determinat-

ion. Although this method is very sensitive and convenient, the aqueous samples frequently give rise to problems.

The proper experimental part has been described in the Chapters 5 and 6. In Section 6.1 various experimental factors which might affect cloud stability have been investigated. Some of the factors investigated had no influence. However, it was found that orange juice activated by PE after pasteurization clarified more rapidly than an identical juice without the heat treatment. A combination of heat treatment, centrifugation and resuspension restored a cloudy appearance to orange juice that had already been clarified. This finding indicates that resuspension experiments are not suitable for studying cloud stability.

In Section 6.2 it is shown that orange juice turbidity develops highly only some time after the extraction of the juice. Correlation between PE action and clarification was significant. If the content of PE in orange juice decreases, it takes longer before clarification occurs. However, then the juice pectin does not need to be saponified to such a great extent as when PE content is higher. Calcium ions were shown to be indispensable for the clarification reaction. Orange juice could be clarified artificially by the addition of pectic acid. Both the pectin adsorbed to the particles and the soluble serum pectin can be attacked by PE. A suspension of cloud particles in a medium containing Ca-ions does clarify upon PE addition. Electron micrographs showed that in a clarified juice all kinds of cloud particles have been encapsulated in clusters, the 'wrapping material' probably being calcium pectate. Hesperidin crystals are very prominent under these particles.

Section 6.3 describes some properties of a yeast polygalacturonase. Clarification of orange juice by PE could be prevented by the addition of this PG. The stabilization requires a certain minimum ratio PG/PE. Similarly cloud could be stabilized by the addition of a purified pectin lyase. If the juice pectin was degraded by PL and this activity was inactivated before the addition of PE, this preincubation appeared to be sufficient to prevent clarification. PG is effective only if it is actively present during the action of PE. By the action of PE the juice pectin is changed in a positive way as a substrate for PG, but in a negative way as a substrate for PL. This explains that PG is more effective than PL in preventing cloud loss of PE active juices. The effect of concentrated sugar solutions and of concentrated serum upon the activity of PG and PL was described in Section 6.4. A model experiment with PG and PL in pectin solutions containing Ca showed

a striking similarity between clarification of orange juice and gelation of pectin solutions at the one side and between preventing of clarification and preventing of gelation at the other side (Section 6.5). A possible effect of protease on the cloud stability has been investigated, but no enhancing effect was found (Section 6.6).

In conclusion Chapter 7 describes a mechanism of cloud loss of orange juice on the basis of the data obtained in this study. The term 'cloud stable' was considered critically.

References

Albersheim, P., H. Neukom & H. Deuel, 1960. Ueber die Bildung von ungesättigten Abbauprodukten durch ein pektinabbauendes Enzym. Helv. chim. Acta 43: 1422-1426.

Attaway, J.A. & R.D. Carter, 1971. Some new analytical indicators of processed orange juice quality. Proc. Fla St. hort. Soc. 84: 200-205.

Ayers, W.A., G.C. Papavizas & R.D. Lumsden, 1969. Purification and properties of the endopolygalacturonase of Aphanomyces euteiches. Phytopath. 59: 925-930

Baker, G.L., 1948. High-polymer pectins and their deesterification. Adv. Fd Res. 1: 395-427.

Baker, R.A. & J.H. Bruemmer, 1969. Cloud stability in the absence of various orange juice soluble components. Proc. Fla St. hort. Soc. 82: 215-220.

Baker, R.A. & J.H. Bruemmer, 1971. Enzymic treatment of orange juice to increase cloud and yield, and decrease sinking pulp level. Proc. Fla St. hort. Soc. 84: 197-200.

Baker, R.A. & J.H. Bruemmer, 1972a. Pectinase stabilization of orange juice cloud. J. agric. Fd Chem. 20: 1169-1173.

Baker, R.A. & J.H. Bruemmer, 1972b. Influence of pectate-hesperidin floc on orange juice clarification. Proc. Fla St. hort. Soc. 85: 225-229.

Baker, R.A. & J.H. Bruemmer, 1973. Protease and pectinase additive to citrus juices. United States Patent 3 754 932.

Bartolome, L.G. & J.E. Hoff, 1972. Gas chromatographic methods for the assay of pectin methylesterase, free methanol, and methoxy groups in plant tissues. J. agric. Fd Chem. 20: 262-270.

Benk, E., 1966. Ueber den Gehalt natürlicher Orangensäfte an Mineralstoffen, insbesondere Natrium. Flüssiges Obst 33: 424-428.

Benk, E., 1968. Beitrag zur Kenntnis des natürlichen Phosphatgehalts von Orangensäften. Flüssiges Obst 35: 98-100.

Benk, E., 1970. Verfälschte Orangen-, Zitronen- und Grapefruitsäfte. Verbraucherdienst, Ausgabe 3: 32-36.

Bielig, H.J. & P.G. Klettner, 1971. Das Pulpe-Serum Ratio bei pulpösen Fruchtprodukten. In: Ber. wiss.-tech. Komm., Int. Fruchtsaft-Union, 11. Juris-Verlag, Zürich, p. 39-53.

Biggs, R.H. & J.E. Pollard, 1970. Effect of cellulase, lipase, pectinase, protease and ribonuclease on the cloud of citrus juice. Proc. Fla St. hort Soc. 83: 314-318.

Bitter, T. & H.M. Muir, 1962. A modified uronic acid carbazole reaction. Analyt. Biochem. 4: 330-334.

Boer, K.A. de, 1972. Gaschromatografische methanolbepaling in sinaasappelsap. M.Sc.-Thesis. Agricultural University, Department of Food Science, Wageningen.

Boos, R.N., 1948. Quantitative colorimetric microdetermination of methanol with chromotropic acid reagent. Analyt. Chem. 20: 964-965.

Chang, L.W.S., L.L. Morita & H.Y. Yamamoto, 1965. Papaya pectinesterase inhibition by sucrose. J. Fd Sci. 30: 218-222.

Cieplinsky, E.W. & S.F. Spencer, 1967. Trace analysis by gas chromatography using porous polymer columns and thermal conductivity detection. Pittsburgh Conf. analyt. chem. appl. Spectrosc.

Clark, E.P., 1932. Die Vieböck and Schwappach method for the determination of methoxyl and ethoxyl groups. J. Ass. off. agric. Chem. 15: 136-140.
Deuel, H., G. Huber & L. Anyas-Weisz, 1950. Ueber 'Salzbrücken' zwischen Makromolekeln von Polyelektrolyten, besonders bei Calciumpektinaten. Helv. chim. Acta 33: 563-567.
Dietz, J.H. & A.H. Rouse, 1953. A rapid method for estimating pectic substances in citrus juices. Fd Res. 18: 169-177.
Doesburg, J.J., 1965. Pectic substances in fresh and preserved fruits and vegetables. IBVT-commun. 25, Institute for research on storage and processing of horticultural produce. Wageningen.
Dupaigne, P., 1960. Détermination du pourcentage de pulpe dans les jus de fruits et de légumes. Fruits d'outre mer 15: 325-327.
Dupaigne, P., 1967. Une méthode objective d'évaluation de la teneur en pulpe en suspension dans les boissons de fruits. In: Ber. wiss.-tech. Komm., Int. Fruchtsaft-Union, 8. Juris-Verlag, Zürich, p. 233-246.
Dupaigne, P., 1970. Détermination du pourcentage de pulpe en suspension dans les jus d'ananas. Fruits d'outre mer 25: 563-564.
Endo, A., 1964. Studies on pectolytic enzymes of molds. Part VII. Turbidimetry of apple juice clarification and its application to determination of enzyme activity. Agric. biol. Chem. 28: 234-238.
Endo, A., 1965a. Studies on pectolytic enzymes of molds. Part XIII. Clarification of apple juice by the joint action of purified pectolytic enzymes. Agric. biol. Chem. 29: 129-136.
Endo, A., 1965b. Studies on pectolytic enzymes of molds. Part XIV. Properties of pectin in apple juice. Agric. biol. Chem. 29: 137-143.
Endo, A., 1965c. Studies on pectolytic enzymes of molds. Part XV. Effects of pH and some chemical agents on the clarification of apple juice. Agric. biol. Chem. 29: 222-228.
Endo, A., 1965d. Studies on pectolytic enzymes of molds. Part XVI. Mechanism of enzymatic clarification of apple juice. Agric. biol. Chem. 29: 229-233.
Faddegon, J.M., 1973. Gaschromatografische methanolbepaling en de cloud stabiliteit van sinaasappelsap. M.Sc.-Thesis. Agricultural University, Department of Food Science, Wageningen.
Florkin, M. & E.H. Stotz (eds), 1965. Enzyme nomenclature. Comprehensive Biochemistry, Vol. 13, 2nd edn, Elseviers Publishing Company, Amsterdam.
Gee, M., R.M. Reeve & R.M. McCready, 1959. Reaction of hydroxylamine with pectinic acids. Chemical studies and histochemical estimation of the degree of esterification of pectic substances in fruit. J. agric. Fd Chem. 7: 34-38.
Gessner, P.K., 1970. Method for the assay of ethanol and other aliphatic alcohols applicable to tissue homogenates and possessing a sensitivity of 1 μg/ml. Analyt. Biochem. 38: 499-505.
Gierschner, K. & G. Baumann, 1969. Neues Verfahren zur Herstellung von trubstabilen Fruchtsäften durch Einsatz von pektolytischen Enzymen. Obst- u. Gemüse-Verw.-Ind. 54: 217-218.
Hasegawa, S. & C.W. Nagel, 1967. Separation of a hydrolase from a pectic acid transeliminase in cell extracts of a Bacillus. Nature, Lond. 213: 207-208.
Hasegawa, S. & C.W. Nagel, 1968. Isolation of an oligogalacturonate hydrolase from a Bacillus specie. Archs Biochem. Biophys. 124: 513-520.
Hatanaka, C. & J. Ozawa, 1970. An oligogalacturonate transeliminase from Erwinia aroideae. Agric. biol. Chem. 34: 1618-1624.
Hendrix, C.M. & J.E. Jefferson, Quality control manual for citrus processing plants. Redd Laboratories Publ., Safety Harbor.

Hollis, O.L. & W.V. Hayes, 1966. Water analysis by gas chromatography using porous polymer columns. J. Gas Chromat. 4: 235-239.
Hultin, H.O. & A.S. Levine, 1963. On the occurrence of multiple molecular forms of pectinesterase. Archs Biochem. Biophys. 101: 396-402.
Hultin, H.O., B. Sun & J. Bulger, 1966. Pectin methyl esterases of the banana. Purification and properties. J. Fd Sci. 31: 320-327.
Ishii, S. & T. Yokotsuka, 1971. Pectin trans-eliminase with fruit juice clarifying activity. J. agric. Fd Chem. 19: 958-961.
Ishii, S. & T. Yokotsuka, 1972. Clarification of fruit juice by pectin trans-eliminase. J. agric. Fd Chem. 20: 787-791.
Ishii, S. & T. Yokotsuka, 1973. Susceptibility of fruit juice to enzymatic clarification by pectin lyase and its relation to pectin in fruit juice. J. agric. Fd Chem. 21: 269-272.
Jansen, E.F., R. Jang & J. Bonner, 1960. Orange pectinesterase binding and activity. Fd Res. 25: 64-72.
Joslyn, M.A., 1962. The chemistry of protopectin: A critical review of historical data and recent developments. Adv. Fd Res. 11: 1-107.
Joslyn, M.A. & H. Deuel, 1963. The extraction of pectins from apple marc preparations. J. Fd Sci. 28: 65-83.
Joslyn, M.A. & W. Pilnik, 1961. Enzymes and enzyme activity. In: The orange, its biochemistry and physiology. W.B. Sinclair (ed.), University of California Printing Department, p. 373-435.
Keegstra, K., K.W. Talmadge, W.D. Bauer & P. Albersheim, 1973. The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cell based on the interconnections of the macromolecular components. Pl. Physiol. 51: 188-196.
Kertesz, Z.I., 1950. A simple turbidity tester. Canner 110: 15-16.
Kertesz, Z.I., 1951. The pectic substances. Interscience Publishers, New York.
Kew, T.J. & M.K. Veldhuis, 1961. Cloud stabilization in citrus juice. United States Patent 2 995 448.
Koch, J. & D. Hess, 1971. Zum Nachweis von verfälschten Orangensäften. Dt. LebensmittRdsch. 67: 185-195.
Koen Mosse, J. & J. Royo Iranzo, 1960. La gelificación en los zumos concentrados de naranja. Influencia de las distintas clases de pectina en concentrados de naranjas sanguinas, vernas y valencias. Revta Ciencia apl. 72: 30-33.
Kohn, R., I. Furda & Z. Kopec, 1968. Distribution of free carboxyl groups in the pectin molecule after treatment with pectin esterase. Colln Czech. chem. Commun. Engl. Edn 33: 264-269.
Koller, A., 1966. Fraktionierung und Charakterisierung der pektolytischen Enzyme von Aspergillus niger. Diss. Nr. 3774. ETH, Zürich.
Koller, A. & H. Neukom, 1969. Untersuchungen über den Abbaumechanismus einer gereinigten Polygalakturonase aus Aspergillus niger. Eur. J. Biochem. 7: 485-489.
Kratzl, K. & K. Gruber, 1958. Zur quantitativen Trennung und Bestimmung verschiedener Alkoxylgruppen mittels Gas-Flüssig-Chromatographie. Mh. Chem. 89: 618-624.
Krop, J.J.P. & W. Pilnik, 1974a. Effect of pectic acid and bivalent cations on cloud loss of citrus juice. Lebensm.-Wiss.u. Technol. 7: 62-63.
Krop, J.J.P. & W. Pilnik, 1974b. Cloud loss studies in citrus juices: Cloud stabilization by a yeast-polygalacturonase. Lebensm.-Wiss. u. Technol. 7: 121-124.
Krop, J.J.P. & W. Pilnik, 1974c. Effect of commercial protease preparations on cloud loss of citrus juice. Lebensm.-Wiss. u. Technol. 7: 125-126.
Krop, J.J.P., W. Pilnik & J.M. Faddegon, 1974. The assay of pectinesterase by a direct gaschromatographic methanol determination -Application to cloud loss studies in citrus juices. Lebensm.-Wiss u. Technol. 7: 50-52.

Lankveld, J.M.G., 1970. Adsorption of polyvinylalcohol on paraffin-water interfaces and the properties of paraffin-in-water emulsions stabilized by polyvinylalcohol. Ph. D. Thesis, also: Meded. Landb. Hogesch. Wageningen, 70-21.

Lankveld, J.M.G., 1973. Fundamental aspects concerning the cloud stability of soft drinks. Lecture International Symposium 'Emulsions and Foams in Food Technology', Ebeltoft, Denmark.

Larsen, S., 1969. Examination of a clouding material prepared from citrus peel. In: Ber. wiss.-tech. Komm., Int. Fruchtsaft-Union, 9. Juris-Verlag, Zürich, p. 109-135.

Lee, M.J., 1969a. Purification, properties, and modes of action of pectinesterase from tomato and Clostridium multifermentans. Ph.D. Thesis. Rutgers University, New Brunswick.

Lee, Y.S., 1969b. Measurement, characterization and evaluation of pectin esterase in apple fruits. Ph.D. Thesis. University of Maryland.

Lee, M. & J.D. Macmillan, 1968. Mode of action of pectic enzymes. I. Purification and certain properties of tomato pectinesterase. Biochemistry 7: 4005-4010.

Lee, M. & J.D. Macmillan, 1970. Mode of action of pectic enzymes. III. Site of initial action of tomato pectinesterase on highly esterified pectin. Biochemistry 9: 1930-1934.

Lee, M., L. Miller & J.D. Macmillan, 1970. Similarities in the action patterns of exopolygalacturonate lyase and pectinesterase from Clostridium multifermentans. J. Bact. 103: 595-600.

Lee, Y.S. & R.C. Wiley, 1970. Measurement and partial characterization of pectinesterase in apple fruits. J. Am. Soc. hort. Sci. 95: 465-468.

Leuprecht, H. & A. Schaller, 1968. Ergebnisse methodologischer Untersuchungen hinsichtlich der Messung der Pektinmethylesterase-Aktivität in aufgetautem Tiefgefrier-Erdbeerpüree. Fruchtsaft-Ind. 13: 50-74.

Lineweaver, H. & G.A. Ballou, 1945. The effect of cations on the activity of alfalfa pectinesterase (pectase). Archs Biochem. 6: 373-387.

Litchmann, M.A. & R.P. Upton, 1972. Gas chromatographic determination of residual methanol in food additives. Analyt. Chem. 44: 1495-1497.

Lockwood, B., 1972. Use of pectin in food processing. Fd process. Ind., Nov.: 47-51.

Loeffler, H.J., 1941. Maintenance of cloud in citrus juices. Proc. Inst. Fd Technol. 29-36.

Lucero, D.P., 1972. Water vapor sensitivity response of hydrogen flame ionization detectors. J. chromat. Sci. 10: 463-467.

Luh, B.S. & H.J. Phaff, 1954. Properties of yeast polygalacturonase. Archs Biochem. Biophys. 48: 23-37.

McColloch, R.J., 1952. Determination of pectic substances and pectic enzymes in citrus and tomato products. USDA-AIC 337.

McComb, E.A. & R.M. McCready, 1957. Determination of acetyl in pectin and in acetylated carbohydrate polymers. Analyt. Chem. 29: 819-821.

McComb, E.A. & R.M. McCready, 1958. Use of the hydroxamic acid reaction for determining pectinesterase activity. Stain Technol. 33: 129-131.

McCready, R.M. & E.A. McComb, 1952. Extraction and determination of total pectic materials in fruits. Analyt. Chem. 24: 1986-1988.

McCready, R.M. & C.G. Seegmiller, 1954. Action of pectic enzymes on oligogalacturonic acids and some of their derivatives. Archs Biochem. Biophys. 50: 440-450.

MacDonnell, L.R., R. Jang, E.F. Jansen & H. Lineweaver, 1950. The specificity of pectinesterases from several sources with some notes on purification of orange pectinesterase. Archivs Biochem. 28: 260-273.

MacDonnell, L.R., E.F. Jansen & H. Lineweaver, 1945. The properties of orange pectinesterase. Archivs Biochem. 6: 389-401.
MacDowell, L.G., 1962. Chemische und physikalische Veränderungen bei der Aufbereitung und Lagerung tiefgekühlter konzentrierter Citrussäfte. In: J. Koch (ed.), Die Fruchtsäfte in chemisch-technologischer und medizinischer Sicht. Ber. Inst. Getränkeforsch. Mainz, Band 1. Gutenberg-Druck, Hofheim/Taunus, 49-64.
Manabe, M., 1973. Saponification of ester derivatives of pectic acid by Natsudaidai pectinesterase (Studies on the derivatives of pectic substances Part V). Nippon Nogei Kagaku Kaishi 47: 385-390.
Mannheim, C.H. & S. Siv, 1969. Occurrence of polygalacturonase in citrus fruit. Fruchtsaft-Ind. 14: 98-103.
Mathers, A.P., 1958. Report on determination of methanol in distilled spirits. J. Ass. off. agric. Chem. 41: 121-122.
Mathers, A.P. & M.J. Pro, 1955. Spectrophotometric determination of methoxyl. Analyt. Chem. 27: 1662-1664.
Miller, L. & J.D. Macmillan, 1971. Purification and pattern of action of pectinesterase from Fusarium oxysporum f. sp. vasinfectum. Biochemistry 10: 570-576.
Mizrahi, S. & Z. Berk, 1970. Physico-chemical characteristics of orange juice cloud. J. Sci. Fd Agric. 21: 250-253.
Monselise, S.P., 1973. Citrusfrüchte als Rohware für die Herstellung von Säften und anderen Erzeugnissen. Verlag Günter Hempel, Braunschweig.
Moran, F., S. Nasuno & M.P. Starr, 1968. Oligogalacturonide trans-eliminase of Erwinia carotovora. Archs Biochem. Biophys. 125: 734-741.
Nederlandse Norm, 1970. Ontwerp 83 428, Nederlands Normalisatie-Instituut, Rijswijk (ZH), the Netherlands, p. 17.
Neukom, H., 1963. Ueber den Abbau von Pektinstoffen. Schweiz. landw. Forsch. 2: 112-122.
Neukom, H., 1967. Pectic substances. Kirk-Othmer Encycl. Chem. Technol. 14: 636-651.
Norman, S., 1970. Comparison of two porous polymer columns for gas chromatographic analysis of acetaldehyde, methanol, ethanol, and other volatiles emanating from intact 'Valencia' oranges. J. Am. Soc. hort. Sci. 95: 777-780.
Peleg, M. & C.H. Mannheim, 1970a. Production of frozen orange-juice concentrate from centrifugally separated serum and pulp. J. Fd Sci. 35: 649-651.
Peleg, M. & C.H. Mannheim, 1970b. Quality of frozen orange juice concentrate made by combining concentrated serum and centrifugally separated pulp. Confructa 15: 360-365.
Phaff, H.J., 1966. α-1,4-Polygalacturonide glycanohydrolase (endo-polygalacturonase) from Saccharomyces fragilis. Meth. Enzym. 8: 636-641.
Pilnik, W., 1958. Die Trubstabilität von Citrus-Konzentraten. In: Ber. wiss.-tech. Komm., Int. Fruchtsaft-Union, 1. Juris-Verlag, Zürich, p. 203-228.
Pilnik, W., 1969. Die Technologie von Riech- und Schmeckstoffen (RS). Dt. LebensmittRdsch. 65: 37-41, 67-74.
Pilnik, W., F.M. Rombouts & A.G.J. Voragen, 1973. On the classification of pectin depolymerases: Activity of pectin depolymerases on glycol esters of pectate as a new classification criterion. Chem. Mikrobiol. Technol. Lebensm. 2: 122-128.
Pilnik, W. & G. Rothschild, 1960. Trubstabilität und Pektinesterase-Restaktivität in pasteurisiertem Orangenkonzentrat. Fruchtsaft-Ind. 5: 131-138.
Pilnik, W. & A.G.J. Voragen, 1970. Pectic substances and other uronides. In: A.C. Hulme (ed.), The biochemistry of fruits and their products. Vol. I. Academic Press, London, 53-87.

Pilnik, W., A.G.J. Voragen & F.M. Rombouts, 1974. Pectic enzymes and their substrates. Lect. IV Int. Congr. Fd Sci. Technol. Madrid.
Pilnik, W. & P. Zwiker, 1970. Pektine. Gordian 70: 202-204, 252-257, 302-305, 343-346.
Pollard, A. & M.E. Kieser, 1951. The pectase activity of apples. J. Sci. Fd Agric. 2: 30-36.
Pressey, R. & J.K. Avants, 1972. Multiple forms of pectinesterase in tomatoes. Phytochemistry 11: 3139-3142.
Primo Yũfera, E., J. Koen Mosse & J. Royo Iranzo, 1961a. Gelification in concentrated orange juices. IV. Enzymatic action in pulp suspensions and its influence on the pectin content and clarification. In: Ber. wiss.-tech. Komm., Int. Fruchtsaft-Union, 3. Juris-Verlag, Zürich, p. 17-31.
Primo Yũfera, E., J. Koen Mosse & J. Royo Iranzo, 1961b. La gelificación en los zumos concentrados de naranja. Modificación de las pectinas en los procesos industriales y en el almacenamiento. Revta Ciencia apl. 79: 126-130.
Primo Yũfera, E., J. Koen Mosse & J. Royo Iranzo, 1963. La gelificación en los zumos concentrados de naranja. VI. Relaciones entre la gelificación, pérdida de turbiedad y evolución de las pectinas en la pulpa. Revta Agroq. Tecn. Alimentos 3. (6p.).
Primo Yũfera, E. & J. Royo Iranzo, 1965. Detection of adulterations in citrus juices-V. Mineral composition of single strength orange juices manufactured in Spain. In: Ber. wiss-tech. Komm., Int. Fruchtsaft-Union, 6. Juris Verlag, Zürich, p. 383-401.
Pritchett, D.E. & H.E. Merchant, 1946. The purification of hesperidin with formamide. J. Am. chem. Soc. 68: 2108-2109.
Procedure for analysis of citrus juices, 1964. USA, Food Machinery Corporation (cited by Mizrahi & Berk, 1970).
Reinert, C., 1973. Beitrag zur Trubstabilisierung von Orangensäften unter besonderer Berücksichtigung des Einflusses von Pektinesterase und Calciumionen. Dissertation. Universität Hohenheim, Polyfoto, Stuttgart.
Resch, A. & A. Schara, 1970. Die anatomischen Elemente der Orangenfrucht in Orangensaftkonzentraten. Confructa 15: 166-178.
Rombouts, F.M., 1972. Occurrence and properties of bacterial pectate lyases. Agric. Res. Rep. 779. Pudoc, Wageningen.
Rombouts, F.M. & W. Pilnik, 1972. Research on pectin depolymerases in the sixties - A literature review- Chem. Rubb. Comp., Crit. Rev. Fd Technol. 3: 1-26.
Rouse, A.H. & C. Atkins, 1955. Pectinesterase and pectin in commercial citrus juices as determined by methods used at the Citrus Experiment Station. Techn. Bull. agric. Exp. Stn Univ. Fla 570.
Royo Iranzo, J., 1971. Detección de adulteraciones en zumos cítricos. -XVII. Contenido en sulfatos, cloruros y nitratos del suero de los zumos de naranja espanoles. Revta Agroq. Tecn. Alimentos 11: 562-567.
Royo Iranzo, J., 1972. Chemische Kennwerte zur Aufdeckung von Verfälschungen bei Orangensaft. Flüssiges Obst 39: 480-488.
Schaller, A. & E. Mihalovics, 1959. Ueber die Bestimmung des suspendierten Fruchtfleischanteiles durch Zentrifugation. Fruchtsaft-Ind. 4: 197-205.
Schultz, T.H., H. Lotzkar, H.S. Owens & W.D. Maclay, 1945. Influence of method of deësterification on the viscosity and acid behavior of pectinic acid solutions. J. phys. Chem., Ithaca 49: 554-563.
Scott, W.C., T.J. Kew & M.K. Veldhuis, 1965. Composition of orange juice cloud. J. Fd Sci. 30: 833-837.
Senn, V.J., M.D. Murray & R.T. O'Connor, 1955. A proposed standard for designation of 'cloud' in citrus juices. USDA, ARS 72-8.

Sinclair, W.B., 1961a. Principal juice constituents. In: W.B. Sinclair (ed.), The orange, its biochemistry and physiology. University of California Printing Department, 131-160.
Sinclair, W.B., 1961b. Pectic substances. In: W.B. Sinclair (ed.), The orange, its biochemistry and physiology. University of California Printing Department, 191-229.
Slavíčková, A., 1961. Die Reaktion vom Pektin mit Ammoniak in Gegenwart von Calziumsalzen. Die Verseifung, Amidierung und Degradation. Scient. Pap. Inst. Chem. Technol., Prague, Fd Technol. 5: 73-158.
Solms, J. & H. Deuel, 1955. Ueber den Mechanismus der enzymatischen Verseifung von Pektinstoffen. Helv. chim. Acta 38: 321-329.
Stegeman, H., 1970. De invloed van elektronenbestraling op de stabiliteit van alkalische fosfatase bij 2°C en de activiteitsbepaling van pektine methylesterase. M.Sc.-Thesis, Agricultural University, Department of Food Science, Wageningen.
Vas, K., M. Nedbalek, H. Scheffer & G. Kovács-Proszt, Methodological investigations on the determination of some pectic enzymes. Fruchtsaft-Ind. 12: 164-184.
Vertalier & Martin, 1958. Microdétermination sélective des groupes alcoxy par chromatographie gaz-liquide. Chim. analyt. 40: 80-86.
Voragen, A.G.J., 1972. Characterization of pectin lyases on pectins and methyl oligogalacturonates. Agric. Res. Rep. 780. Pudoc, Wageningen.
Voragen, A.G.J. & W. Pilnik, 1970a. Pectin research in the sixties. Dt. Lebensmitt. Rdsch. 66: 325-329.
Voragen, A.G.J. & W. Pilnik, 1970b. Pektindepolymerasen. I. Mitteilung. Einteilung der Pektinsäuredepolymerasen nach ihrer Wirkung auf oligomere Galakturonsäuren. Z. Lebensmittelunters. u. -Forsch. 142: 346-359.
Voragen, A.G.J., F.M. Rombouts & W. Pilnik, 1971. The influence of the degree of esterification on the activity of pectin- and pectate-lyases. Lebensm.-Wiss. u. Technol. 4: 126-128.
Walstra, P., 1964. Approximation formulae for the light scattering coefficient of dielectric spheres. Br. J. appl. Phys. 15: 1545-1552.
Weast, R.C. (ed.), 1971-1972. Handbook of chemistry and physics, 52nd edn. The Chemical Rubber Company Co., Cleveland.
Weiss, J. & H. Sämann, 1972. Ergebnisse von Untersuchungen über die Trubstabilität von Marillennektar. Mitt. Rebe Wein Obstbau Früchteverwertung 22: 177-184.
Wolford, R.W., M.H. Dougherty & D.R. Petrus, 1969. Citrus juice essences. In: Ber. wiss.-tech. Komm., Int. Fruchtsaft-Union, 9. Juris-Verlag, Zürich, p. 151-169.
Wood, P.J. & I.R. Siddiqui, 1971. Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. Analyt. Biochem. 39: 418-428.
Yamasaki, M., A. Kato, S.-Y. Chu & K. Arima, 1967. Pectic enzymes in the clarification of apple juice. Part II. The mechanism of clarification. Agr. biol. Chem. 31: 552-560.
Yamasaki, M., T. Yasui & K. Arima, 1964. Pectic enzymes in the clarification of apple juice. Part I. Study on the clarification reaction in a simplified model. Agr. biol. Chem. 28: 779-787.