Starch Identification and Determination in Sweetened Fruit Preparations

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Starch is used as a thickening and gelling agent in sweetened food products such as sweetened fruit preparations (SFP). Due to a lack of efficient methods in the literature for identification and measurement of starch in such samples, this study was deemed necessary. Dialysis was used as a purification technique that allowed the preservation of starch while simultaneously eliminating saccharose. The study of experimental parameters such as dialysis membrane cutoff, treatment time, temperature, and water renewal leads to a yield of saccharose elimination of 99.4% ($\sigma\%$ = 0.1%) for strawberry SFP. Enzymatic hydrolysis of starch has been done by α-amylase and amyloglucosidase. A research strategy relying on the use of Rechtschaffner's matrix was achieved for hydrolysis conditions study. Previous gelatinization−alkalinization treatment, starch modification, hydrolysis time, and pH have great effect on the efficacy of hydrolysis. The determination of glucose released by hydrolysis has been done with hexokinase and glucose-6-phosphate dehydrogenase. The use of this method on strawberry SFP containing 3.0% (w/w) acetylated distarch adipate leads to the determination of 92% (relative standard deviation $\sigma\%$ = 3.1%) starch. This method can be considered to be efficient, specific, and reproducible for this starch in these samples.

Keywords: *Sweetened fruit preparations; starch identification and determination*

INTRODUCTION

The use of polysaccharides as functional additives in food products, coupled with regulatory controls, has created a need for effective methods of separation, identification, and quantitation. The determination of starch content has always difficult for the analyst, requiring indirect techniques. However, many different methods (Bernetti et al., 1990) have been developed for the identification and quantitation of starch: optical and electronic microscopy, enzymology, rheology, chromatography, NMR, and FT-IR. Most of these techniques have been used on products containing high levels of starch (cereals, flours, breads, etc.) or directly on pure starches.

The increasing use of sweetened fruit preparations in dairy food (Millet, 1994) requires a better knowledge of their composition to control technological, commercial, and legal aspects.

The case of fruit preparation with sugar is a particularly intricate problem, indeed; these preparations contain high concentrations of saccharose and sometimes other kinds of polysaccharides that interfere with starch determination.

The presence of fruit pieces and the colored medium are additional obstacles for a quantitative assay. Thus, a number of classical methods become unserviceable.

The aim of this work becomes a real challenge: to develop reliable and accurate methods with good reproducibility for the identification and quantitative determination of starch in fruit preparation.

MATERIALS AND METHODS

Samples. Four types of modified starches and two types of pectins have been used: acetylated distarch adipate, Colflo 067, National Starch; distarch phosphate, Cleargel A, National Starch; acetylated distarch phosphate, C Tex 06306, Cerestar; hydroxypropylated distarch phosphate, VA 70, Farinex; pectins, Genu 101 and 104 AS, Copenhagen Pectin.

Fructose (ref 47740), saccharose (ref 84105), and glucose (ref 49140) were obtained from Fluka (Buchs, Switzerland).

Solutions of saccharose (46% w/w), glucose (0.5 g/L), fructose (0.5 g/L), and pectins (0.4% w/w) were prepared by heat solubilization (40 °C) with strong agitation. Test solution (S1) was prepared with saccharose (60.00% w/w), water (39.25% w/w), acetylated distarch adipate (0.50% w/w), and pectins (0.25% w/w). Starch solutions (5.0 g/L) and S1 were gelatinized during 30 min at 70 °C with strong agitation.

The sweetened fruit preparations (SFP) were obtained from International Research Center Daniel Carasso, groupe DANONE (Le Plessis Robinson, France). SFP were not pasteurized, and potassium sorbate was used as preservative. Their compositions are shown in Table 1.

Table 1. Composition of Sweetened Fruit Preparations (SFP) Used in the Study

	SFP1 (% w/w)	SFP2 (% w/w)	SFP3 (% w/w)	SFP4 (% w/w)	SFP5 (% w/w)
strawberry	50.0	50.0	50.0	50.0	50.0
saccharose	46.0	46.0	46.0	46.0	46.0
pectins[a]	0.3	0.3	0.3	0.3	0.3
starch[b]	0.0	0.5	1.0	2.0	3.0
water	3.6	3.1	2.6	1.6	0.6
potassium sorbate	0.1	0.1	0.1	0.1	0.1

[a] Genu LM 101 and 104 AS. [b] Colflo 067, acetylated distarch adipate.

Moisture Content Measurement. Moisture contents of starch samples were determined according to the French standard method NF V-03 707 (AFNOR, 1982).

Sample Purification by Dialysis. Samples were placed in dialysis membranes made of cellulose acetate (ref SPECTRA POR 6, cutoff 2000 Da, Spectrum, Houston, TX). SFP were diluted (25.0 g in 50 mL of water).

Membranes were maintained during 48 h at 45 °C in a water bath with a continuous renewal of water at a 128 mL/min rate.

The yield of elimination of saccharose ($T\%$) allowed estimation of the efficiency of dialysis. $T\%$ is calculated from

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0021-8561/96/1444-0502$12.00/0

Table 2. Experimental Field

factors	lower limit (−)	upper limit (+)
U1, α-amylase	Sigma A3403	Sigma A0273
U2, amyloglucosidase	Boehringer B208469	Boehringer B1202332
U3, pH	citrate buffer (pH 4.6)	phosphate buffer (pH 6.9)
U4, temperature (°C)	25	55
U5, hydrolysis time (h)	4	24
U6, gelatinization	without alcalinization (water)	with alcalinization (NaOH)
U7, modified starch	DP[a]	ADP[b]

[a] DP, Distarch phosphate. [b] ADP, acetylated distarch phosphate.

saccharose concentration before and after dialysis according to

$$T\% = \frac{[\text{saccharose}]_{\text{initial}} - [\text{saccharose}]_{\text{final}}}{[\text{saccharose}]_{\text{initial}}} \times 100 \quad (1)$$

Starch Identification. Starch was precipitated by ethanol from aqueous dialyzed samples (final ethanol concentration >80% w/w). The samples were then centrifuged during 10 min at 600*g*.

Starch staining was obtained by dispersion of a few milligrams of centrifugal pellet in a droplet of water and a droplet of lugol (1 g of I_2 in 100 mL of KI at 2% w/v in water). Microscopic observation was done at magnification ×40.

Hydrolysis Procedure. The two α-amylases (ref A3403 and A 0273) and the β-fructosidase (ref I 4504) used were obtained from Sigma (St. Louis, MO). The two amyloglucosidases (ref B1202332 and B208469) were Boehringer products (Mannheim, Germany).

The pH values of phosphate and citrate buffers were, respectively, 4.6 and 6.9. Gelatinization and alkalinization were done by adding 2 mL of NaOH (5 M) to 100 mL of dialyzed sample. This solution was kept at 70 °C for 30 min under strong agitation.

After cooling, the samples were neutralized with HCl (5 M). The starch was hydrolyzed in screwed tubes in an oven during 24 h at 55 °C.

Tube 1 for the starch and saccharose hydrolysis contained 0.2 mL (360 UI) of α-amylase, 1.0 mL (24 UI) of amyloglucosidase, 1.0 mL (400 UI) of β-fructosidase, 2.5 mL of phosphate or citrate buffer, and 5.0 mL of sample.

Tube 2 for the saccharose hydrolysis contains 1.0 mL (400 UI) of β-fructosidase, 3.7 mL of phosphate or citrate buffer, and 5.0 mL of sample.

Experimental Design. An experimental strategy (Box et al., 1978; Fargin et al., 1985) relying on the use of factorial design was achieved to improve hydrolysis conditions of starch in sweetened fruit preparations. This made data handling easier.

All factors able to modify the hydrolysis efficiency were identified and estimated from literature data and personal results.

In Table 2, the experimental field lists the main factors (**U*i***) factors) and describes the upper and lower limits of the studied intervals.

Theoretically, 128 experiments are required to evaluate 7 factors. Using Rechtschaffner's experimental design (Rechtschaffner, 1967) only 29 experiments are required.

The influence of each factor (**U*i***) on hydrolysis efficiency was estimated by working out its main effect (b_i) by multilinear regression of data obtained from experiments and Rechtschaffner's matrix (Rechtschaffner, 1967).

In the same way, antagonistic and synergistic effects between factors have been determined by working out the first-order interaction effects (b_{ij}). Relevant interaction effects were studied thanks to interaction diagrams (Charts 1 and 2) showing the *R*% average of each possible combination of two factors.

The interactions between more than two factors were considered to be negligible.

The experimental parameters of the necessary 29 experiments were established from experimental limits and from the structure of Rechtschaffner's experimental design (Rechtschaffner, 1967).

Chart 1. Study of Interaction b_{67}

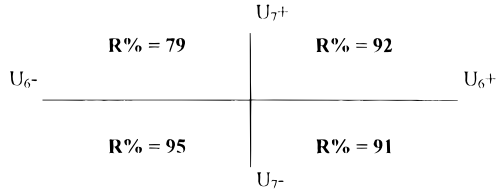
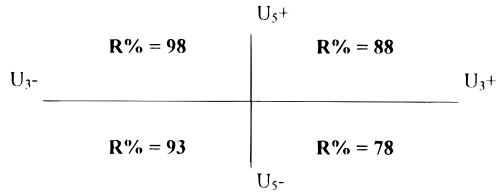


Chart 2. Study of Interaction b_{35}



The experiments have been done in randomized order to avoid block effects. The starch concentrations consider the moisture content (*M*%) of the samples.

The results were expressed in percentage of recovery of starch (*R*%) and were calculated according to

$$R\% = \frac{[\text{starch}]_{\text{measured}}}{[\text{starch}]_{\text{effective}}} \times 100 \quad (2)$$

Determination Protocol. The enzymatic assay of glucose by the glucose-6-phosphate dehydrogenase/hexokinase system was determined with a spectrophotometer (Shimadzu UV 120) at 340 nm and the glucose kit Boehringer (ref 716251).

RESULTS AND DISCUSSION

Dialysis Step and Identification. The saccharose, largely added, carbohydrates, and oligosaccharides from fruits and other possible thickening and gelling agents (TGA) are the main compounds that may significantly interfere with starch determination in SFP.

A previous purification step is required to eliminate large quantities of saccharose while preserving starch. The literature (Glicksman, 1969; BeMiller et al., 1973; Colonna and Thibault, 1986) recommends the extraction of starch by hydroalcoholic precipitation. This technique is not suitable for quantitative determination because coprecipitation of other TGA occurs, in particular pectins.

The dialysis, cited by other authors (Bernetti et al., 1990; Southgate, 1991), has been chosen in this study for sample purification. Its efficiency has been calculated by measuring the yield of elimination of saccharose (*T*%) on a test solution (S1) containing saccharose, starch, and pectins. S1 does not contain fruit to avoid interference phenomena with solid particles. The saccharose concentration is higher than in fruit preparations to increase responses and to interpret more easily the results.

Twenty-two analyses using S1 solution allowed the determination of the main parameters of the dialysis, which are membrane cutoff, water renewal of dialysis bath, dialysis time, and temperature of the bath. A *T*% of 99.6% is obtained for a cutoff of 2000 Da (Spectra Por 6 MWCO 2000 D), 48 h, 45 °C, and a water renewal of 128 mL/min.

The lack of stray hydrolysis phenomena during dialysis has been proved with these experimental conditions applied to solutions of acetylated distarch adipate (0.5% w/w), acetylated distarch phosphate (0.5% w/w), distarch phosphate (0.5% w/w), hydroxypropylated distarch phosphate (0.5% w/w), saccharose (46% w/w), and pectins (0.4% w/w).

The mean yield of elimination was 99.4% ($\sigma\% = 0.1\%$) for 50 dialyses made on 4 SFP (SFP2−SFP5). This result is similar to that obtained with the test solution S1. It proves the efficiency of the dialysis method proposed despite the gellified and heterogeneous properties of SFP.

Before quantitative determination, starch has to be identified by a simple, rapid, and accurate method. The literature (Flint, 1990) recommends microscopic observation after iodine staining. In SFP, many interferences make difficult the starch identification by this technique.

The use of dialysis has permitted the elimination of colors from fruits (strawberry) and cellular fragments. After alcohol precipitation, easy observation by optical microscopy was possible. Experiments with several strawberry SFP (SFP1−SFP5) containing or not starch at various concentration (0.0−3.0% w/w) prove the efficiency of this technique of identification. The presence or the lack of starch has been checked in all cases.

The concentration of starch in SFP has no influence on the microscopic observation. The required quantities of starch, for microscopic observation, can be obtained by increasing amounts of SFP in treated samples. The other TGAs (polysaccharides) in the SFP do not interfere in microscopic identification because of the high specificity of iodine staining.

Choice of Enzymatic System for Glucose Determination. Because of residual saccharose and other TGAs (polysaccharides) in the sample after dialysis, the method used for starch determination must be very specific. The enzymatic method seems to be the most suitable. The literature (Beutler, 1978; Ettel, 1981; Karkalas, 1985, 1991; Henri et al., 1990; Southgate 1991) recommends the use of the enzymatic assay systems glucose-6-phosphate dehydrogenase/hexokinase and glucose oxidase/peroxidase.

Preliminary tests done on solutions of glucose, fructose, saccharose, pectins, modified starches, and test solution S1 show the lack of reproducibility and specificity of the glucose oxidase/peroxidase system and the stray determination of fructose of a glucose-6-phosphate dehydrogenase/hexokinase system commercial kit. These analyses prove; on the other hand, the reproducibility and the specificity of the glucose-6-phosphate dehydrogenase/hexokinase system of the Boehringer kit (ref 716251). The accuracy of the method of determination has been evaluated on a series of solutions of glucose at concentrations between 0.5 and 5.0 g/L. The results lead to undervaluation lower than 3% with relative standard deviation σ% between 1% and 2%.

Choice of Enzymatic System for Starch Hydrolysis. The good accuracy of the assay method permits the study of starch hydrolysis conditions. Starch hydrolysis must be specific because of the possible saccharose hydrolysis in samples.

The release of glucose from saccharose leads to a stray signal, preventing a good determination. Chemical modifications give higher stability of starches to hydrolysis. Acidic hydrolysis as often described in the literature (Nittler et al., 1980; Kartchner and Theurer, 1981; Faithfull, 1990; Rose et al., 1991) is not suitable in these samples because it induces hydrolysis of saccharose and other carbohydrates contained in SFP.

The enzymatic method is longer but more specific. The literature recommends the enzymes α-amylase and amyloglucosidase (Karkalas, 1985; Henri et al., 1990; Rose et al., 1991). A range of experiments with α-amylase (Sigma A3403) and amyloglucosidase (Boehringer B208469) demonstrated the lack of affinity of these enzymes for pectins and stray hydrolysis of saccharose. Similar results have been obtained with other enzymes (α-amylase, Sigma A0273; amyloglucosidase, Boehringer B1202332).

Blank determination on saccharose and starch in hydrolysis conditions without enzymes proved the enzymatic origin of saccharose hydrolysis.

β-Fructosidase permits enzymatic hydrolysis of saccharose in glucose and fructose. Its specificity has been verified on solutions of starches, pectins, and saccharose. Its use before starch hydrolysis allows the determination of the glucose from saccharose. The dialysis sufficiently reduces the saccharose content to obtain a significative difference between the two glucose origins.

Study of Hydrolysis Parameters. The hydrolysis parameters described in Table 2 were studied according to an experimental strategy relying on Rechtschaffner's experimental design (Rechtschaffner, 1967). The pH, enzyme sources, duration, and temperature are the studied parameters for the hydrolysis method. The gelatinization degree and the kind of starch modification are specific parameters of samples. The aim of this work is to define optimum hydrolysis and gelatinization methods and to evaluate the effect of acetylation on hydrolysis efficiency.

Twenty-nine experiments have been realized on solutions (5.0 g/L) of distarch phosphate and acetylated distarch phosphate. The yields (*R*%) of starch recovery were between 66% and 98%.

The main (b_i) and interaction (b_{ij}) effects of the studied factors calculated from the results (*R*%) by multilinear regression (Rechtschaffner, 1967) are represented in Figure 1.

Each main effect (b_i) visualized in Figure 1 provides two pieces of information. The absolute value of (b_i) reveals the relative influence of the factor (**U*i***), and the algebraic sign points out what limit (+ or −) will improve the hydrolysis efficiency. The interaction between two factors is revealed by the level of the absolute value of the interaction effect (b_{ij}).

Figure 1 shows the influence of the interaction effects b_{67} (gelatinization/modified starch) and b_{35} (pH/hydrolysis time) on the hydrolysis efficiency. These different parameters have to be studied with their interaction effects taken into account.

b_{67} (**U6**, alkalinization/**U7**, starch modification) is the most influential effect. The interaction diagram in Chart 1 allows a more detailed study of this interaction. It shows that the recovery yield (*R*%) is virtually the same for the distarch phosphate (**U7**−) with (**U6**+) or without (**U6**−) alkalinization. Concerning the acetylated distarch phosphate (**U7**+), the alkalinization

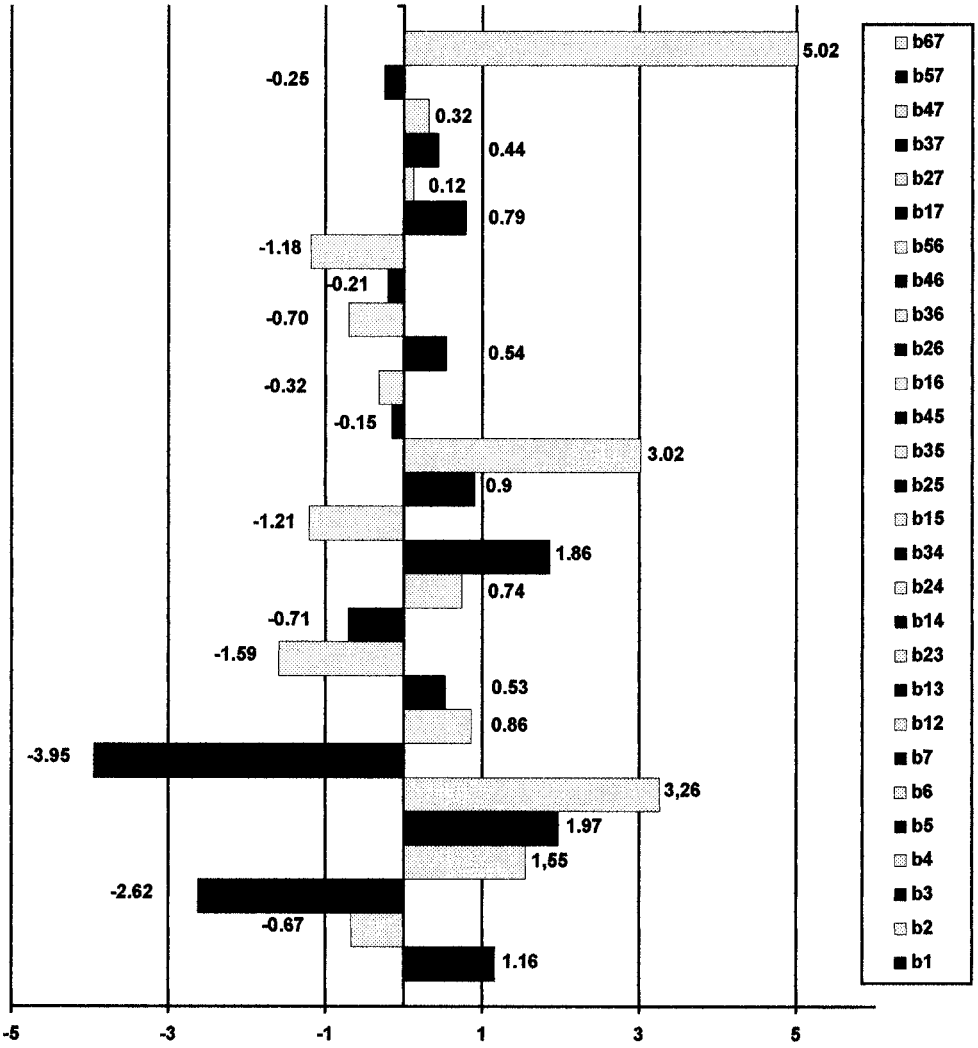


Figure 1. Graphic analysis of the effects (b_i and b_{ij}).

(**U6**+) increases *R*% from 79% to 92%. This means that the alkalinization decreases the resistance to enzymatic hydrolysis of acetylated distarch phosphate by removing acetyl groups from glucose units. This justifies the choice of a systematic alkalinization of the samples.

Figure 1 brings to light the effect of the interaction b_{35} (**U3**, pH/**U5**, hydrolysis time) on the hydrolysis efficiency. b_{35} is described by the interaction diagram shown in Chart 2.

Chart 2 shows clearly that the hydrolysis time (**U5**) has a weak influence on the starch recovery yield (*R*%) at pH 4.6 (**U3**−) and an important one at pH 6.9 (**U3**+).

It can be also observed that *R*% is greater in 4 h at pH 4.6 (*R*% = 93%) than in 24 h at pH 6.9 (*R*% = 88%), which means that the enzyme activities are strongly influenced by the pH. The experimental conditions recommended by the interaction diagram of Chart 2 are therefore **U3**− (pH 4.6) and **U5**+ (24 h).

With an effect value smaller than 2, the other interaction effects represented in Figure 1 appeared to be insignificant.

Figure 1 shows that temperature (factor **U4**) is independent of other parameters, so its influence on hydrolysis efficiency is described by only the main effect (b_4). This one shows that a temperature of 55 °C (**U4**+) favors the starch recovery yield. In the same way, it can be observed that **U1**+ (α-amylase, Sigma A0273) and **U2**− (amyloglucosidase, Boehringer B208469) weakly improve the hydrolysis efficiency.

Thus, the selected protocol is a first step of alkalinization−gelatinization of samples followed by a starch hydrolysis with α-amylase (Sigma A0273) and amyloglucosidase (Boehringer 208469) in a citrate buffer (pH 4.6) during 24 h at 55 °C.

The reproducibility of hydrolysis has been estimated by solutions of acetylated distarch adipate (Colflo 067), distarch phosphate (Cleargel A), and hydroxypropylated distarch phosphate (Farinex VA70). Six series of analyses result in recovery yields (*R*%) of 93% (σ% = 2.1%) for Colflo 067, 96% (σ% = 0.5%) for Cleargel A, and 49% (σ% = 2.7%) for Farinex VA70. The last result is weak but in agreement with the literature (Blake and Coveney, 1979). It constitutes an exception regarding the results on modified starches.

Efficiency of the Method. The study of the dialysis and the optimization of hydrolysis parameters allowed efficient determination of acetylated distarch adipate in S1.

The experimental conditions previously defined have been applied to four SFP (SFP2−SFP5) containing COLFLO 067 to verify the efficiency of the overall method. The results are given in Table 3.

According to the relative standard deviation (σ%, Table 3), the method is reproducible for the whole range of concentrations from 0.5% to 3.0% (w/w). We also see that standard deviations obtained with SFP (Table 3) are closely related to the one obtained with S1 (σ% = 2.1%).

Table 3. Determination of Acetylated Distarch Adipate in SFP

SFP	starch concentration in SFP (% w/w)	starch determination[a] (% w/w)	SD (σ%)
2	0.50	0.41	4.2
3	1.00	0.84	4.1
4	2.00	1.84	3.9
5	3.00	2.74	3.1

[a] Average value of 15 determinations.

The linear regression between experimental and real values on the 60 experiments of Table 3 demonstrates the good accuracy of the method ($\rho_{xy} = 0.99899$).

Conclusion. A method of identification and determination of acetylated distarch adipate in SFP has been developed. The use of dialysis as a method of purification led to a significant reduction of interference due to high concentrations of saccharose.

Optical microscopy allowed a rapid and accurate identification of starch in dialyzed samples.

The enzymatic hydrolysis of starch was the more difficult step of the assay. The lack of specificity of amylolytic enzymes led us to use β-fructosidase to determine the proportion of glucose coming from saccharose. A study, using experimental design, of all the parameters improved obviously the accuracy of this step.

The use of an efficient and specific enzymatic kit of glucose determination led to reliable and reproducible quantitation of starch in SFP.

ACKNOWLEDGMENT

We thank Madame M. Piaia of the International Research Center Daniel Carasso (Le Plessis Robinson, France) for the SFP preparation and Dr. M. Sergent of the Laboratoire de Prospective Réactionnelle et d'Analyse de l'Information (Université d'Aix-Marseille III, France) for stimulating discussion.

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Received for review March 1, 1995. Revised manuscript received October 2, 1995. Accepted October 13, 1995.[⊗]

JF950126T

[⊗] Abstract published in *Advance ACS Abstracts*, December 15, 1995.