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THE ROLE OF PHYTASE AND LIGNIN IN DECORTICATED DRY BEAN (*PHASEOLUS VULGARIS*) HARDENING DURING STORAGE

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ABSTRACT

*The lignification-like mechanism and that involving phytic acid degradation by phytase were evaluated in the hard-to-cook phenomenon of decorticated Malawian white and red bean genotypes (*Phaseolus vulgaris*). Samples were stored under various temperatures (16C; 35C) a_w; (0.55 a_w; 0.85 a_w) and time periods (4 and 8 months) compared to the control group (2C; 0.30 a_w; zero months). Phytase activities (Pi), phytic acid, calcium and magnesium ions, water soluble pectic substances and lignin concentrations were determined spectrophotometrically.*

Elevated phytase activities and slight, but nonsignificant increase in lignin levels were produced in both bean genotypes maintained under adverse storage conditions for extended time periods. Positive correlations between phytase activities and cooked white bean hardness for the 4 month ($r^2 = 0.844$) ($p \leqslant 0.01$) and 8 month ($r^2 = 0.689$) storage periods were found. Lignin content of red beans was significantly ($p \leqslant 0.01$) correlated ($r^2 = 0.669$) with hardness for the extended (8 month) storage period. However, no clear relationships were found between lignin levels and the 4 month stored red ($r^2 = 0.232$) and white ($r^2 = 0.210$) bean hardness, and between lignin concentrations and the 8 month ($r^2 = 0.232$) stored white bean hardness. The mechanism involving phytic acid degradation appeared to be the dominant system influencing the hard-to-cook defect in the white, and to some extent the red bean genotypes for the storage period 0–8 months.

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INTRODUCTION

Research has established that legume seeds stored at elevated humidities ($\geqslant$ 70% RH) or containing high moisture ($\geqslant$ 14%) and kept at high temperatures ($\geqslant$ 21C) fail to soften during cooking, resulting from the hard-to-cook defect (Kon and Sanshuck 1981; Moscoso *et al.* 1984). This defect, which prevents cotyledons from softening sufficiently during normal atmospheric cooking, even after imbibing water (Jones and Boulter 1983a; Vindiola *et al.* 1986), has contributed to increased cooking time and processing energy consumption and reduced protein digestibility of the stored seeds (Burr *et al.* 1968; Kon and Sanshuck 1981; Sievewright and Shipe 1986).

The hard-to-cook condition is expected and widely found in tropical genotypic environments of the world with elevated temperatures and humidities ($\geqslant$ 21C and $\geqslant$ 79% RH) (Burr *et al.* 1968; Malawi Meteology Dept. 1986). Two hard-to-cook defect mechanisms have been postulated. Supporting literature is reviewed.

A proposed dual-enzyme mechanism involving phytase and pectinmethylesterase (PME) includes the hydrolysis of phytic acid by phytase (myoinositol-hexakis-phosphate phosphohydrolase, 3.1.3) thus releasing inorganic phosphate (Pi), magnesium and calcium ions within cotyledon cells. Concomitantly, within the middle lamella, pectin methyesterase (PME) hydrolyzes pectin to pectinic acid and methanol. The calcium and magnesium ions are considered to traverse from the cotyledon to the middle lamella to produce calcium and magnesium pectates that subsequently restrict cell separation. The role of PME in the postulated dual enzyme mechanism remains to be determined (Mattson *et al.* 1951; Lolas and Markakis, 1975; Kon and Sanshuck 1981; Jones and Boulter 1983a,b; Hincks and Stanley 1986; Vindiola *et al.* 1986). A negative correlation between phytic acid/calcium ratios and length of dry bean cooking was established, and cooking time was reduced by steeping hard-to-cook beans in phytic acid and EDTA, although seed hardness was not reversed (Kon and Sunshuck 1981). Kon and Sunshuck (1981) deduced that another system, possibly a lignification-like mechanism, must exist besides that involving phytic acid degradation by phytase in the legume seed hard-to-cook phenomenon, because of the incomplete reversal of the hardness when seeds were soaked in solutions containing metal chelators. However, a study by Hentges *et al.* (1990) suggested that the hard-to-cook defect of beans could be reversed through low (6.5C) temperature and high (71% RH) humidity storage. Interpretation of the Hentges data is, however, limited in the absence of supportive cell wall microscopic examinations to demonstrate the complete reversibility in the hard-to-cook conditions of beans.

Several researchers have attempted to explain the involvement of a lignification-like process in the hard-to-cook phenomenon of beans. Stanley's group has hypothesized that small polypeptides and free aromatic amino acids were hydro-

lyzed, leading to polyphenol synthesis. The phenolic substances traverse from cotyledon to the middle lamella where the phenolic compounds are lignified by peroxidase (Hincks and Stanley 1986; Hohlberg and Stanley 1987).

Histological research has revealed hard-to-cook black beans to have reduced cell separations, high lignin content in cell wall corners, secondary cell walls and middle lamella, and increased lignified cotyledon proteins. In addition, there have been reductions in phaseolin protein, phytic acid and extractable phenol levels, raised small polypeptide and free aromatic amino acid concentrations and elevated phytase activities (Molina *et al.* 1976; Hincks and Stanley 1986, 1987; Hohlberg and Stanley 1987). The reduction in extractable phenols may be partially due to increased phenol polymerization (Hincks and Stanley 1986). Within the bean cotyledon during suboptimal storage conditions, protein hydrolysis products, in the presence of free aromatic amino acids and enzymatic activities, may represent polymerization and/or lignification reactions in plant tissues (Hohlberg and Stanley 1987). Srisuma *et al.* (1989) found no quantitative differences in the 9 month stored navy bean cotyledon and seed coat lignin levels and concluded that enhanced legume seed lignification was not a major determinant or the hard-to-cook defect.

The dual-enzyme activity mechanism involving phytase and PME seems to predominate during early storage periods (2–4 months). In contrast, the lignification-like mechanism appears to prevail during later storage periods (> 8 months). The two mechanisms have been advocated to influence the hard-to-cook condition of dry legume seeds during 4–8 month storage (Hincks and Stanley 1986, 1987); however, minimal quantitative data are available to critically evaluate this proposition.

The current study examined several indicators in the proposed hard-to-cook defect theories in order to elucidate the contributions of these indicators in the development of legume seed hardening: phytase activities and lignin, phytic acid, water soluble pectic substances, calcium and magnesium ion concentrations.

The approach followed in the phytase assay in the present study was to determine enzyme activity by measuring the amount of product, inorganic phosphate (Pi), in decorticated beans following seed exposure to specific temperatures, water activity (a_w) and storage time periods. We hypothesized that decorticated beans held under suboptimal environmental conditions (35C; 0.85 a_w) would have higher phytase activities and lignin concentrations compared to the controls stored at 2C and 0.3 a_w with increase in storage time (0–8 months). There would also be a decrease in cotyledon water soluble pectic substances with an accompanying increase in cell wall calcium and magnesium ion concentrations, resulting in increased bean hardness. It was also postulated that both the red and white dry bean genotypes would exhibit similarities in hardness alterations when maintained under the same storage conditions. Finally, it was hypothesized that the hard-to-cook

defect developed under suboptimal storage conditions would be caused predominately by the mechanism involving phytic acid degradation, as opposed to the lignification-like mechanism during the storage period (0–8 months).

MATERIALS AND METHODS

Environmental Conditions

Controlled environmental cubicles in theDepartment of Food Science and Human nutrition at Michigan State University were adjusted to 2C, 16C and 35C ± 2C. The 18.925 L high-density polyethylene (HDPE) containers with tight fitting lids (Cole-Palmer Instrument Co., Chicago, IL) were placed in the cubicles. Preliminary studies, over three months, demonstrated that a_w within the HDPE containers held in the 2C cubicles averaged 0.30 a_w, which was in equilibrium with 10% moisture beans. Saturated solutions of potassium chloride and magnesium nitrate (certified ACS) were prepared according to the Labuza (1984) method and used to maintain a_w at 0.85 and 0.55 ± 0.05, respectively. A digital hygrometer-thermometer (Fisher Scientific Co., model no. 11-661-71) was used to measure a_w and temperature within the containers via a resealable opening and using a rubber stopper. Measurements were taken following initial sample equilibration weekly for the first month, twice per month the second and the third months, and once per month thereafter.

Sample Preparation

Two Malawian bean landraces (genotypes), one a small red bean (Acc: 6-5) with a thick seed coat and the other a large white bean (Acc: 2-10) with a thin seed coat were grown at Bunda College, Malawi (Africa) and generously provided by the Malawi/Michigan State University Bean/Cowpea Collaborative Research project at Michigan State University, East Lansing, MI.

Beans were decorticated to eliminate variation due to seed coat differences. A single layer of beans (500 g) was placed in each of a series of steel wire net baskets constructed in the laboratory. The baskets were suspended approximately 10 cm over excess deionized water in desiccators (one basket per desiccator) for 3–4 days, and then sprinkled with deionized water (ambient temperature) to enhance loosening the seed coats. The beans were decorticated manually with the aid of a surgical blade, and air dried at room temperature for three days. A preliminary study had demonstrated that previously humidified/decorticated/dried beans were similar in moisture content (9.5–10%) to untreated decorticated beans. The treated seeds were held in the sealed HDPE containers at 2C until initiation of the storage study.

TABLE 1.

EXPERIMENTAL STORAGE TREATMENT ARRANGEMENT OF DECORTIFICATED BEANS

Landrace	Storage time (mos)	Temperature (°C)	Water activity (a_w)
White			
Control [1]	0	2	0.30
Treatments			
1	4	16	0.55
2	4	16	0.85
3	4	35	0.55
4	4	35	0.85
5	8	16	0.55
6	8	16	0.85
7	8	35	0.55
8	8	35	0.85
Red			
Control [1]	0	2	0.30
Treatments			
9	4	16	0.55
10	4	16	0.85
11	4	35	0.55
12	4	35	0.85
13	8	16	0.55
14	8	16	0.85
15	8	35	0.55
16	8	35	0.85

[1] Control was not part of the factorial arrangement. The control treatment was compared to means in the factorial arrangement using the least significant difference (LSD) test.

Experimental Design

The experimental design was a four factor factorial (2^4) model at two levels (Table 1) in a completely randomized design where the factors: storage time, temperature, a_w and bean genotypes were fixed. The control was not part of the factorial arrangement. The control was compared to means within the factorial arrangement using the least significant difference (LSD) test (Steel and Torrie 1980). The treatment combinations for each genotype were replicated twice.

Thirty-six, 400 g decorticated dry bean samples (18 red, 18 white) were weighed, put into low density polyethylene (LDPE) Zip-loc™ coded bags (16.5 × 14 cm, 1.15 mil thick), and randomly assigned to the treatment combinations. Samples were then treated with approximately 5 g Captan™ dust to control/inhibit mold growth prior to initiation of the storage study. Preliminary work showed no differences between Pi from Captan™ treated decorticated beans and untreated seeds held at 2C for 3 months. Samples were analyzed for phytase activity and lignin, phytic acid, water soluble pectic substances, calcium and magnesium ion concentrations at (zero control), 4 and 8 months storage.

Bean Processing/Hardness Determination

Decorticated beans were used in this investigation, thus conventional canning procedures could not be employed as processing conditions were greatly lower. Bean processing specifications and conditions have been published previously (Mafuleka *et al.* 1991).

The 100 g bean samples in 264 ml pyrex glass jars containing deionized water were soaked in boiling water bath for 30 min, exhausted for 5 min, then processed at 5 psig (109C) for 5 min. A bean was considered cooked upon yielding to slight pressure when individually squeezed between the forefinger and thumb (Jones and Boulter 1983a). Cooked bean hardness (100g) was measured using a Kramer shear press (Food Technology Corp., Reston, VA, model no. t-2100-C) with a standard shear-compression cell (model no., CS-1) at ⅓ range setting and 3000 lb transducer force. Shear force was calculated by the method of Binder and Rockland (1964).

Bean Flour Preparation

All hard-to-cook indicators were determined using bean flour samples. Legume seed flour was obtained by grinding a 50 g dry bean sample for 5 min (80 mesh) using a Braun mill (Model No. KSM2, Lynnfield, MA).

[2]Captan [1,2,3,6, tetrahydro-N-(trichloromethylthio) phthalimidine] is a fungicide with protective and curative action used to treat seeds for control of *Pythium, Phoma, Rhizonctonia species* (Hartley and Hamish 1987).

Phytase Assay

Phytase activities were measured indirectly by determining Pi ion concentrations extracted from 0.5 g bean cotyledon flour following the methods of Watanabe and Oslen (1965), Murphy and Riley (1962), and Pons and Guthrie (1946). Chemicals, supplies and equipment included ammonium molybdate (ACS certified); antimony potassium tartrate, ascorbic acid (USP); H_2SO_4 (ACS certified, EM Industries, Inc., Gibbstown, NJ); trichloroacetic (TCA) acid (ACS certified); phosphorus (20 mg/ml soln.); and a spectrophotometer (Spectronic model 21D, Milton Roy Co., Rochester, NH). Pi absorbance was measured spectrophotometrically at 730 nm. TCA (0.75N) served as the sample blank (Pons and Guthrie 1946).

Phytic Acid

Chemicals and supplies used for the phytic acid determination included: TCA (ACS certified); hydroxylamine hydrochloride (ACS certified); NaOH (1N); HCl (ACS certified); orthophenanthroline (ACS certified); ferrous ammonium sulphate and ferric nitrate (Pfs). Phytic acid compounds were extracted and analyzed following the methods of Wheeler and Ferrel (1971) as modified by Lolas and Markakis (1975). Phytic acid concentrations were determined spectrophotometrically at 510 nm, using a UV/Visible spectrophotometer (Model 4050, KLB Biotechnology Inc.,) and following the procedure of Wheeler and Ferrel (1971).

Calcium and Magnesium

Chemicals, supplies and equipment for the bean cation determinations included:HCl and mannitol (ACS certified); lanthanum chloride (ACS certified); calcium (Atomic absorption standard: 1000 mg/ml 1% HCl) and magnesium (atomic absorption standard: 1015 mg/ml 1% HNO]; pfs, Sigma Chem. Co.); spectrophotometer (Atomic absorption, model 2380, Perkin-Elmer Co.); centrifuge (model HN S11, International Equipment Co.); vacuum drying oven (model FF3174X, Labline, Inc.); and a muffle furnace (Model FF470/471, Thermolyne Corporation).

Bean calcium and magnesium ions were extracted and analyzed from legume seed flour following the methods of Jones and Boulter (1983a). The minerals were determined spectrophotometrically at 422.7 nm and 285.2 nm for calcium and magnesium ions, respectively. Deionized water served as a sample blank.

TABLE 2.

ANALYSIS OF VARIANCE[1] FOR Pi (μg/ml), PHYTIC ACID (mg/ml), CALCIUM (μg/ml), PHYTIC ACID/CALCIUM RATIO, MAGNESIUM (μg/ml), WATER SOLUBLE PECTIC SUBSTANCES (mg/ml) AND LIGNIN (μg/ml) OF STORED DECORTICATED BEANS FOR TIME, TEMPERATURE, a_w AND GENOTYPE

		Mean Square						
Source	df	Inorganic Phosphate (Pi)	Phytic Acid	Calcium	Phytic Acid/ Calcium Ratio (10^5)	Magnesium	Water Soluble Pectic Substances	Lignin
Storage (S)	1	0.716**	0.097	0.000*	1.5**	0.004	0.011*	27**
Temperature (T)	1	3.311**	0.216	0.026*	17.8**	0.002	0.016*	7*
S x T	1	0.311	0.029	0.013*	0.8*	0.001	0.000	2
A_w	1	0.001	0.016	0.027*	6.6*	0.002	0.002	4
S x A_w	1	0.014	0.001	0.000	0.3	0.003	0.000	0.8
T x A_w	1	0.011	0.001	0.000	2.2	0.001	0.002	6*
S x T x A_w	1	0.003	0.011	0.000	0.5	0.000	0.000	5*
Genotype (G)	1	0.907**	0.828	0.002	10.8*	0.016**	0.024**	24**
S x G	1	0.150**	0.022	0.000	0.02	0.005	0.000	0.3
T x G	1	0.372**	0.005	0.017	3.5	0.000	0.005	2
S x T x G	1	0.103*	0.005	0.011	1.5	0.000	0.000	3
A_w x G	1	0.105*	0.004	0.001	0.06	0.000	0.000	0.7
S x A_w x G	1	0.004	0.000	0.005	0.8	0.001	0.000	1
T x A_w x G	1	0.034	0.011	0.008	0.07	0.002	0.000	0
S x T x A_w x G	1	0.000	0.000	0.002	0.5	0.004	0.000	0.4
Error	16	0.015	0.092	0.005	1.5	0.002	0.002	1.0

1 Factor Factorial ANOVA with four factors at two levels (2^4).

*, ** Significant at the 5% and 1% probability levels, respectively.

Total and Water Soluble Pectic Substances

Using a method of Owen *et al.* (1952), bean water soluble pectic substances were determined from the total pectic substances extracted from 1 g of cotyledon flour and spectrophotometrically analyzed at 520 nm. Chemicals, supplies and equipment for the legume seed total and water soluble pectic substances method included: Calgon (Beechum Products);HCl (ACS certified) and Celite analytical filter aid, (Fisher Scientific Co.); ground pulp (Scheicher and Schuell, Inc.), and macroporous filter paper (pore size: 10–2000 μm, Spectrum Medical Industries, Inc.); NaOH (1 N); H_2SO_4 (ACS certified); sodium tetraborate (CAS, electrophoresis grade); meta-hydroxydiphenyl (Eastman Kodak Co., Rochester, NY) and a digital pH meter (Corning model no. 610A). Absorbances of water soluble pectic substances were read at 520 nm (UV/Visible, model 4050, LKB, Biotechnology, Inc., Gaithersberg, MD). NaOH (0.5%) was used for the blank sample.

Lignin

Chemicals, supplies and equipment for the lignin determination included: methanol (Absolute, ACS certified): NaOH (1N): HCl (ACS certified); thioglycolic acid (70% Grade IV, Sigma Chem. Co.); centrifuge (Sorvall model RC2-B, Du Pont Co.); and spectrophotometer (UV/Visible, Model 4050, LKB Biotechnology, Inc.). Legume seed lignin was extracted from 2 g bean flour and measured spectrophotometrically at 280 nm following the method of Hammerschidt (1984). A lignin standard curve was developed relating relative lignin absorbance at 280 nm (potato suberin) to its concentration. NaOH (0.5 N) served as the sample blank.

RESULTS AND DISCUSSION

Phytase Activity (Pi)

Analyses of variance from stored decorticated beans showed that Pi was significantly ($p \leqslant 0.01$) influenced by storage time, temperature and genotype factors (Table 2). Furthermore, there were significant ($p \leqslant 0.05$) time $\times$ temperature $\times$ a_w $\times$ genotype interactions. The variations in Pi concentrations for beans held at different environmental conditions in the present research were expected to be primarily due to differences in phytase activity levels, since phytic acid phosphorus constitutes about 70% of total dry bean seed phosphorus (Lolas and Markakis 1975). Bean storage conditions (time, temperature and a_w) influenced phytase activities in bean cotyledons. These findings agree with Hincks and Stanley's (1986) data, which revealed increased phytase activities as time advanced

TABLE 3.

INFLUENCE OF BEAN TYPE, STORAGE TIME, TEMPERATURE AND a_w ON MOISTURE CONTENT, COOKED BEAN HARDNESS, Pi, PHYTIC ACID, CALCIUM, PHYTIC ACID/CALCIUM RATIO, MAGNESIUM, WATER SOLUBLE PECTIC SUBSTANCES AND LIGNIN OF STORED DECORTICATED BEANS

Bean seed coat color		Moisture Content (%)	Bean Hardness (g force/100 g)	Phytic Acid (mg/ml)	Pi (ug/ml)	Ca (ug/ml)	Phytic Acid/ Ca ratio (10^3)	Mg (ug/ml)	Water soluble pectic substances (mg/ml)	Lignin (mg/ml)
White										
Control		10.5	954	3.06	0.65	0.81	3.8	0.17	0.43	4.23
Treatments	1	10.5	759	2.85	0.68	0.67	4.2	0.19	0.42	3.60
	2	11.6	826	2.73	0.70	0.77	3.6	0.15	0.42	3.33
	3	10.4	1379	2.66	1.11	0.84	3.1	0.18	0.39	5.62
	4	14.1	1867	2.71	1.31	0.96	2.9	0.19	0.34	4.55
	5	10.7	993	2.83	0.77	0.80	3.4	0.17	0.39	2.57
	6	12.3	929	2.80	0.81	0.79	3.5	0.19	0.40	3.31
	7	10.5	1474	2.54	1.85	0.78	3.3	0.20	0.33	3.15
	8	15.6	3319	2.54	2.12	0.86	3.0	0.17	0.30	3.50
Red										
Control		9.5	2545	2.76	1.23	0.85	3.2	0.19	0.37	5.49
Treatments	9	10.6	2332	2.57	1.32	0.76	3.4	0.23	0.35	4.65
	10	11.3	2057	2.47	1.21	0.81	3.1	0.23	0.34	8.38
	11	11.0	2783	2.45	1.69	0.80	3.1	0.32	0.34	6.93
	12	13.7	3021	2.38	1.48	0.81	2.9	0.22	0.32	6.78
	13	10.3	2375	2.39	1.33	0.73	3.3	0.19	0.31	3.39
	14	11.8	2217	2.40	1.30	0.85	2.9	0.21	0.31	3.57
	15	10.6	2634	2.27	1.88	0.79	2.9	0.20	0.30	5.67
	16	15.4	4220	2.16	1.83	0.80	2.7	0.20	0.27	6.09

(0–10 mo) in black beans stored at 30C and 0.85 a_w compared to the control group held at 15C and 0.36a_w.

Data for legume seed moisture content in equilibrium with a_w for the different treatments of the current study indicated that the relationship between phytase activities and storage conditions cannot be attributed to either time, temperature or a_w in isolation (Table 3). The variations in Pi concentrations due to variety may not reflect differences in phytase activity rates per se, but probably reflect dissimilarities in initial substrate (phytic acid) concentrations. Red bean phytic acid concentrations were lower ($p \leqslant 0.01$) than in white bean genotype. Genotypic differences in phytic acid content were also observed in faba beans (*Vinia faba*) by Hussein *et al.* (1989).

The relationships between several storage parameters and Pi levels of white and red decorticated means are illustrated (Table 3). White beans stored for 4 and 8 months at two a_w levels (0.55 a_w and 0.85 a_w) demonstrated significantly ($p \leqslant 0.05$) greater Pi accumulation at higher storage temperature (35C) than under mild (16C) conditions. These results agree with data by Kon (1979) who demonstrated increased phytase activities in black beans steeped (16 h) at high temperatures (40–60C) as compared to seeds at 20C. Legume seeds stored at 35C, 0.85 a_w for 4–8 months exhibited significantly ($p \leqslant 0.05$) higher Pi levels than samples maintained at the same temperature, but lower a_w (0.55 a_w) and control seeds held at 2C and 0.30 a_w. The higher Pi concentrations and consequently the greater phytase activities were produced in beans stored under the maximum time, temperature and a_w conditions.

Red beans (Table 3) maintained at 0.55 a_w and 0.85 a_w for 4 and 8 months exhibited significantly ($p \leqslant 0.05$) higher Pi contents at higher (35C) than at lower (16C) temperature and the control group (2C and 0.30 a_w). Greater Pi concentrations were evident for red beans held under the maximum time (8 months), temperature (35C) and both a_w (0.55 a_w, 0.85 a_w) conditions as opposed to the shorter storage period (4 months).

It can be deduced that the higher phytase activities in decorticated white beans maintained under high temperature and high a_w stored for 8 months contributed greatly to the bean hard-to-cook defect since these seeds also exhibited increased cooked hardness (Table 3). Cooked bean hardness data have been reported in Table 4. The phytase showed a similar trend in the red beans and the white beans stored under the same conditions, but the differences between treatments were not as apparent.

Phytic Acid

Phytic acid levels were significantly ($p \leqslant 0.01$) influenced by the bean genotype (Table 2). Since the white bean genotype had a greater phytic acid concentration

TABLE 4.

INFLUENCE OF BEAN TYPE, STORAGE TIME, TEMPERATURE AND a_w ON COTYLEDON HARDNESS

Bean Type	Storage Time (Months)	Storage Temperature (C)	a_w	Cotyledon Hardness (g force/100 g)
White	0	2	0.30	954
	4	16	0.55	759
	4	16	0.85	826
	4	35	0.55	1379
	4	35	0.85	1867
	8	16	0.55	993
	8	16	0.85	929
	8	35	0.55	1474
	8	35	0.85	3319
Red	0	2	0.30	2545
	4	16	0.55	2332
	4	16	0.85	2057
	4	35	0.55	2783
	4	35	0.85	3021
	8	16	0.55	2375
	8	16	0.85	2217
	8	35	0.55	2634
	8	35	0.85	4220

than the red bean ones, the differences in phytase activity noted between the two genotypes were probably due to the phytic acid differences (Table 3). The variances in Pi concentrations due to genotype may not reflect differences in phytase activities per se, but most likely reflect dissimilarities in initial substrate concentrations. Genotypic differences in phytic acid content have been reported to exist in common beans (Blaisdell and Hosfield 1988).

Calcium

Cell wall calcium concentrations in stored decorticated red and white beans were significantly ($p \leqslant 0.05$) influenced by storage temperatures and a_w (Table 2). The cell wall calcium concentrations were similar in both genotypes. Legume seeds stored at 35C, 0.85 a_w were similar ($p \leqslant 0.05$) in cell wall calcium contents (Table 3) to the control held at 2C, 0.30 a_w, but were greater ($p \leqslant 0.05$) in calcium concentrations than seeds stored under low temperature and a_w conditions (16C, 0.55 a_w). These data concur with others (Moscoso *et al.* 1984). Moscoso *et al.* (1984) found that bean calcium contents were slightly higher (0.49 mg/g) in seeds stored for 9 months at 32C, 17.9% moisture than controls (0.47 mg/g) held at 2C and 12.5% moisture.

Phytic Acid/ Calcium Ratios

Phytic acid/calcium ratios of stored decorticated beans were significantly influenced by storage temperature ($p \leqslant 0.01$), a_w ($p \leqslant 0.05$) and genotype ($p \leqslant 0.05$) (Table 2). Beans stored at the highest temperature and a_w levels (35C, 0.85 a_w) exhibited significantly ($p \leqslant 0.05$) lower phytic acid/calcium ratios as compared to other storage conditions (16C, 0.55 a_w; 2C, 0.30 a_w) (Table 3). These data lend support to the Pi results in that a decrease in phytic acid/calcium ratios within each genotype under adverse storage conditions implied advanced phytic acid degradation with concomitant elevation of calcium. Phytase degrades phytic acid to release Pi, calcium and magnesium (Peers 1953; Lolas and Markakis 1975; Kon and Sanshuck 1981; Moscoso *et al.* 1984; Henderson and Ankrah 1985). A decline in phytic acid/calcium ratios in beans stored under high temperature (32C) and moisture (16%) over 10 months was reported (Kon and Sanshuck 1981).

Genotype differences in phytic acid/calcium ratios, may not reflect differences in phytase activity levels but probably reflect dissimilarities in initial phytic acid/calcium ratios. Bean genotypic differences in phytic acid/calcium ratios have been noted by Kon and Sanshuck (1981). Low ratios have been associated with increased cooked bean hardness (Kon and Sanshuck 1981; Hussein *et al.* 1989). The phytic acid calcium rations observed in this study agree with Kon and Sanshuck (1981) and the work of Hussein *et al.* (1989). A significant ($p \leqslant 0.05$)

negative correlation ($r^2 = 0.71$) was found between white bean hardness and phytic acid/calcium ratios under low and high temperatures (16C, 35C) and a_w (0.55, 0.85 a_w) during the 0–8 month storage period. Likewise, a significant ($p \leqslant 0.05$) negative correlation ($r^2 = -0.66$) was observed between red bean hardness and the phytic acid/calcium ratios under the same storage conditions.

Magnesium

Cell wall magnesium data (Table 2) from decorticated beans revealed that magnesium levels differed ($p \leqslant 0.01$) between bean genotypes. The red genotype had a greater magnesium concentration than the white bean genotype (Table 3). Nonsignificant genotypic differences in bean magnesium contents (0.133–0.168%) have been demonstrated (Kon and Sanshuck 1981). Storage conditions did not affect bean magnesium levels (Table 2). No literature was found to support or refute these data.

Correlation of Cooked Bean Hardness and Phytase Activity

Phytase activities (Cotyledon Pi levels) were significantly ($p \leqslant 0.01$) correlated with white cooked bean hardness for the 4 month ($r^2 = 0.831$) stored beans under two storage temperatures (16C, 35C) and a_w (0.55 a_w, 0.85 a_w). Similar results were reported by Kon (1979) who found a high correlation ($r^2 = 0.77$) between length of cooking and phytase activities as steeping temperature increased from 40C to 70C. Hardness/Pi correlations at 4 months for the present study were $r^2 = 0.759$ at 0.55 a_w and $r^2 = 0.877$ at 0.85 a_w. Under long (8 months) storage, the correlations were $r^2 = 0.873$ at 0.55 a_w and $r^2 = 0.935$ at 0.85 a_w.

A significant ($p \leqslant 0.01$) correlation ($r^2 = 0.689$) was found between hardness and Pi content of the 8 month, but not the 4 month ($r^2 = 0.444$) stored red beans. With increased storage, the relationship was significant at both the 0.85 a_w ($r^2 = 0.907$) and the 0.55 a_w ($r^2 = 0.745$).

The hardness/Pi correlations demonstrate that phytase activity is of great importance in the white bean hard-to-cook phenomenon up to 8 months of storage, although the impact declined slightly with time when temperature and a_w were combined. Phytase activity is important in the hard-to-cook defect of red beans under advanced (8 month) storage period, although the enzyme activity data alone cannot fully explain the defect.

Water Soluble Pectic Substances

Water soluble pectic substance concentrations in beans were significantly influenced by storage time ($p \leqslant 0.05$), temperature ($p \leqslant 0.05$) and genotype

($p \leqslant 0.01$) parameters (Table 2). Higher ($p \leqslant 0.05$) water soluble pectic substance concentrations were measured from beans held at the high temperature (35C) (Table 3) for the longer storage time period (8 months) in contrast to legume seeds stored for less time and at lower temperatures (16C for 4 months; 2C). These results agree with Jones and Boulter (1983a) and Moscoso *et al.* (1984) data. Jones and Boulter (1983a) postulated that the reduction in water soluble pectic substance levels was due to phytic acid degradation by phytase, which released calcium and magnesium ions. Subsequently, cation bridges were formed within the pectinaceous middle lamella, rendering the pectin insoluble. Pectin desolubilization was thought to be facilitated from pectin deesterification by the action of PME, which increased the availability of free carboxyl sites (Jones and Boulter 1983a).

In support of the pectin desolubilization theory, relationships were found between decreased phytic acid/calcium ratios and decreased water soluble pectic substance concentrations for white ($r = 0.78$) ($p \leqslant 0.01$) and red beans ($r = 0.72$) ($p \leqslant 0.05$) during the 8 months of storage at both the low and high temperature and a_w levels. These relationships implied that the desolubilization of water soluble pectic substances from stored legume seeds (4 and 8 months) was associated with depressed phytic acid levels with an accompanying elevation in cell wall calcium ion concentrations. The increased pectic desolubilization due to calcium and magnesium cation accumulation in the middle lamella has been suggested as a mechanism to restrict cell separation and result in hardened beans (Kon and Sanshuck 1981). The reduction in cell separation of the hard-to-cook beans implied that the middle lamella pectins were modified compared to non hard-to-cook beans. Negative correlations between cooked bean hardness and bean water soluble pectic substance concentrations for white ($r^2 = 0.88$) ($p \leqslant 0.01$) and red ($r^2 = 0.73$) ($p \leqslant 0.05$) beans during storage (0–8 months) under both low and high temperatures and a_w levels were found in the current study.

Genotypic differences ($p \leqslant 0.01$) among beans in water soluble pectic substance contents could be accounted for by the high and low phytic acid/calcium ratios between white and red beans, respectively, rather than differences in their phytase activities. The increases in bean Pi and cooked bean hardness and decreases in water soluble pectic substance contents and phytic acid/calcium ratios support the mechanism involving phytic acid degradation by phytase as influencing the subsequent hardness (hard-to-cook defect) of the white and red bean genotypes during storage. The effect of the system involving phytic acid degradation on legume seed hardness was greater in the white bean genotype, which had higher initial phytic acid contents as compared to the red bean genotype. Despite the greater contribution of the mechanism involving phytic acid degradation towards the development of the hard-to-cook defect on white beans, the red legume seeds remained harder following heat treatment ($p \leqslant 0.05$) than the white beans.

Lignin

The ANOVA (Table 2) revealed that lignin concentrations in decorticated beans were significantly influenced by storage time ($p \leqslant 0.01$), temperature ($p \leqslant 0.05$) and genotypic effects ($p \leqslant 0.05$). In addition, there were significant temperature $\times$ a_w ($p \leqslant 0.05$) and time $\times$ temperature $\times$ a_w ($p \leqslant 0.05$) interactions.

The relationship between storage time, temperature, a_w and genotype factors, and bean lignin contents indicated that white beans stored for 4 months at 0.55 a_w exhibited significant lignin concentration increases ($p \leqslant 0.05$) at high temperature (35C) as compared to the low (16C) temperature (Table 3). Moreover, bean lignin contents also increased at low (16C) temperature and at high 0.85 a_w as compared to the low a_w ($0.55a_w$) following storage at 4 months (Table 3). Although it was not significant, a general decline occurred in lignin levels of decorticated white beans as storage time, temperature and a_w increased. The decrease in lignin levels from white decorticated beans stored at high temperature and a_w for 8 months was not expected and did not agree with the qualitative studies by Hincks and Stanley (1987) who demonstrated an increased cell wall and middle lamella lignin deposition in hard-to-cook beans stored at 30C, 0.85 a_w for a maximum of 10 months. The lignin studies of Hincks and Stanley (1987) did not involve extraction of lignin, but rather the staining of lignin in intact cell walls and middle lamellae. In the present study, the decline in lignin levels for the 8 month stored beans may be attributed to lignin-protein cross-linking (Whitmore 1978), which would not be quantitatively measured following the thioglycolic lignin determination used in this research.

Increased ($p \leqslant 0.05$) lignin concentrations in the red beans were apparent at the high a_w ($0.85a_w$) compared to the low a_w (0.55) while maintaining 16C temperature following a 4 month storage period. At 8 months of storage, beans held at 0.55 a_w and 0.85 a_w showed significant ($p \leqslant 0.05$) increases in extractable bean lignin at high (35C) temperature compared to the low (16C) temperature. However, for the red bean genotype and similar to the white bean one, there was a general, but nonsignificant decline in extractable lignin levels as storage time increased. After 4 months of storage, legume seed lignin levels tended to decline at high a_w (0.85 a_w) and high (35C) temperature as compared to the lower (16C) temperature. These findings agree with the results of Srisuma *et al.* (1989) on seed coat and cotyledon lignification of navy beans (*Phaseolus vulgaris* var. seafare). There was a slight, but nonsignificant decline in relative seed coat lignin concentrations between partially hard (4.84 absorbance/g) beans stored at 20C, 0.73 a_w and hard (454 absorbance/g) beans kept at 35C, 0.80 a_w, for 9 months. A similar trend was observed for the cotyledon lignin, although the concentrations were much lower. Srisuma and co-workers (1989) used the thioglycolic method of lignin determination.

Extractable lignin contents from both the red and white bean genotypes following an 8 month storage period in the present study were similar to their legume controls. These results agree with data reported by Srisuma *et al.* (1989) who found no significant differences in lignin levels among beans stored under three conditions (5C, 0.40 a_w, ; 20C, 0.73 a_w, 35C, 0.80 a_w) for 9 months.

Genotypic differences in lignin concentrations were apparent (Table 3). The red bean genotype exhibited significantly ($p \leqslant 0.01$) higher lignin contents compared to the white bean genotype and may explain why the red beans showed higher hardness values than the white beans.

Correlations of Cooked Bean Hardness and Lignin

Lignin content in red beans correlated ($r^2 = 0.669$) significantly ($p \leqslant 0.01$) with hardness during the longer (8 month) storage period at the two storage temperatures (16C, 35C) and a_w levels (0.55 a_w, 0.85 a_w). During this advanced (8 month) stage, correlations were significant at 0.85 a_w ($r^2 = 0.821$), but not at 0.55 a_w ($r^2 = 0.560$, ($p \leqslant 0.136$). Lignin was not significantly correlated with hardness at 4 months ($r^2 = 0.232$). In addition, no significant correlations were found between white bean hardness and lignin levels at both the 4 month ($r^2 = 0.210$) and the 8 month ($r^2 = 0.232$) period. The significance of lignin in the hard-to-cook condition development has partially been revealed through correlations in red beans under extended (8 month) storage period. The role of lignin in the white bean defect has been minimal during this time, although the impact slightly increased with time. More research needs to be conducted to develop better quantitative methods to determine lignin. Further more, research needs to be carried out to quantitatively determine lignin's contribution to the legume seed hard-to-cook phenomenon during the 0–8 month time period, and following 8 months of storage to substantiate our findings.

CONCLUSIONS

The hard-to-cook defect developed in the two Malawian decorticated bean genotypes (white and red) used in the study as storage time increased (0–8 months) and when stored under the most severe conditions (35C, 0.85 a_w). In both the genotypes, the mechanism involving phytic acid degradation appeared to be the major system influencing the hard-to-cook defect. The effect of a lignification mechanism was partially revealed in the hard-to-cook defect of red beans after an extended (8 month) storage period. The role of lignin was not clearly shown in the 4 month stored red and 0–8 month stored white beans. The trends of lignin

development with storage noted in the experiment suggested that lignin formation influenced the hardness of the red bean genotype more than the white one, despite the minimal effect of lignification on the hard-to-cook phenomena. Lignin concentrations may have a major influence on varietal differences in hardness between the red and white beans soon after harvest, as opposed to the hard-to-cook defect that develops during adverse storage conditions. Further studies are needed in this area to quantify lignin concentrations in several bean genotypes using protein-free samples stored under conditions to produce the hard-to-cook defect.

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INHIBITION OF APPLE POLYPHENOLOXIDASE (PPO) BY ASCORBIC ACID, CITRIC ACID AND SODIUM CHLORIDE

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ABSTRACT

The inhibiting effect of ascorbic acid, citric acid and sodium chloride on Polyphenoloxidase (PPO) of Golden Delicious apple cubes was studied.

Dipping in ascorbic acid (0.2–10 g/L range) and in NaCl (0.2–1 g/L range) solutions for 5 min increases the PPO activity. Citric acid solutions (0.2–10 g/L range) have little or no inhibition of PPO.

A 90–100% PPO inhibition was obtained with a 5 min dip in mixtures of ascorbic acid and citric acid (10 + 2 g/L), and of ascorbic acid and sodium chloride (10 + 0.5 g/L).

INTRODUCTION

Browning occurs during fruit processing. At least five causes of browning in processed and/or stored fruit and vegetables are known: enzymatic browning of the phenols, Maillard reaction, ascorbic acid oxidation, caramelization and formation of browned polymers by oxidized lipids.

The oxidation of the o-diphenols to o-quinones by polyphenoloxidase (E.C. 1.14.18.1:usually named PPO) is the most important cause of the change in color as the o-quinones quickly polymerize and produce brown pigments (melanin) (Mayer and Harel 1979; Vamos-Vigyazo 1981). Enzymatic browning also causes a loss in the nutritional value through oxidation of ascorbic acid.

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In the food industry, enzymatic browning can be avoided by using thermal inactivation of PPO, but heat can cause softening.

Instead of blanching, chemical additives can be used to prevent enzymatic browning: bisulfite (Ponting 1960; Walker 1977; Sayavedra-Soto and Montgomery 1986), ascorbic acid and its analogs (Bauernfeind and Pinkert 1970; Eskin *et al.* 1971; Walker 1977; Sapers and Ziolkowski 1987; Hsu *et al.* 1988), and cysteine as reducing agent (Walker and Reddish 1964; Montgomery 1983; Dudley and Hotchkiss 1989).

The chemical action of the bisulfites is to react with the o-quinones forming colorless complex compounds (Embs and Markakis 1965; Lu Valle 1952; Wedzicha 1984). Ascorbic acid reduces the o-quinones to colorless dihydroxyphenols (Varoquaux and Sarris 1979; Golan-Goldhirsh and Whitaker 1984).

Although the bisulfites are efficient, they are banned in the USA for use in raw fruit and vegetables by a ruling of the FDA (1986). The presence of bisulfites can be dangerous to human health, especially in asthmatic patients (Taylor and Bush 1986), so alternative chemical additives are needed that are without toxic effects.

Langdon (1987) showed that different combinations of ascorbic and citric acid prevent enzymatic browning of sliced potatoes. Santerre *et al.* (1988) confirmed that combinations of ascorbic acid, erythorbic acid and citric acid were efficient in preventing browning of sliced apples. According to Ponting *et al.* (1972) sliced Golden Delicious apples could be protected from browning by using a mixture of ascorbic acid (0.5%) and calcium chloride (0.05%) at pH 7. De Poix *et al.* (1980), in a study on the action of sodium and calcium chloride on preventing browning of apple puree, pointed out that the addition of chlorides delays the occurrence of browning. For a given chloride concentration, the latent period before the advent of browning is proportional to the amount of ascorbic acid added.

Sapers and Douglas (1987) showed that the treatment of cut apple surfaces with 1% citric acid monohydrate solutions containing 0.4, 0.8, 1.6 or 3.2% ascorbic acid, is effective in the inhibition of browning.

The phosphate esters of ascorbic acid (ascorbic acid-2-phosphate and ascorbic acid-2-triphosphate) were investigated as alternative sources to ascorbic acid for the inhibition of browning at the cut surfaces of raw apples (Sapers *et al.* 1989). The phosphate esters were more effective than similar concentrations of ascorbic acid in the prevention of browning in Red Delicious and Winesap apples.

Vacuum and pressure infiltration of ascorbic and erythorbic acid into the cut surfaces of raw apples improved the efficiency of inhibitors (2.25% sodium ascorbate or erythorbate and 0.2% calcium chloride) (Sapers *et al.* 1990).

The influence of ascorbic acid on PPO activity is still controversial. Varoquaux and Sarris (1979) reported that ascorbic acid neither prevented nor activated PPO. Activation of PPO by ascorbic acid has been reported (Krueger 1950). Golan-

Goldhirsh and Whitaker (1984) showed there was rapid inactivation of the mushroom PPO incubated with ascorbic acid without the phenolic substrate. Recently, Hsu *et al.* (1988) reported an inhibiting effect of ascorbic acid on the PPO of mushrooms.

In the present work the inhibiting effect of ascorbic acid, citric acid and sodium chloride, used alone or in mixtures, on the apple PPO activity was studied. As a comparison the PPO inhibition obtained using potassium metabisulfite was also analyzed.

MATERIALS AND METHODS

Raw Materials

Fresh apples (*Malus domestica* Borkh., cv. Golden Delicious) were obtained from Valtellina (Italy), picked at commercial maturity and stored at 2–4C.

After washing in running water the apples were peeled, cored and mechanically diced (14 mm).

PPO Inhibition

Inhibition of PPO in apple cubes was obtained by:

(1) Dipping in aqueous solutions of: (a) L (+) ascorbic acid (AA), (b) citric acid (CA); (c) sodium chloride (NaCl); (d) potassium metabisulfite (PBS). The reagents were of analytical grade (Merck, Darmstadt, Germany). The concentrations used were: (a) 0.2–1.0–2.0–10.0 g/L of ascorbic acid; (b) 0.2–1.0–2.0–10.0 g/L of citric acid; (c) 0.2–0.5–1.0 g/L of sodium chloride; (d) 0.1–0.3–0.5–1.0 g/L of potassium metabisulfite.

(2) Dipping in aqueous solutions of the following mixtures: (a) L (+) ascorbic acid and citric acid (AA + CA); (b) potassium metabisulfite and citric acid (PBS + CA); (c) L (+) ascorbic acid and sodium chloride (AA + NaCl).

The concentrations of the mixtures were: (a) AA 1 g/L and CA 1 g/L; AA 1 g/L and CA 2 g/L; AA 10 g/L and CA 1g/L; AA 10 g/L and CA 2 g/L; (b) PBS 0.1 g/L and CA 1 g/L; PBS 0.1 g/L and CA 2 g/L; PBS 0.3 g/L and CA 1 g/L; PBS 0.3 g/L and CA 2 g/L; (c) AA 10 g/L and NaCl 0.2 g/L; AA 10 g/L and NaCl 0.5 g/L; AA 10 g/L and NaCl 1 g/L.

The solid/liquid ratio was 1:5 and deionized water at 16–18C was used.

The dipping time was 5 min and the apple cubes were stirred every 30 s with a plastic spatula. Lengthening the dipping time to 15 min did not significantly influence the PPO inhibition (data not reported). After dipping, the apple cubes were left to drip for 2–3 min and kept at 2C prior to analysis. Apple cubes dipped in deionized water for 5 min, were used as control.

TABLE 1.
ASCORBIC ACID AND POTASSIUM METABISULFITE INHIBITION OF APPLE PPO ACTIVITY

Ascorbic acid				Potassium metabisulfite			
	activity ($0.001\Delta A_{420} min^{-1} ml^{-1}$)				activity ($0.001\Delta A_{420} min^{-1} ml^{-1}$)		
Conc. (g/L)	X	s.d.	Inhibition %	Conc. (g/L)	X	s.d.	Inhibition %
0	594	30	0	0	605	38	0
0.2	880	41	-48*	0.1	516	32	15
1.0	670	31	-13	0.3	120	8	80
2.0	780	33	-31	0.5	27	0.9	96
10.0	890	36	-50	1.0	0	-	100

X = means of 5 replicates
s.d. = standard deviation
(*) = negative values indicate PPO activation

PPO Activity

The apple cubes were immersed in liquid nitrogen and ground immediately in a stainless steel blender.

A 10 g aliquot of the ground apple was mixed with 10 ml of citric-phosphate buffer (McIlvaine) pH 6.5 using a Ultra-Turrax T25 (Janke & Kunkel) homogenizer (20500 rpm) for 60 s. The homogenate was centrifuged (45000 × g) at 4C for 30 min (Beckman J2-21 centrifuge).

The supernatant was filtered on saturated paper (Schleicher & Schull GmbH, 589) and analyzed for PPO activity at 420 nm and 25C. One milliliter catechol solution (0.175 M) and 2 ml of citric-phosphate buffer (McIlvaine) pH 6.5 were added to 0.5 ml of PPO extract (Pifferi and Cultrera 1972). The enzyme activity was calculated on the basis of the slope of the linear portion of the curve plotted with ΔA_{420} against time (up to 3 and 6 min in untreated and treated samples, respectively). One unit of enzyme activity was defined as 0.001 ΔA_{420} min^{-1} $(ml\ of\ extract)^{-1}$.

TABLE 2.
CITRIC ACID INHIBITION OF APPLE PPO ACTIVITY

Conc. (g/L)	activity ($0.001\,\Delta A_{420} min^{-1} ml^{-1}$) X	s.d.	Inhibition %
0	597	33	0
0.2	624	32	-4.5*
1.0	666	34	-11.5
2.0	574	27	3.8
10.0	492	20	17.6

X = means of 5 replicates
s.d. = standard deviation
* = negative values indicate PPO activation

TABLE 3.
SODIUM CHLORIDE INHIBITION OF APPLE PPO ACTIVITY

Conc. (g/L)	activity ($0.001\,\Delta A_{420} min^{-1} ml^{-1}$) X	d.s.	Inhibition %
0	784	31	0
0.2	1027	46	-31*
0.5	1480	50	-88
1.0	1483	50	-89

X = means of 5 replicates
s.d. = standard deviation
* = negative values indicate PPO activation

Residual PPO activity was expressed as ratio of treated sample versus its control. pH was determined according to the AOAC Methods (1980).

RESULTS AND DISCUSSION

PPO Inhibition Using Ascorbic Acid, Citric Acid and Sodium Chloride

The inhibition of PPO activity in apple cubes after dipping in ascorbic acid, potassium metabisulfite, citric acid and sodium chloride solutions is reported in Table 1, 2 and 3, respectively. When the concentrations of ascorbic acid, citric acid and sodium chloride in the solutions were increased, the pH of the solutions and the pH of apple cubes after dipping did not significantly change (data not reported). Concentrations between 0.2 and 10 g/L of ascorbic acid did not inhibit, but activated PPO. The increase of PPO activity could be due to an insufficient concentration of the ascorbic acid, which at low concentrations might act as a prooxidant (Kanner *et al.* 1977; Vamos-Vigyazo 1981).

The inhibition of the PPO was correlated to the amount of potassium metabisulfite; 1g/L completely inhibited PPO activity (Table 1).

Citric acid in concentrations between 0.2 and 10g/L had a low inhibiting effect and only at the maximum concentration (Table 2). Citric acid is not an antioxidant agent but its inhibiting effect could be related to the phenolase Cu-chelating power.

Sodium chloride in concentrations between 0.2 and 1 g/l activated PPO (Table 3): 1g/L increased PPO activity of about 90%. Concentrations between 0.5 and 1% of sodium chloride had an inhibiting effect on the enzymatic browning of whole apples or apple pieces (Taufel and Voigt 1964) but only concentrations of about 20% inactivated PPO isolated from the apple (Ponting and Joslyn 1948). The inhibitory effect of sodium chloride is attributed to the anion chloride: the action is of the noncompetitive type, as shown for purified PPO from apples (Sharon and Mayer 1967; Janovitz-Klapp *et al.* 1990). The increase of PPO activity observed in this study could be related to conformational changes of the enzyme or protein association or dissociation due to the modification of the ionic strength.

PPO Inhibition Using Binary Mixtures

(1) Mixtures of L (+)-Ascorbic Acid and Citric Acid (AA + CA). In Table 4 the inhibitory effect of mixtures of ascorbic acid and citric acid on apple PPO is reported. As a comparison the PPO inhibition obtained using mixtures of potassium metabisulfite and citric acid (PBS + CA) is also reported.

Ascorbic acid and citric acid inhibited PPO activity, and citric acid increased the inhibiting effect of ascorbic acid. When 2 g/L of citric acid instead of 1 g/L

TABLE 4.
ASCORBIC ACID AND CITRIC ACID, AND POTASSIUM METABISULFITE AND CITRIC ACID INHIBITION OF APPLE PPO ACTIVITY

Ascorbic acid + Citric acid				Potassium metabisulfite + Citric acid			
	activity ($0.001\Delta A_{420}min^{-1}ml^{-1}$)				activity ($0.001\Delta A_{420}min^{-1}ml^{-1}$)		
Conc. (g/L)	X	s.d.	Inhibition %	Conc. (g/L)	X	s.d.	Inhibition %
0	899	35	0	0	899	35	0
1+1	471	22	47.6	0.1+1	310	18	65.5
1+2	577	25	35.8	0.1+2	304	17	66.2
10+1	573	24	36.3	0.3+1	53	2	94.1
10+2	116	8	87.1	0.3+2	34	2	96.2

X = means of 5 replicates
s.d. = standard deviation

were added to 10 g/L of ascorbic acid the PPO inhibition increased from 36.3% to 87.1%. Citric acid also increased the inhibiting effect of metabisulfite but indepedelty from the concentration.

(2) Mixture of L (+)-Ascorbic Acid and Sodium Chloride (AA + NaCl). The inhibition of PPO using mixtures of ascorbic acid and sodium chloride is reported in Table 5. Ten grams/L of ascorbic acid with 0.5 g/L of sodium chloride completely inhibited the PPO activity. Sodium chloride is more efficient than citric acid, as by adding 0.5 g/L of NaCl instead of 2 g/L of citric acid to 10 g/L of ascorbic acid, the PPO inhibition was 100% compared to 87%.

After the treatments using the most efficient mixtures, i.e., ascorbic acid and citric acid (10 + 2 g/L) and ascorbic acid and sodium chloride (10 + 0.5 g/L), the pH values of the apple cubes were only slightly lower than the values of the untreated apples (data not reported). Therefore, in this case, the pH could be excluded from playing a part in the PPO inhibition.

The explanation of De Poix *et al.* (1980) of the synergic phenomena between ascorbic acid and sodium chloride is that the ascorbic acid reduces the quinones enzymatically formed and delays browning without altering the enzymatic activity, whereas the anion chloride directly inhibits the PPO.

TABLE 5.
ASCORBIC ACID AND SODIUM CHLORIDE INHIBITION OF APPLE PPO ACTIVITY

Conc. (g/L)	activity ($0.001\Delta A_{420} min^{-1} ml^{-1}$) X	s.d.	Inhibition %
0	1057	43	0
10+0.2	563	23	46.8
10+0.5	0	--	100.0
10+1	0	--	100.0

X = means of 5 replicates
s.d. = standard deviation

In this study PPO activity was increased by ascorbic acid alone (0.2–10 g/L) or by sodium chloride alone (0.2–1 g/L), on the contrary PPO activity was inhibited by the mixture of ascorbic acid and sodium chloride and the inhibition was directly related to the concentration of sodium chloride. This suggests that a more complex mechanism could be involved in the enhancement of the inhibitory action of ascorbic acid due to sodium chloride.

CONCLUSIONS

A 90–100% inhibition of PPO in apple cubes is obtained by using mixtures of ascorbic acid and citric acid (10 + 2 g/L) and ascorbic acid and sodium chloride (10 + 0.5 g/L). A similar inhibition is obtainable with 0.5% potassium metabisulfite or potassium metabisulfite and citric acid (0.3 + 2 g/L) mixture.

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APPLICATION OF THE WLF EQUATION TO DESCRIBE THE COMBINED EFFECTS OF MOISTURE AND TEMPERATURE ON NONENZYMATIC BROWNING RATES IN FOOD SYSTEMS

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ABSTRACT

Nonenzymatic browning rates of several vegetables, dairy products and model food systems stored at different moisture contents and temperatures were analyzed and related to their glass transition temperature (T_g). The data analyzed corresponded to a region of moisture content in which effects due to reactant diffusion could be expected. As changes in diffusion constants may in turn, be related to glass transition, the Williams-Landel-Ferry (1955) (WLF) equation was used to describe the combined effects of moisture and temperature on the nonenzymatic browning rate constants. Ferry's (1980) procedure of reduced variables utilizing a reference temperature (T_0) was applied, the T_0 selected within the experimental range. In this procedure, the equation coefficients are calculated for the equation using T_0. Then they are recalculated by shifting the selected reference temperature to T_g to obtain the coefficients with reference to T_g. The resulting equation can be applied to relate the browning rate constants to temperature, moisture and T_g. The equation has predictive value and the method avoids extrapolations when data at T_g are not available.

INTRODUCTION

The effect of moisture (m) water activity (A_w) and temperature (T) on the rates of nonenzymatic browning (NEB) was investigated by many authors (Legault *et*

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al. 1951; Hendel *et al.* 1955; Labuza and Kamman 1978; Resnik and Chirife 1979; Samaniego *et al.* 1991). Different models that combine the effects of those variables were proposed to predict reaction rates at given conditions for particular foods and model systems (Mizrahi *et al.* 1970; Franzen *et al.* 1990).

Typically, the maximal NEB rates in foods and model systems occur in a water activity range of 0.5–0.75 (Labuza and Saltmarch 1981), depending on the composition of the system. At low a_w values, usually below $a_w = 0.5$, the decrease in reaction rate is attributed to diffusional limitations (Labuza and Saltmarch 1981). Above the maximum, the decrease was related to dilution effect and to inhibition by water, which is one reaction products of NEB (Eichner and Karel 1972).

The kinetics of NEB was also related to physical aspects such as crystallization (Vuataz 1988; Saltmarch *et al.* 1981; Kim *et al.* 1981) and viscosity. Glass transitions were found to affect the rate of NEB reactions (Karmas *et al.* 1992). The amorphous food matrix may exist as a very viscous glass, or a more liquid-like "rubber." The change from the glassy to the "rubbery" state occurs as a second order phase transition at a specific temperature (T_g, the glass transition temperature) for each amorphous material. T_g is strongly dependent on the concentration of water and other plasticizers (Levine and Slade 1986; Roos and Karel 1990). Changes in diffusion coefficients were related to glass transition (Karel and Saguy 1991), and when NEB reactions take place in the diffusion limited region, changes in the diffusion coefficients may affect browning rate.

A mathematical expression known as the Williams-Landel-Ferry (WLF) equation (Williams *et al.* 1955) was originally applied to relate time for mechanical relaxation processes to the difference between the actual temperature of the material and T_g ($T - T_g$). The WLF equation was developed to describe viscosity changes of synthetic polymers as a function of ($T - T_g$), and was demonstrated as useful in describing changes in viscosity in solution of sugar mixtures (Soesanto and Williams 1981) and changes in time for sugar crystallization (Roos and Karel 1990, 1991a). The WLF equation was used extensively by Levine and Slade (Levine and Slade 1986, 1989; Slade and Levine 1991; Slade *et al.* 1986) to describe various changes in viscosity, diffusivity, or flexibility in foods. If the following assumptions hold true: that the rates of NEB, below $(a_w)_{max}$ decrease because of diffusional limitations of the reactants in a highly viscous matrix, the diffusion coefficient inversely proportional to viscosity, and the viscosity of the food model system decreases drastically above T_g, then the rates of browning should show a strong dependence on ($T - T_g$) (Karel *et al.* 1992). If in addition, in the system studied, viscosity decreases in accordance with the WLF equation, then the reaction rates may also be described with this equation (Slade *et al.* 1989). A break and a curvature in the Arrhenius plot for the temperature dependence of diffusivity were observed in polymers (Kovarskii *et al.* 1978; Hori *et al.* 1986). Karmas *et al.* (1992) observed that the glass transition affected nonenzymatic

browning rates of some vegetables and model systems, but the overall effects of temperature and moisture could not be related simply to $(T - T_g)$. Changes of activation energy with temperature, qualitatively similar to those expected for diffusion in polymers, were observed for browning reactions close to the glass transition temperature in model system (Karmas *et al.* 1992). However, the activation energies obtained in the vicinity of T_g were not as high as predicted by the WLF equation employing the original equation coefficients used by Williams *et al.* (1955). The purpose of this paper was to investigate the applicability of the WLF equation in describing the combined effect of m, T and T_g on nonenzymatic browning rates, using Ferry's method of reduced variable (Ferry 1980), which allows the calculation of the equation coefficients on the basis of experimental data.

MATERIALS AND METHODS

Data from literature (for vegetables and dairy products) and from experimental model systems were analyzed. In order to relate data of browning rates to the T_g of these materials, the experimental determination of T_g was performed both for the experimental model systems and for selected vegetables, since the original studies on their NEB do not provide this information. The glass transition temperature for selected vegetables was reported in a previous paper (Karmas *et al.* 1992). For each vegetable, T_g was determined at various moisture content, obtained by equilibration of freeze-dried samples at various relative humidities (from 0 to 75%). The glass transition temperature of dairy products was observed to be similar to that of lactose (Karmas *et al.* 1992) and the T_gs were obtained from the isotherm for lactose (Roos and Karel 1991[a]).

Literature data for NEB rates for carrot (Legault *et al.* 1951), potato (Hendel *et al.* 1955), cabbage (Mizrahi *et al.* 1970), milk (Franzen *et al.* 1990), apple (Resnik and Chirife 1979), whey powder (Saltmarch *et al.* 1981) and onion (Samaniego *et al.* 1991) were analyzed in relation to the experimentally determined glass transition temperature.

Preparation of Food Model Systems

The model systems were composed of amino acids and sugar reacting in a matrix of poly(vinylpirrolidone) (PVP). Table 1 shows the composition of the two food model systems analyzed. Low concentrations of amino acid and sugars were used to enhance diffusion effects (at high concentration the reacting molecules require minimal diffusion for reaction due to their proximity). Amorphous samples were prepared by freeze-drying 20% (w/w solids) solutions (Table 1). Then 0.5 ml

TABLE 1.
COMPOSITION OF MODEL FOOD SYSTEMS [a]

Model system	Matrix	Reducing sugar	Amino acid	M.C[b]
I	PVP[c] (99)	glucose (0.5)	glycine (0.5)	5.9
II	PVP[d] (98)	xylose (1)	lysine (1)	7.3

(a) Numbers in brackets represent percent of each component in the dry sample.
(b) Moisture content (dry basis) after equilibration at a relative humidy of 22%.
(c) PVP (polyvinylpyrrolidone) of molecular weight= 10,000.
(d) PVP of molecular weight= 24,000.

aliquots of each solution, in 2 ml glass vials were frozen for 24 h at −35C, stored over dry ice [as recommended for amorphous glass formation by Roos and Karel, 1991a)], and freeze-dried for 48 h in a freeze-dryer (VirTis Benchtop 3L). Following freeze-drying, model systems were further dried in an evacuated desiccator over P_2O_5 for one week. These samples were considered to be anhydrous. Samples were then equilibrated further in an evacuated desiccator over a saturated salt solution of $K^+CH_3COO^-$, maintaining $a_w = 0.22$. The moisture content was determined gravimetrically after equilibration, and it was 5.9% (dry basis) for model system I and 7.3% for model system II.

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC), was used to determine the glass transition of amorphous matrices. The DSC used was a Mettler TA 4000 thermal analysis system, with a DSC30S DSC cell. The instrument was calibrated as reported by Roos and Karel (1991b). Thermograms were analyzed using Mettler GraphWare TA72PS.1 software. An empty aluminum pan was used as a reference in all measurements. All samples were measured in DSC pans (Mettler aluminum pan, 40 μl). The glass transition temperature was taken as the onset of the change in specific heat observed as an endothermic shift in the baseline on the thermogram of a dynamic scan done at 5C/min. Samples were scanned twice to eliminate hysteresis effects of thermal relaxations, typical of glass transitions (Roos and Karel 1991a,b). All samples measured were done in triplicate and their average reported as the T_g.

Determination of Nonenzymatic Browning Rates in the Model Food Systems

Following equilibration of the food model systems to the desired moisture contents, sample vials were hermetically stoppered and incubated at fixed temperatures

(45, 55, 60, 65, 70, 80 and 90C). At fixed time intervals, samples were removed and refrigerated until analysis of the extent of browning was done. Absorbance at 420 nm, which was observed to be a good index to follow the extent of NEB reactions in several foods and model systems, including those containing PVP (Karmas *et al.* 1992), was used to determine the extent of browning. In order to be in the dynamic range of the spectrophotometer's (HP Diode Array UV/VIS 8452A) absorbance, samples were adequately diluted with water. Under our conditions, this range proved to be between 0.02 to 2.

Williams-Landel-Ferry (WLF) Equation

The Williams-Landel-Ferry (WLF) equation relates relaxation times of mechanical properties to the temperature above T_g. This equation was obtained empirically to describe temperature dependence of viscosity of amorphous materials (Williams *et al.* 1955), but the model is not limited to viscosity and it can accommodate other relaxation or rate parameters, such as compliance (Ferry 1080). The temperature dependence of viscosity at temperatures above T_g can be written as follows:

$$\log_{10}\frac{\eta}{\eta_g} = \frac{C_1(T-T_g)}{C_2+(T-T_g)} \tag{1}$$

where:
T: Temperature
T_g: Glass transition temperature
η: Viscosity at T (Pa·s)
η_g: Viscosity at T = T_g (Pa·s)
C_1;C_2: Constants

Williams *et al.* (1955), reported "universal" values for the constants to be -17.44 and 51.6 for C_1 and C_2, respectively, and to apply for many materials. These values were obtained by averaging the coefficients calculated for different synthetic polymers. The same "universal" coefficients were also adequate to describe the temperature dependence of viscosity of sugar solutions (Soesanto and Williams 1981) and time to crystallization of amorphous sugars at temperatures above their T_g (Roos and Karel 1990, 1991a,b). However, the authors of the WLF model advise against the use of these "universal" coefficients (Williams *et al.* 1955), and Ferry (1980) stated that these values can only be used as a last resort, when no reliable data are available from another source.

RESULTS AND DISCUSSION

Previous observations (Karmas *et al.* 1992), indicated that a break in the Arrhenius plot takes place in the vicinity of the glass transition for NEB reactions occurring in model food matrices. Changes in the activation energies (E_a) were also detected close to T_g. The temperature dependence of diffusivity of small molecules in amorphous polymers showed the same behavior (Kovarskii *et al.* 1978; Hori *et al.* 1986). The appearance of a break in the Arrhenius plot for NEB in amorphous model systems may indicate that diffusion is affecting the NEB reaction rate. If the reaction is subjected to diffusional limitations, and the diffusion coefficient is inversely proportional to viscosity, then the break may be due to glass transition and the WLF equation could be applied to describe the dependence of the NEB rate constants on temperature and T_g. As the T_g depends on the moisture content of the material, the equation would integrate both temperature and moisture dependence.

The application of Eq. (1) requires the use of experimental values of the viscosity (η) at T_g, or in its vicinity to obtain η_g. Since viscosity or translational relaxation times are extremely high close to T_g, these values are difficult to obtain experimentally and thus are usually unknown. Therefore, extrapolations are employed and estimated values for η_g are used in Eq. (1) (Soesanto and Williams 1981; Roos and Karel, 1991b). Ferry (1980) noted that better correlations between observed and predicted values for viscosity in polymers at temperatures above T_g were, in many cases, obtained by using a reference temperature different than T_g,and C_1 and C_2 different from the originally proposed constants. Peleg (1992), prepared a critique of the use of the WLF equation by a number of investigators (Slade and Levine 1991; Roos and Karel 1990). His recommendation was to use the WLF equation in its general form, with coefficients calculated for each material. Ferry (1980) proposed the method of reduced variables for the evaluation of the coefficients. The main advantage of this method is that extrapolations are avoided. The procedure qualified by Ferry as being "more objective" was employed as follows:

The WLF equation in its general form, as applied to kinetic reaction rate constants (k), assuming that diffusion is the limiting factor for the reaction to occur and that k is proportional to $1/\eta$, reads

$$\log\frac{k}{k_0} = \frac{-C_1^0 (T-T_0)}{C_2^0 + (T-T_0)} \tag{2}$$

where:
T: Temperature (C, K)
T_0: Reference temperature (arbitrarily selected) (C, K)
k: Browning rate constant at T (1/h)
k_0: Browning rate constant at T_0
C_1^0, C_2^0: Numerical coefficients obtained at $T = T_0$

ERRATA

"Application of the WLF Equation to Describe the Combined Effects of Moisture and Temperature of Nonenzymatic Browning Rates in Food Systems" by Maria Del Pilar Buera and Marcus Karel — Journal of Food Processing and Preservation (1993) *17*, 34–45.

(1) In equations 3, 5 and 9 there should be *no* minus sign before $C°_1$ in the numerator.

(2) The denominator in equation 9 should read:

$$T - T_g + T_{go}$$

and *not*

$$T + T_g + T_{go}.$$

We apologize for the inadvertent transposition of signs. The calculations reported in the paper are correct.

Then C_1^0 and C_2^0 can be evaluated as follows: Defining T_∞ as a fixed temperature at which, regardless of the choice of T_0, the rate constant k_∞ becomes vanishingly small, Eq. (2) can be written:

$$\log \frac{k_\infty}{k_0} = \frac{-C_1^0 (T_\infty - T_0)}{C_2^0 + (T_\infty - T_0)} \tag{3}$$

If k_∞ approaches zero, then the logarithm of k_∞ approaches $(-\infty)$ and:

$$C_2^0 = T_0 - T_\infty \tag{4}$$

when Eq. (4) is combined with Eq. 2 it follows that:

$$\log \frac{k}{k_0} = \frac{-C_1^0 (T - T_0)}{(T - T_\infty)} \tag{5}$$

A plot of $\log_{10}$ versus $(T - T_0)/(T - T_\infty)$ is linear through the origin if T_∞ is chosen correctly. Ferry (1980) suggested trials with different values of T_∞ until curvature is eliminated, starting with the "rule of thumb" that T_∞ is usually 50 K below T_g.

In the model systems used in the present study, straight lines were obtained when absorbance was plotted versus time (plot not shown). Zero order kinetics were assumed and the slopes were taken as reaction constants. The literature NEB data also followed zero order kinetics.

Both literature and experimental data were plotted using Eq. (5), with $T_\infty = T_g - 50K$, and choosing the reference temperature (T_0), within the experimental range. Good linear correlations between $\log(k/k_0)$ and $(T - T_0)/(T - T_\infty)$ were obtained, with correlation coefficients higher than 0.98 for all foods and model systems with $T_\infty \leqslant T_g - 50K$. Figure 1 shows the results for cabbage, carrot, onion and apple. The T_g at zero moisture (T_{g0}), for these vegetables was close to 50C. For potato and dairy products, which had higher values of T_{g0}, it was observed that the number to subtracted from T_g to obtain T_∞ was higher than 50K. On the basis of these observations it was decided to set T_∞ to be equal to $(T_g - T_{g0}]$ and in this way all data points for all food systems could be superimposed on the same line as shown in Fig. 2.

The coefficient C_1^0 was obtained from the slope of the linear correlation between $\log (k/K_0)$ and $(T - T_0)/(T - T_\infty)$ and C_2^0 from Eq. (3). The values obtained for these coefficients are summarized in Table 2. The coefficient C_2^0 is a function of T_∞, and T_∞ depends on T_g. Therefore C_2^0 is a function of moisture content. The average of the value of C_1^0 obtained from all the values for the different foods and model systems also represent the slope of the curve in Fig. 2 and was equal to 7.9. Coefficient C_2^0 however is dependent on the selected reference temperature. Both C_1^0 and C_2^0 can be normalized in order to be com-

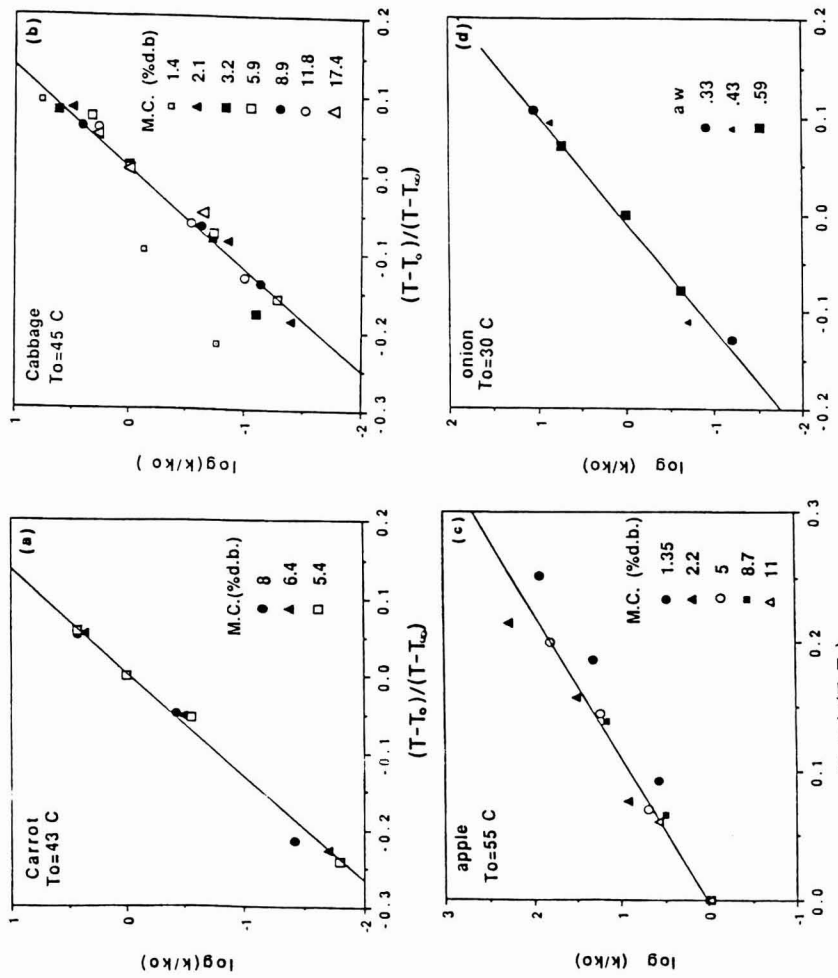

FIG. 1. LINEARIZED WLF PLOT FOR VEGETABLES $T_\infty = T_g - 50C$
(a) Carrot. Data from Legault *et al.* (1951). (b) Cabbage. Data from Mizrahi *et al.* (1970).
(c) Apple. Data from Resnik and Chirife (1979). (d) Onion. Data from Samaniego *et al.* (1990).

pared with the "universal" coefficients. Recently, the same procedure was successfully applied to polystyrene melt viscoelasticity by Lomellini (1992). Once the coefficients were calculated with T_0, the reference temeprature was shifted to T_g and the coefficients were recalculated as follows:

TABLE 2.
COEFFICIENTS c_1^0 AND c_2^0 AND THE CORRESPONDENT c_1^g AND c_2^g CALCULATED BY SHIFTING THE REFERENCE TEMPERATURE FROM T_0 TO T_g. THE REFERENCE TEMPERATURE, T_0, WAS SELECTED IN THE MIDDLE OF THE RANGE OF THE DATA AVAILABLE. T_∞ WAS SET AS EQUAL TO $T_g - T_{g0}$

	T_∞, T_0, T.r.[a]	M.C.[b]	c_1^0	c_2^0(C)	c_1^g	c_2^g(C)
Apple	T_∞=55 C T_0 T.r.:55/83 C $T_\infty = T_g$-50 C	1.3 2.2 5. 8.7 11. 17.	8.8	83 103 112 118 129 143	14.6 18.2 19.7 20.7 22.7 25.1	50
Cabbage	T_∞=45 C T_0 T.r.:30/52 C $T_\infty = T_g$-50 C	1.4 2.1 3.2 5.6 8.9 11.7 17.9	7.8	80 90 94 103 115 121 153	12.5 14.1 14.7 16.1 18.0 18.9 23.9	50
Carrot	T_∞=43 C T_0 T.r.:20/50 C $T_\infty = T_g$-50 C	5.4 6.2 8.0	7.4	98 103 108	14.6 15.3 16.1	50
Milk	T_∞=90 C T_0 T.r.:70/130 $T_\infty = T_g$-100 C	0.[c] 1.2[c] 5.9[c]	8.1	89 125 146	7.2 10.1 11.8	100
		3.[d] 4.[d] 5.[d]	6.8	140 145 150	9.5 9.9 10.2	100
Onion	T_∞=30 C T_0 T.r.:20/40 $T_\infty = T_g$-50 C	5.6 8.9 18.9	8.8	88 100 138	15.9 18.1 24.5	50
Potato	T_∞=50 C T_0 T.r.:40/80 C $T_\infty = T_g$-65 C	4.9 9.4 15. 20.	7.9	85 95 120 130	10.4 11.6 14.6 15.8	65
Whey powder	T_∞=35 C T_0 T.r.:30/40 C $T_\infty = T_g$-100 C	5.9 8.0	8.4	106 117	9.0 9.9	100
M.S.I[e]	T_∞=45 C; T_0 T.r.:45/90 C $T_\infty = T_g$-90 C	5.9	8.3	90	8.3	90
M.S.II[f]	T_∞=55 C T_0 T.r.:45/90 C $T_\infty = T_g$-120 C	7.3	6.9	135	7.8	120

(a) T.r.: temperature range of data employed in the calculations.
(b) M.C.: moisture content in dry basis.
(c), (d) Nonfat milk data from Flink et al., 1974, and Franzen et al., 1990, respectively.
(e) M.S.I: model food system I:PVP10/Glu/Gly (99:0.5:0.5)
(f) M.S.II: model food system II:PVP24/xyl/lys (98:1:1)

The difference between the reference temperature and T_g is defined as:

$$\delta = T_0 - T_g \tag{6}$$

(Ferry 1980; Peleg 1992)

$$C_1^g = \frac{C_1^0 C_2^0}{C_2^0 - \delta} \tag{7}$$

and

$$C_2^g = C_2^0 - \delta \tag{8}$$

where C_1^g and C_2^g are the WLF coefficients when $T_0 = T_g$.

The values of the calculated C_1^g and C_2^g were also included in Table 2. It can be observed that the values for C_1^g varied from 7 to 23; hence they were in the range of the "universal" coefficient values of C_1, but did vary with moisture content. As a consequence, no single "universal" coefficient can be assigned, but it must be calculated for each system. The dependence of C_1^g on the moisture content of each system was almost linear for moisture contents higher than 3% (dry basis) as shown in Fig. 3. The coefficients C_1^g were independent of the selected reference temperature (Fig. 4).

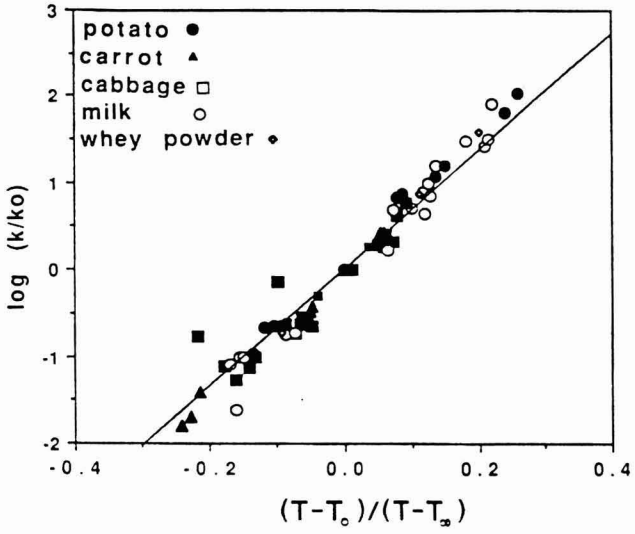

FIG. 2. LINEARIZED WLF PLOT FOR VEGETABLES AND DAIRY PRODUCTS, NORMALIZED BY SETTING $T_\infty = T_g - T_{g0}$

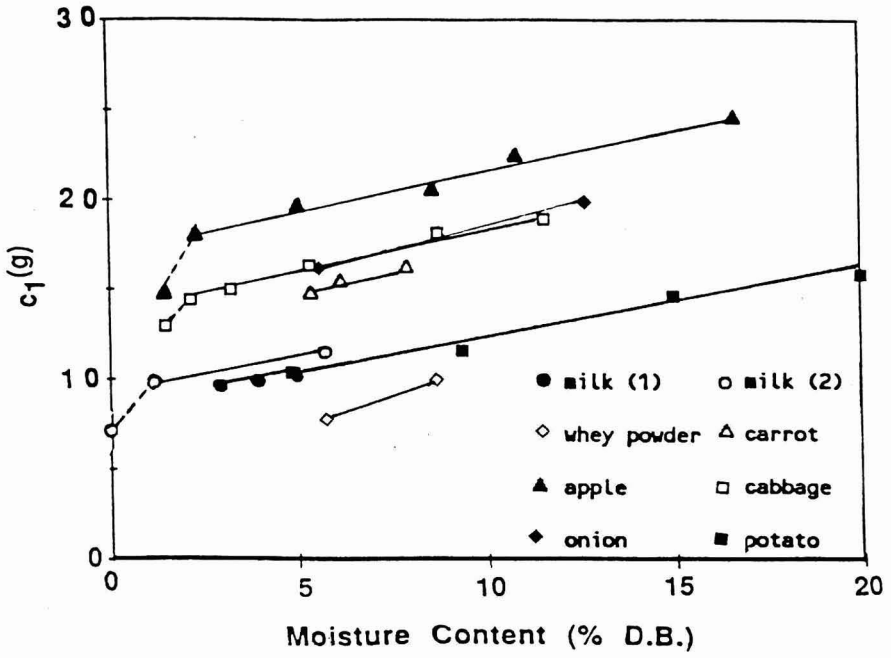

FIG. 3. DEPENDENCE OF c_1^g ON MOISTURE CONTENT FOR SEVERAL VEGETABLES AND DAIRY PRODUCTS $T_\infty = zt_g - T_{g0}$

For the food model systems, C_1^g were obtained from results at a single moisture content and exactly the same values were obtained when reference temperature was changed. Though small variations in C_1^g were observed in food systems in which data was obtained at various moisture contents, the observations oscillated around the same value, as shown in Fig. 4 for vegetables. Coefficients C_2^g, which represent the differential between T_∞ and T_g, was constant for each system and by the definition used in our procedure, corresponded to the glass transition temperature of the system at zero moisture content (T_{g0}).

CONCLUSIONS

(a) An equation based on the WLF equation, adapted for reaction rate constants, can be applied to relate NEB rate constants to the variables temperature, moisture and T_g.

(b) The method of reduced variables proposed by Ferry (1980), proved to be suitable for estimation of the coefficients C_1 and C_2 of the WLF equation. The selection of a reference temperature (T_0) to calculate C_1^0 and C_2^0 within the ex-

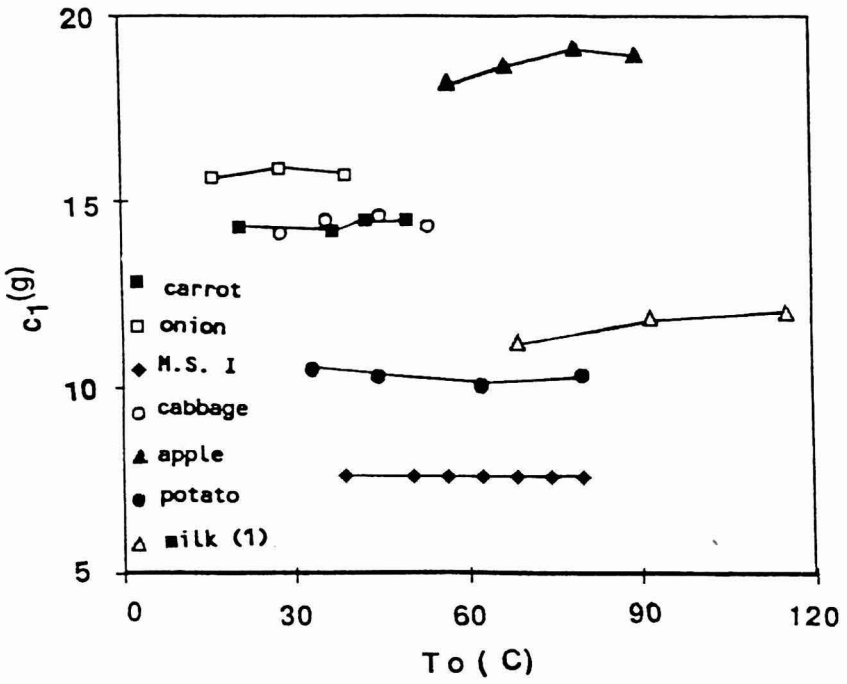

FIG. 4. CHANGE IN THE REFERENCE TEMPERATURE AND THE VALUE OF c_1^g

perimental range, for which the rate constant is known, avoids extrapolations needed when T_g is selected as the reference temperature.

(c) Following the calculation of the coefficients at the reference temperature T_0, values were shifted and recalculated to C_1^g and C_2^g and compared with the corresponding "universal" coefficients. The coefficient C_1^g for all the systems studied, was within the range of the "universal" value of the "universal" C_1. However these values were dependent on the moisture content of the system. A linear relationship was observed between C_1^g and moisture content over a wide range. The coefficient C_1^g was found to be independent of the selected reference temperature.

(d) The coefficient C_2^g was related to the glass transition temperature at "zero" moisture content, which was between 45 and 124C, depending on the food or model system. When the T_∞ was fixed at the value $(T_g - T_{g0})$, which corresponds to $C_2^g = T_{g0}$, the points for the different foods and model systems fell on the same line in the plot of log (k/k_0) vs $(T - T_0)/(T - T_\infty)$.

(e) The use of the WLF equation allowed to combine the effects of m, and T in a single relation. However an independent experimental determination of T_g is required to normalize the temperature–moisture effects.

(f) A general equation applicable to several foods and model systems is:

$$\log k = \log k_0 + \frac{-C_1^0 (T - T_0)}{T + T_g + T_{g0}} \tag{9}$$

T_0 was chosen in the temperature range in which reaction rates were available. For these systems C_1^0 averaged 7.9. This value can be used as first approximation and a better fit is obtained when the value of C_1^0 is determined for each particular system. Caution should be exercised when applying the model to evaluate the system in a range of temperatures too wide from the experimental values used to calculate the coefficients (Ferry 1980).

(g) The equation has predictive value. The main value it has over other proposed equations that combine effects of temperature and moisture, is that the coefficients can be estimated easily, providing the T_gs are known and kinetic data at several temperatures are available. The dependence of the NEB rate constants on moisture content is implied by the inclusion of T_g in the equation.

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MICROBIAL INACTIVATION OF FOODS BY PULSED ELECTRIC FIELDS

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ABSTRACT

Pulses of high voltage electric fields (PEF) are potentially a most important cold pasteurization/sterilization food preservation technique to replace or partially substitute for thermal processes. During the PEF process, lysis of microorganisms is caused by irreversible structural changes in the membranes, leading to pore formation and destruction of the semipermeable barrier of the membrane. Theories explaining electroporation of the cell membrane and applications of the nonthermal PEF process are reviewed in this paper.

INTRODUCTION

In the past decade a variety of papers have dealt with the effects of electric pulses of high voltage field strength on cells, especially on microorganisms. Under an external electric field of short duration (μs), inactivation of microorganisms occur, and potentially results in an alternative technique to thermal sterilization or pasteurization of foods.

In a suspension of cells, an electric field causes a potential difference across the membrane and induces a sharp increase in membrane conductivity and permeability. Membrane destruction occurs when the induced membrane potential exceeds a critical value of 1 Volt in many cellular systems, corresponding to an external field of roughly 10 kV/cm for *E. coli* (Sale and Hamilton 1967).

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The increase in membrane permeability and cytoplasm conductivity, termed "dielectric breakdown" by Zimmermann *et al.* (1980) is explained by the creation of pores due to local electromechanical instability of cell membranes. Sale and Hamilton (1967, 1968) studied the effects of applying intense (25 kV/cm) electric pulses from microseconds to milliseconds and concluded that ". . . the application of excessive potential difference across the membrane may lead to irreversible changes in the ordered structure to give rise to the observed forms of membrane breakdown." "In microorganisms, the structural change due to a potential difference of about 1 V gives rise to irreversible loss of membranes' function as the semipermeable barrier between the cell and its environment" (Sale and Hamilton (1967, 1968).

Promising preliminary results on the inactivation of microorganisms in a pulsed electric field has promoted an increasing interest to develop a nonthermal process for the pasteurization or commercial sterilization of foods. One of the advantages of using pulsed electric field technology over thermal processing methods to achieve commercial sterility in foods is the low quantity of heat produced.

As a result of the small temperature increase during processing, the food retains its "fresh" physical, chemical, and nutritional characteristics and possesses a satisfactory ambient shelf-life. This paper reviews the developing theories that explain the effects of high voltage electric fields on microorganisms, and the application of pulsed high voltage electric fields in food processing.

High Voltage Electric Fields and Microorganisms

Sale and Hamilton (1967) reported that electric pulses of high voltage electric fields up to 25kV inactivate *Escherichia coli, Staphylococcus aureus, Micrococcus lysodeikticus, Sarcina lutea, Bacillus subtilis, B. cereus, B. megatherium, Clostridium welchii*, a pseudomonad, *Saccharomyces cerevisiae*, and *Candida utilis*. Electrical fields up to 25 kV/cm were applied as a series of direct current pulses from 2 to 20 μs to suspensions of microorganisms. The inactivation of microorganisms was a result of the electrical pulses, not due to the chemical products of electrolysis or increase in temperature. The maximum temperature rise of 10C occurred during 10 pulses of 20 μs at 19.5 kV. Inactivation of the microbial population was dependent on the electric field strength and the total time of treatment. The sensitivity of microorganisms was variable depending on electric field strength and the total time of the treatment, and was also dependent on the concentration and nature of cells treated, yeasts being more sensitive than vegetative bacteria (Fig. 1).

The mechanisms by which high voltage electrical field strength inactivate vegetative bacterial cells in suspension was studied by Hamilton and Sale (1967). They described membrane damage as the lysis of protoplasts, the leakage of in-

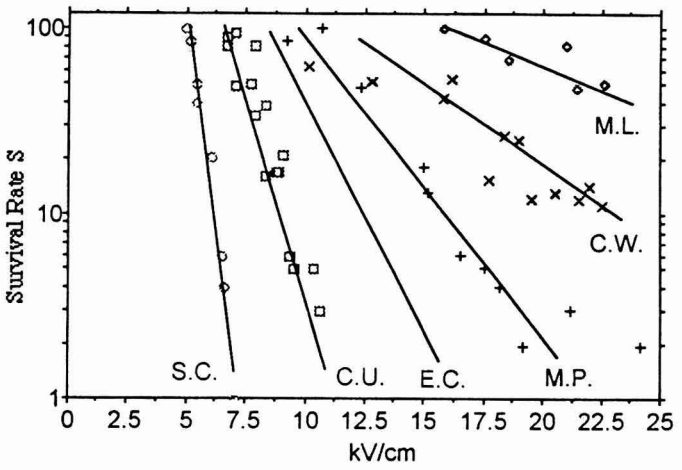

FIG. 1. RELATIONSHIP BETWEEN DEGREE OF MICROBIAL INACTIVATION AND ELECTRIC FIELD STRENGTH
Ten pulses of 20 μs.S.C., *Saccharomyces cerevisiae*; C.U., *Candida utilis*; E.C., *Escherichia coli*; M.P., motile pseudomonad; C.W., *Clostridium welchii*; M.L., *Micrococcus lysodeikticus* (Sale and Hamilton 1967).

tracellular contents, the loss of ability of *E. coli* to plasmolyze in the hypertonic medium (20 mM phosphate buffer, pH 7.2 + 10% sucrose) and the loss of β-galactosidase activity in a permease-negative mutant of *E. coli*.

Membrane damage is the direct cause of cell inactivity. There is a similarity between the number of cells inactivated by a given treatment and the number of cells capable of forming spheroblasts (Hamilton and Sale 1967). Suspensions of *Staphylococcus aureus* were first subjected to direct current pulses of 25 kV/cm of field strength, and subsequently treated with the cell wall dissolving enzyme lysostaphin in a hypertonic medium. The direct relationship between the effects of the pulse treatment on cell inactivity and membrane damage, as measured by poor to no spheroblast formation, demonstrates that cell inactivation is a result of membrane damage (Table 1).

Bacteria and yeasts in liquids are inactivated by pulsed high voltage electric fields applied to the liquid. Using high voltage electric fields of up to 25 kV/cm for short times (μs), inactivation rates of more than 99.9% were demonstrated (Sale and Hamilton 1967; Hülsheger and Niemann 1980; Hülsheger *et al.* 1981, 1983; Jacob *et al.* 1981; Mizuno and Hori 1988).

The effects of the pulsed electric fields on membranes of viable cells are defined by the potential theory (Schwan 1977, cited by Hülsheger *et al.* 1983). The potential theory implies that induced transmembrane potential depends upon cell size (Dao-Sheng *et al.* 1990).

TABLE 1.
Staphylococcus aureus ACTIVITY AFTER PULSED ELECTRIC FIELD TREATMENT (Hamilton and Sale 1967)

Electrical field kV/cm	Survivors (%)	Protoplasts not lysed (%)
0.00	100.0	100.0
9.25	100.0	100.0
14.25	35.0	43.0
19.50	0.9	16.0
24.00	0.3	3.0
27.50	0.6	1.5

For spherical cells surrounded by nonconducting membranes, the induced potential is given by the equation:

$$V_m = f\ a\ E_c \tag{1}$$

Where V_m = membrane potential induced by the external field strength E_c; a = cell radius; and f = form factor for spherical shape. Zimmermann *et al.* (1974) derived a mathematical equation to calculate the membrane potential V_m for nonspherical cells.

The equation is based on the assumption that the cell shape consists of a cylinder with two hemispheres at each end. The form factor f for rod-shaped microorganisms is given by:

$$f = L/(1 - 0.33\ d) \tag{2}$$

with **L** = length of particle and d = diameter. Substitution of the f factor in Eq. (1) allows an approximate calculation of the induced potential for rod-shaped bacteria. Table 2 presents the resulting V_m values calculated by Eq. (1). A critical membrane potential of about 1 V is necessary for inactivation of bacteria in the stationary growth phase. When the transmembrane potential reaches a critical value of 1 V, the potential creates pores in the membrane which remain open after the removal of the applied potential (Kinosita and Tsong 1977a,b). A relatively high potential of more than 2 V is obtained for the critical membrane potential of *Candida albicans* with a large mean cell diameter of 4 μm. Hülsheger *et al.* (1983) reported that bacteria and yeast do not exhibit the same membrane characteristics when comparing cell inactivation in electric fields. Provided that the field induced

membrane processes are responsible for cell inactivation, yeast cell membranes exhibit greater stability than bacterial cell membranes. Hülsheger *et al.* (1983) results are contrary to the results of Sale and Hamilton (1967) that concluded yeast were more sensitive to electric fields than vegetative bacteria. The contradictory results are explained by the electric field strength (25 kV/cm) applied by Sale and Hamilton (1967) and the electric field strength (20 kV/cm) applied by Hülsheger *et al.* (1983). The pulse treatment of the yeast leads to greater inactivation when a large number of pulses (30) or high voltage (25 kV/cm) are applied.

Allen and Soike (1966) reported that *Saccharomyces cerevisiae* were more resistant to electrohydraulic treatment than *E. coli*, spores of *Bacillus subtilis*, and bacteriophage T2. Electrohydraulic treatment is a process in which high voltage electricity is discharged across an electrode gap beneath the surface of a liquid medium. Cell inactivation is governed primarily by the field strength and discharge time. Gilliland and Speck (1967a) reported inactivation of *Escherichia coli, Streptococcus faecalis,* vegetative cells and spores of *Bacillus subtilis*, and *S. cremoris* bacteriophage suspended in an aqueous media. The inactivation was accomplished by a electrohydraulic treatment of 8–15 kV, 6–24 μf capacitance, across an electrode gap of 0.125 in. The addition of 0.05% Bovine Serum Albumin in distilled water to the microbial suspension resulted in reduced inactivation. Hülsheger and Niemann (1980) and Hülsheger *et al.* (1981) demonstrated that electrolytically produced chlorine acts as an additional toxic agent when chloride is present in the treated medium. They recommended avoidance of chloride compounds for electrolytic treatment solutions.

Divalent cations such as Ca^{2+} and Mg^{2+} reportedly play a role in the integrity of bacterial cell membranes (Asbell and Eagon 1966, cited by Hülsheger *et al.*

TABLE 2.
CELL SIZE AND INDUCED MEMBRANE POTENTIAL OF STUDIED MICROORGANISMS: d = mean diameter, l = mean length, V = mean volume, f = shape factor, V_m = membrane potential induced by an external field E_c under the assumption of a parallel long particle axis and field vector (Hülsheger et al. 1983)

	d (μm)	l (μm)	V (μm^3)	f	V_m (V)
E. coli (4 h)	1.15	6.9	7.2	1.06	0.26
E. coli (30 h)	0.88	2.2	1.4	1.15	1.06
K. pseudomonia	0.83	3.2	1.7	1.09	1.26
P. aeruginosa	0.73	3.9	1.6	1.07	1.25
S. aureus	1.03	-	0.6	1.50	1.00
L. monocytogenes	0.76	1.7	0.8	1.70	0.99
C. albicans	4.15	-	38.0	1.50	2.63

1981). Ca^{2+} and Mg^{2+} in the treated suspensions induce a protective mechanism against electrical treatments in bacteria cell membranes (Hülsheger *et al.* 1981).

The results of the study of Gilliland and Speck (1967b) on inactivation of microorganisms reported that electrohydraulic treatment accomplished by discharging high-voltage electricity of 10 kV, 6 and 24 μf capacitance across an electrode gap of 0.125 in. produced oxidation reactions that inactivated lactic dehydrogenase, trypsin, and proteinases of *Bacillus subtilis*.

Enzymes which contain or require free sulfhydryl groups such as reduced glutathione were most susceptible to electrohydraulic inactivation. Free sulfhydryl groups and reduced nicotinamide adenine dinucleotide (NAD) were oxidized during electrohydraulic treatment (Gilliland and Speck 1967b). Hamilton and Sale (1967) studied NADH dehydrogenase (EC I.6.99.3) as a measure of the activity of the electron transport system in extracts from pulsed electric field treated and untreated *E. coli* 8196. Succinic dehydrogenase (EC I.3.99.I) and hexokinase (EC 2.7.II) activities were also assayed. Although the pulsed electric field treatments were sufficient to inactivate more than 90% of the cell population, no significant inhibition of individual enzyme activity was achieved.

Hamilton and Sale (1967) using transmission electron microscopy observed unimpaired membranes in erythrocytes and *E. coli* after treatment with 10 pulses, each of 20 μs of a high voltage electric field up to 25 kV/cm. Destruction of the characteristic bilayer structure of the membranes was not observed. However, electron micrographs of erythrocytes showed that partial breakdown occurred at particular areas of membranes. Stable pores on the order of the 1–3 nm occupying 0.01–0.1% of the membrane were created by PEF (Tsong 1990). No perceptible damage to the cell wall was observed when bacterial cells were examined with a light microscope after electrohydraulic treatment (Allen and Soike 1966). No significant amount of cell breakage was observed by phase microscope, nor was cell damage observed in cell stain preparations (Gilliland and Speck 1967b) in suspensions of *E. coli, Streptococcus faecalis, B. subtilis* spores or vegetative cells subjected to electrohydraulic treatment (discharge voltage, 10 kV; capacitance, 24 μf; electrode gap, 0.125 in.). *Saccharomyces cerevisiae* cells treated with 2400 pulses of 15 kV, a capacitance of 0.01 μf and a frequency of 4 Hz were observed with a scanning electron microscope. The treated yeast cells appeared to be like deflated balloons compared to untreated yeast cells, perhaps the result of leakage of the cell contents after damage to the cell walls or membranes by the applied electrical fields (Sato *et al.* 1988).

Sytnik and Sytnik (1976), cited by Palaniappan *et al.* (1990), studied the effect of electrohydraulic shock of 40 kV after 50 pulses on *Candida utilis, C. guilliermondii* and *S. cerevisiae*, and observed remnants of inactive cells. Cell inactivation increased in proportion to the number of electrical discharges. Yeast and Gram-positive bacteria were less sensitive to electrical pulse treatment than Gram-

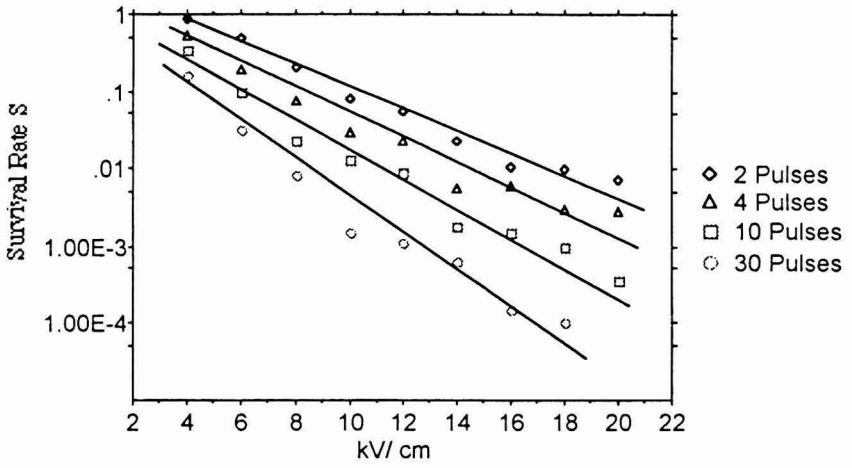

FIG. 2A. SURVIVAL RATE FOR *E. COLI* IN THE LOGARITHMIC GROWTH PHASE, 4 H CULTURE
(Hülsheger *et al.* 1983)

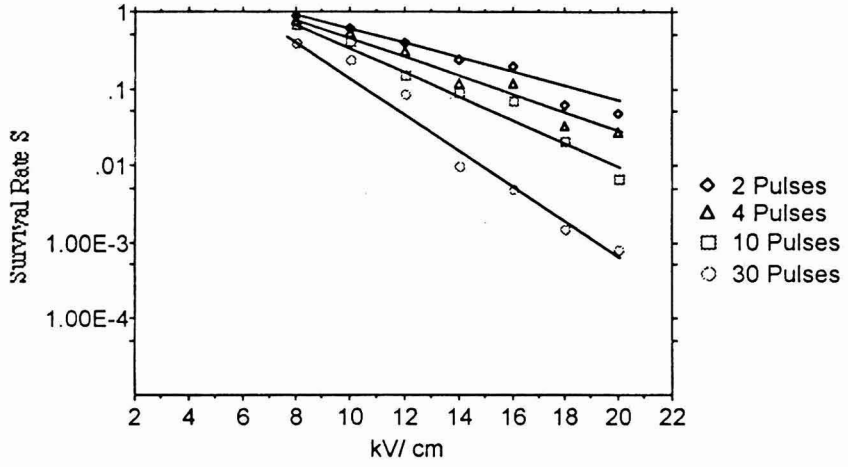

FIG.2B. SURVIVAL RATE FOR *E. COLI* IN THE STATIONARY GROWTH PHASE, 30 H CULTURE
(Hülsheger *et al.* 1983)

negative bacteria when 2–4 pulses of 36 μs were applied to the following organisms by Hülsheger *et al.* (1983): *E. coli* K12, *Klebsiella pneumoniae* ATCC 27736, *Pseudomonas aeruginosa* Pa-103, *S. aureus*, ATCC 25923, *Listeria monocytogenes* I (serovar 1/2a) 85 H, *Listeria monocytogenes II* (serovar 1/2a) 47 J

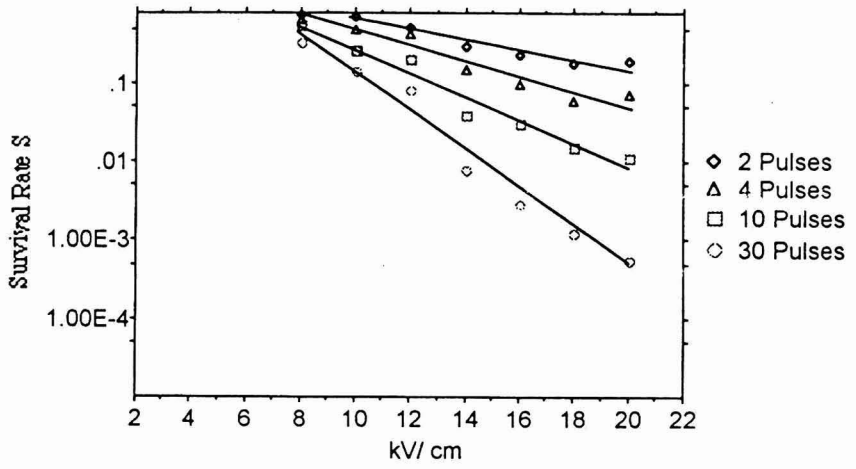

FIG. 3. SURVIVAL RATES FOR *PSEUDOMONAS AERUGINOSA* FROM THE STATIONARY GROWTH PHASE, 30 H CULTURE
(Hülsheger *et al.* 1983)

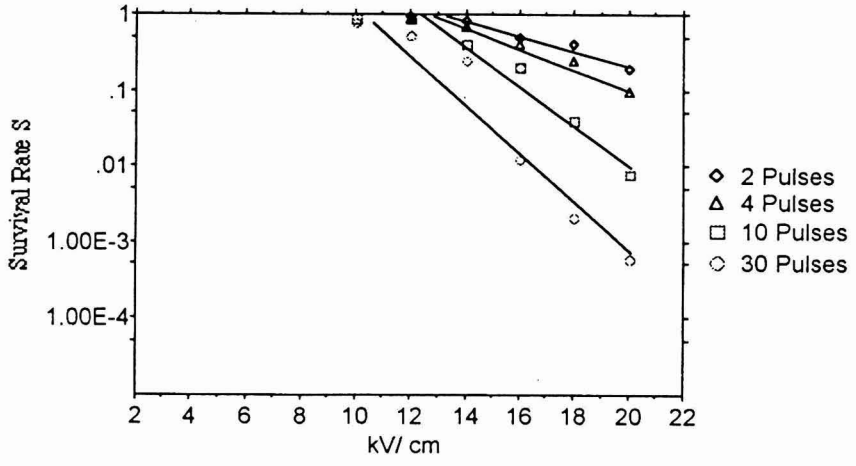

FIG. 4. SURVIVAL RATES FOR *STAPHYLOCOCCUS AUREUS* FROM THE STATIONARY GROWTH PHASE, 30 H CULTURE
(Hülsheger *et al.* 1983)

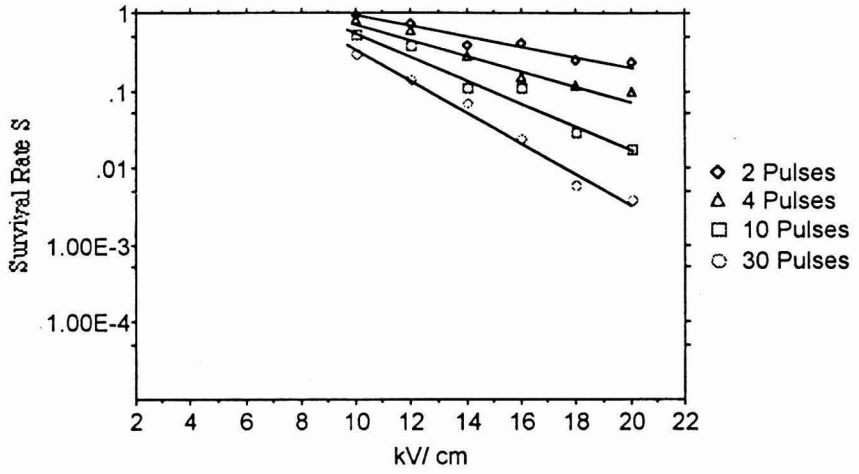

FIG. 5. SURVIVAL RATES FOR *LISTERIA MONOCYTOGENES* I (SMOOTH FORM) FROM THE STATIONARY GROWTH PHASE, 16 H CULTURE
(Hülsheger *et al.* 1983)

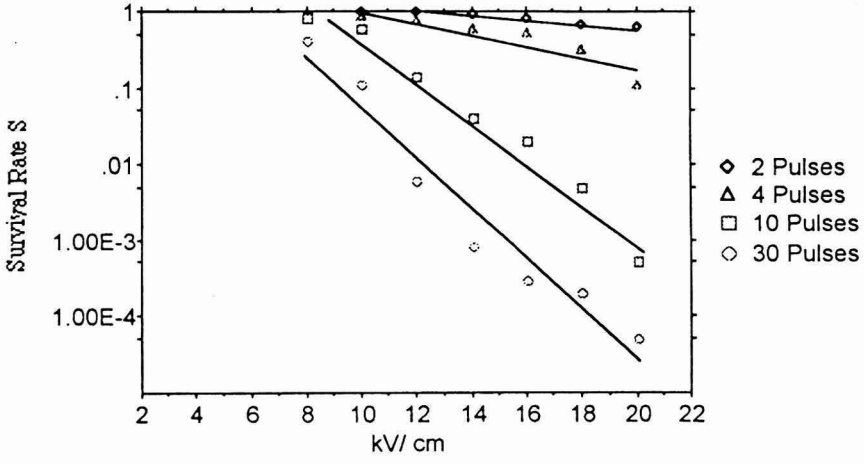

FIG. 6. SURVIVAL RATES FOR *CANDIDA ALBICANS* FROM THE STATIONARY GROWTH PHASE, 30 H CULTURE
(Hülsheger *et al.* 1983)

and *Candida albicans* 2648. The results obtained with selected microorganisms are presented in Fig. 2 through 6. The kinetics of the survival rates is dependent on both the electric field strength and the number of pulses. The field strength was varied in steps of 2 kV/cm with 2–30 pulses over 36 μs pulse time. The treatment time is the product of the pulse duration and the number of pulses. Microorganisms in a logarithmic growth phase are inactivated at a markedly greater rate than microorganisms harvested from a stationary growth phase (Jacob *et al.* 1981; Hülsheger *et al.* 1983). Microbial inactivation by pulsed electric fields is primarily governed by the intrinsic properties of the microbial cells such as electrical resistance, and membrane potential of the microorganisms (Zimmermann *et al.* 1976; Kinosita and Tsong 1979a,b).

The inactivation of yeast cells from logarithmic or stationary growth phases by 1 to 8 electric field pulses of 35 kV/cm for 20 μs each was investigated by Jacob *et al.* (1981). Cells of *Saccharomyces cerevisiae* in the logarithmic growth phase were more sensitive to pulsed electric fields than cells in the stationary phase. The growth region incorporating separation of mother and daughter yeast cells during budding, were especially sensitive to pulsed electric fields.

Insignificance of Temperature Increase

Sale and Hamilton (1967) reported that the cooling water system supplied during the treatment by the high voltage treatment apparatus reduced heating of the suspension, Sale and Hamilton (1967) reported a maximum temperature rise of 10C when *E. coli* was exposed to 10 pulses of 20 μs each at 4.9 to 18.5 kV/cm electric field strengths (Table 3).

Neumann and Rosenheck (1972) and Neumann (1989) reported that electric fields on the order of 20 kV/cm and a pulse time of 120 μs were discharged to membranes of vesicles storing biogenic amines. The electric field was established instantaneously. The dissipation of the electric energy occurs in picoseconds; thus, no local overheating occurred. Pulsed electric field application to ionic solutions causes a temperature increase, mainly due to Joule heating (electrical energy dissipated as heat in a resistor). The differential increase dT of the temperature is given by:

$$d\mathrm{T} = \mathrm{U}^2 d\mathrm{t}/\mathrm{R}c_p p\mathrm{V} \qquad (3)$$

where U is the applied voltage, dt is the pulse duration, R is the resistance, V is the volume of the chamber, c_p is the specific heat of the aqueous solution (c_p = 4.18 J/gK), and p is the specific mass of the solution (p = 1 g/cm^3). Neumann and Rosenheck (1972) reported a temperature increase of 6C in an experiment having the following conditions: 20 kV/cm, 0.05 μf, 3,030 ohms, specific heat

TABLE 3.

ELECTRIC FIELD STRENGTH, TEMPERATURE RISE, AND INACTIVATION OF *E.coli* (Sale and Hamilton 1967)

Electric field (kV/cm)	Temperature rise (C)	Survivors (%)
4.9	0	100
10.5	5	1
14.5	5	8
18.5	10	<1

Treatment conditions: 10 pulses of 20 μs at room temperature. Conductivity: 3.2 millimho.

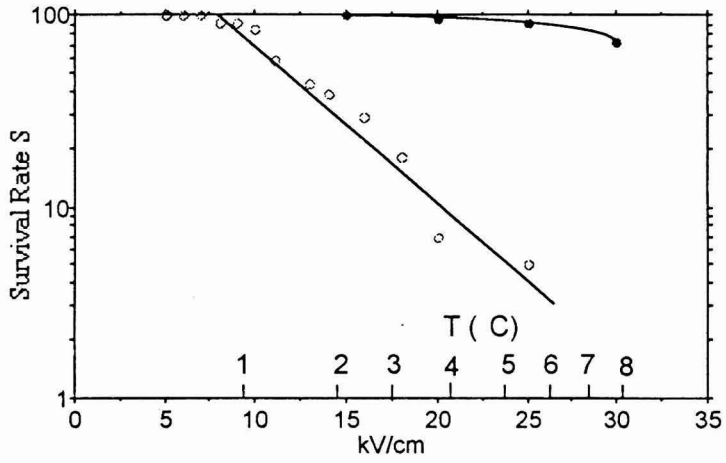

FIG. 7. INACTIVATION OF *SACCHAROMYCES CEREVISIAE* BY PULSED ELECTRIC FIELDS OF DIFFERENT STRENGTH E_O
Open circles: logarithmic growth phase; closed circles: stationary growth phase. The corresponding temperature changes during the application of 25 kV/cm are indicated (Jacob *et al.* 1981)

1 cal/g C, and a density of 1.05 g/cm^3. The initial temperature of the solution was 0C.

Hülsheger *et al.* (1981) reported an increase of 10C after pulsed electric field treatment of *E. coli* with the following parameters: 10 pulses of 30 μs, 12 kV/cm, capacitance 1 μf, repetition rate one pulse per 5 s, and a bacterial concentration of 10^8 cells/ml in NaCl, 17.1 mM/L. Jacob *et al.* (1981) reported an 8C increase in temperature after applying 1–8 pulses of 20 μs of 30 kV/cm to *Saccharomyces cerevisiae* (Fig. 7). Zimmermann *et al.* (1974) concluded that the thermal effect has little role in *E. coli* inactivation during high voltage electric field treatment of 2 and 7 kV. The resistance of the microbial suspension was 10 ohms and the electrical decay constant was 27 μs. A temperature increase of 3–5C was reported.

Although the heat generated during the high voltage electric pulse treatment is not significant, the temperature during the processing is very important. The critical membrane breakdown potential decreases towards high temperatures of the solution, normally by a factor of two between 3 and 25C (Coster and Zimmermann 1975; Kinosita and Tsong 1977a,b; Hofmann 1986; Zimmermann 1986). The breakdown voltage is about 1.2 V at 4C, and about 0.6 V at 30C.

Tsong (1990) reported that the repair of perforated membranes after pulsed electric field treatment also depends on temperature. At 4C, repair is slow and takes hours or days. At 37C, repair is complete within minutes to hours. Cell membrane breakdown usually occurs at the lipid-protein junction or at areas of large portion concentrations (Zimmermann *et al.* 1980).

PULSED ELECTRIC FIELDS AND CELL MEMBRANES

Several publications appeared in the 1950s and 1960s demonstrating that an externally applied electric field can induce a large membrane potential at two poles within a cell (Cole 1968). Sale and Hamilton (1967) reported that an excessively high voltage electric field, such as 25 kV/cm, causes cell lysis. By the early 1970s, *dielectric breakdown* of cell membranes was related to the induced membrane potential critical value of approximately 1 V for bimolecular lipid membranes, experimental models of biological membranes, and for *E. coli*, human and bovine red blood cells (Crowley 1973; Zimmerman *et al.* 1974). Electrical modification of the cell membrane permeability is reversible (Cole 1968). Coster (1975) called the reversible electrical modification of cell membrane "*reversible electrical punch through.*" By late 1970, the concept of membrane *pore* formation or membrane destabilization was a recognized result of dielectric breakdown of the cell membrane (Kinosita and Tsong 1977a,b).

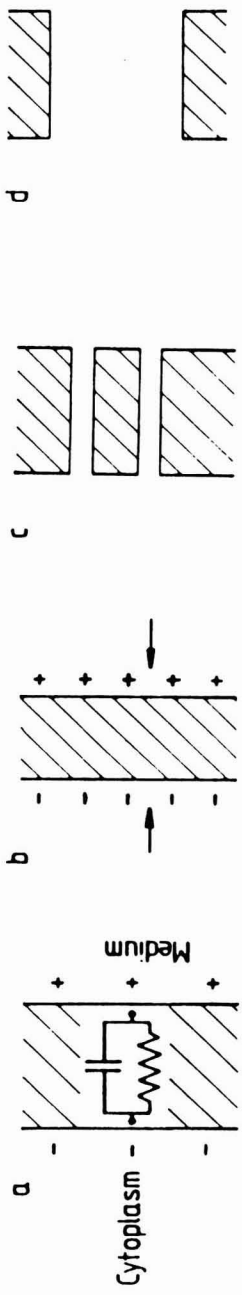

FIG. 8. DIAGRAM OF REVERSIBLE AND IRREVERSIBLE ELECTRIC BREAKDOWN
(a) Cell membrane with a potential V'_m, (b) membrane compression, (c) pore formation, reversible breakdown, (d) large pores are formed, irreversible breakdown (From Zimmermann 1986)

Dielectric Breakdown

Strong polarization of viable cells by an external electric field leads to an increase in cell membrane conductance and permeability (Coster 1965). According to Zimmermann *et al.* (1973), when a cell membrane is exposed to an electric field pulse intensity of about 1–10 kV/cm with a duration of 20 nanoseconds to 10 milliseconds, reversible electric breakdown of the cell membrane is observed. When the electric field is switched off, the membrane will return from a conducting state to the initial and normal state. If duration of the electric field pulse is longer than 10–15 milliseconds, irreversible damage to the membrane cells occurs (Zimmermann *et al.* 1976). Electric fields of 1–10 kV/cm correspond to a membrane potential of 1 V (Zimmerman *et al.* 1976).

Dielectric breakdown of the cell membrane is observed when cell suspensions are subjected to external field pulses of 2–20 kV/cm for short time intervals, nanoseconds to milliseconds. When a membrane potential of about 1 V is reached in response to an electric external field, the membrane breaks down. The breakdown of the membrane is associated with a reversible permeability increase of the cell membrane. The extent of permeability depends on the strength and duration of the electric field pulse (Zimmermann *et al.* 1980). The electric breakdown of the cell membrane is schematically depicted in Fig. 8.

There are a number of references dealing with dielectric breakdown (Crowley 1973; Zimmermann *et al.* 1974; Coster and Zimmermann 1975; Benz *et al.* 1979; Sugar and Neumann 1984). Dielectric breakdown leads to localized instabilities in the membrane by way of electromechanical compression and electrical field induced tension, forming pores, as illustrated in Fig. 8 (Zimmermann *et al.* 1974; Coster and Zimmermann 1975; Benz *et al.* 1979; Benz and Zimmermann 1980; Zimmermann 1986).

Membranes can be regarded as a capacitor. The bilayer structure of a cell membrane is a dielectric material with small dielectric constant (K) on the order of 2, compared to the dielectric constant of 80 for water. As a result, free charges can be accumulated at both membrane surfaces, but cannot exist in high concentration in the membranes, at least not within the membrane hydrocarbon layer. The application of an electric field pulse leads to an increase in the transmembrane potential. The generated charges at the two membrane surfaces are opposite and attract each other. This attraction gives rise to a compression which acts toward the membrane surface and reduces the membrane thickness. The distance between the opposing charges on the two membrane surfaces is thus reduced, and the compression is correspondingly increased because the electric forces increase with reduced separation. An elastic or viscoelastic restoring force opposes the electrocompression of the membrane thickness. Since the electric compressive forces increase more rapidly with decreasing membrane thickness

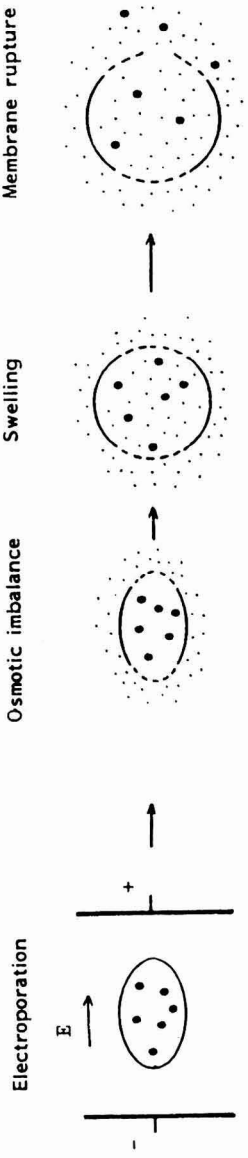

FIG. 9. ELECTROPORATION OF THE CELL MEMBRANE
(From Tsong 1991)

than elastic restoring compression forces, a local breakdown of membranes occurs at an external electrical field strength of 2–20 kV/cm (Zimmermann 1981).

Electroporation

Electroporation is the phenomenon in which a cell exposed to high voltage electric field pulses temporarily destabilizes the lipid bilayer and proteins of cell membranes (Chang *et al.* 1992). Application of high voltage electric field pulses to suspensions of cells induces formation of pores in the cell membranes (Tsong 1983; Dimitrov 1984; Sowers and Lieber 1986; Neumann 1989; Sowers 1989; Dimitrov and Sowers 1990). As a result of the formation of pores, the normal permeation barrier of the cell membrane is partially or completely impaired (Neumann 1989).

Kinosita and Tsong (1977a, 1979) demonstrated that an electric field of 2.2 kV/cm induced pores in human erythrocytes. The pores were approximately 1 nm in diameter. Kinosita and Tsong (1977a) suggested a two step mechanism for pore formation: the initial perforation is a response to a suprathreshold electric potential followed by time dependent expansion of the pore size. Large pores are obtained either by using a higher field intensity, by increasing the pulse duration, or by reducing the ionic strength of the pulsation medium. The process of pore formation is relatively insensitive to temperature in contrast to the marked temperature dependence of the repair process (Kinosita and Tsong 1977b). Pulsation in 150 mM NaCl solution yields human erythrocyte membranes of greater permeability than pulsation in a 272 mM sucrose solution. Inclusion of sucrose in media retarded hemolysis, suggesting less membrane permeation to sucrose than to NaCl. When the suspension media contains both sucrose and NaCl, the equilibrium partitioning of the NaCl across the cell membrane is stabilized, resulting in the shrinkage of the cell. Gradual entry of the sucrose reexpands the cells, Fig. 9, leading to the cell lysis. The change in cell volume was monitored by measurement of light scattering (Kinosita and Tsong 1977a; Tsong 1991).

In a cell membrane, protein channels, pores, and pumps are present (Vassilev and Tien 1985). The opening and closing of many channels constituted by proteins is dependent on transmembrane electric potential (Tsong 1990). The gating potential of the channel constituted by proteins are in the 50 mV range. The gating potential is smaller than the dielectric strength of a lipid bilayer. Thus, when a pulsed electric field is applied, many voltage sensitive protein channels will open before the transmembrane electric potential reaches the breakdown potential of the lipid bilayer, i.e., 150–500 mV. Protein channels once open may experience current much larger than the channels are designed to conduct. As a result, protein channels may be irreversibly denatured by Joule heating or electric modification of functional groups (Tsong 1991).

Lipid bilayers are susceptible to applied electric fields due to the electric charges of the lipid molecule and the permeability of the bilayer to ions. Electric charges will cause lipid molecules to reorient under intense electric field strength, thus, creating hydrophilic pores and impairing the bilayer barrier against ions. Hydrophilic pores conduct current, thus, generating a local Joule heating and inducing thermal phase transitions of the lipid bilayer (Tsong 1991). Thus, electroporation in a cell membrane can occur both in protein channels and lipid bilayers.

PULSED ELECTRIC FIELDS AND FOOD PROCESSING

Electro-Pure Process

The Electro-Pure process was one of the first attempts to use electrical fields to inactivate microorganisms. The electrical field was small, 220 V alternating electric current and was not pulsed. Microbial inactivation occurred as a result of ohmic heating. Fetterman (1928) reported the use of electrical conductivity known as the Electro-Pure Process to increase the temperature of milk to 160F in 15 s (Getchell 1935). After the electric treatment, the milk was cooled to 34F. The Electro-Pure Process inactivated *Tubercle bacilli* and *E. coli* (Fetterman 1928), provided uniform generation of heat and adequately pasteurized milk. The Electro-Pure Process was claimed to be an economical, fast, simple, flexible milk pasteurization system (Getchell 1935). The electrical pasteurizing equipment consists of a vertical, rectangular chamber, two sides of carbon and two sides of glass. The milk is pumped upward through the chamber as the conductor of electric current between the two carbon plates producing rapid heating (Moses 1938). Tracy (1932) reported that passing 120 volt, alternating current through *Saccharomyces ellipsoideus* cell suspensions in grape juice at nonlethal temperatures of 108F inactivated 96% of the viable yeast cells, indicating that an alternating current of 60 Hz for 60 s exerted a lethal action to the yeast cells independent of temperature. Grape juice treated electrically for 30 min was not lacking in nutrient value. The gases evolved from electrolysis were not toxic to yeast cells. Moses (1938) reported that the Electro-Pure Process was under investigation by health departments in New York, Pennsylvania, Connecticut, Illinois, Michigan, and Maryland. Moses (1938) estimated that at least 200 million quarts of electrically pasteurized milk was consumed without detrimental effects upon the health of consumers. However, the Electro-Pure Process fell into disfavor. Hall and Trout (1968) reported that the process was not used in the dairy industry by the 1960s. It is not clear why processors stopped using the Electro-Pure Process (Palaniappan *et al.* 1990).

TABLE 4.
FOOD PROCESSED BY PULSED ELECTRIC FIELDS

Reference	Treat-ment	Dose	Food	Micro-organism	Treatment results			Treat. temp C	Store temp. C
					Time after Treat. h	Control cfu/ml	Treated cfu/ml		
Dunn and Pearlman 1987	High Voltage Electric Field	35 pulses 33.6-33.7 kV/cm 5.6-9 10^3 A	Orange juice	Naturally occurring yeast, mold and bacteria	0 142.5 199 241.5	1.3×10^7	5 5 32 1.8×10^4	42-65	9 +/- 2
		23 pulses 28.5-43 kV 3.7-8 10^3 A	Milk	*Escherichia coli* ATCC 10536	0 19 45 95 140	8.1×10^6 7.6×10^6 9.1×10^6 4.7×10^7 6.2×10^6	7.4×10^3 5.8×10^3 3.6×10^3 3.1×10^3 $1.4 \times 10^{5*}$	17-43	7-9
		40 pulses 36.7 kV 6 -11 10^3 A	Milk	*Salmonella dublin*	0 24 72 144 192	3.8×10^3S** 4.6×10^3S+B 1.2×10^6S+B 2.7×10^7B 10^7 B	0 S 20 B 6 B*** 1×10^2 B 1×10^2 B 4×10^2 B	63	7- 9
			Milk	*Salmonella dublin*	0 3 22 46	4.1×10^3 5.4×10^3 4.6×10^2 2.6×10^5	1.4×10^3 1.4×10^3 1.0×10^3 9.6×10^2	40	4
			Milk	*Salmonella dublin*	0 3 22 46	5.2×10^3 1.8×10^2 9.8×10^7 TNTC	1.4×10^3 2.7×10^3 1.2×10^4 TNTC	40	19-22

			Yogurt	*L. bulgaricus* (L) *S. cerevisiae* (Y)	0 48 92 143 191****	47 Y 650 L 77 Y 650 L 68 Y 650 L 82 Y 3.9×10^4 Y	0.1 Y 23 L 0.1 Y 5 L 0.1 Y 6 L 0.1 Y - 40 Y -	23-28	4
Sitzmann 1990	High Voltage Electric Field	20 pulses 20 kV/cm	Milk	*L. brevis*					
		19 kV/cm	Orange juice	*S. cerevisiae* *L. brevis*					
Bach 1975	UHF Electro-magnetic Field	1. 13.56 or 27.12 mHz 2. 915 or 2450 mHz. 200 V/cm	Yogurt	Psychrotrophs, molds and yeast					
		13.56 and 915 mHz	Beer	Yeast Sarcina Lactic acid bacteria		$6x10^3$ - $3x10^4$ $4x10^3$ - $2x10^4$ $2x10^4$ - $1x10^5$	0 0 0	<100	30
Fetterman 1928	Electro-Pure Process	220 V 15 kW	Milk	*Tubercle bacilli* *E.coli*				65-71	
Getchell 1935	Electro-Pure Process		Milk	*E.coli* Others		$1x10^3$ - $2x10^6$ $2x10^3$ - $5x10^5$	3.6×10^2 3×10^3	71 (15 s)	
Tracy 1932	Electro-Pure Process	20 V 0.31 A	Grape juice	*Saccharomyces ellipsoideus*		1.5×10^6		42	

* This count reprents a mixed population of *E. coli* and milk bacteria. ** S :*Salmonella dublin* *** B: Milk bacteria.. **** Room temperature.
TNTC to numerous to count.

ELSTERIL

ELSTERIL is a new process developed by Krupp Maschinentechnik GmbH, Hamburg, Germany, for inactivating microorganisms in fluid media (Sitzmann 1990; Grahl *et al.* 1992). In the ELSTERIL process, a high-voltage generator charging capacitor of 5 microfarads with 5–15 kV is discharged through a treatment chamber. The duration of the electric pulse is characterized by a decay time constant (t). The treatment chamber is composed of two carbon electrodes of 50 cm^2 area with a electrode gap of 5 or 12 mm. The treated fluid material is exposed to the high voltage pulses in the electrode gap (Sitzmann 1990; Grahl *et al.* 1992; Mertens and Knorr 1992).

Lactobacillus brevis suspended in milk was treated with 20 pulses of 20 μs of electric current with 20 kV/cm, (Fig. 10, Table 4), *Saccharomyces cerevisiae* suspended in orange juice was treated with 5 pulses of 20 μs duration at 4.7 kV/cm; and *Escherichia coli* suspended in sodium alginate was treated with 5 pulses of 20 μs duration at 14 kV/cm. The viable cell counts of the microorganisms were reduced by more than four log cycles (Sitzmann 1990; Grahl *et al.* 1992). The inactivation of endospores of *Clostridium tyrobutyricum* by ESTERIL was negligible. The inactivation of endospores of *Bacillus cerus* and ascospores of *B. nivea* were not observed (Grahl *et al.* 1992; Grahl 1992).

The energy used for the inactivation of *S. cerevisiae* cells was much smaller than energy used to inactivate *L. brevis*. Grahl *et al.* (1992) attributed the necessary energy difference to the larger diameter of yeast cells and the low pH (3.3) of orange juice.

Vitamin C (ascorbic acid) concentrations in milk were decreased by 90%, and lipase in milk was substantially inactivated with the pulsed electric field treatment. Sensory evaluations of milk and orange juice indicated that the taste did not deteriorate significantly during the pulsed electric field treatment (Grahl *et al.* 1992; Grahl 1992).

Elcrack

Elcrack (Krupp Maschinentechnik GmbH, Hamburg, Germany) is a system that industrially applies the dielectric breakdown concept of cell membranes. In the Elcrack system, a slurry of comminuted fish or slaughterhouse offal is pumped through one or more electrical chambers and treated with high voltage electric pulses. Cell membranes are disrupted allowing the fat to escape from the animal cell giving a rise to efficient fat recovery during a mechanical separation step. Using pulses of high voltage electric fields, the detrimental effects, such as, protein denaturation and degradation of vitamins associated with thermal processes are avoided (Mertens and Knorr 1992).

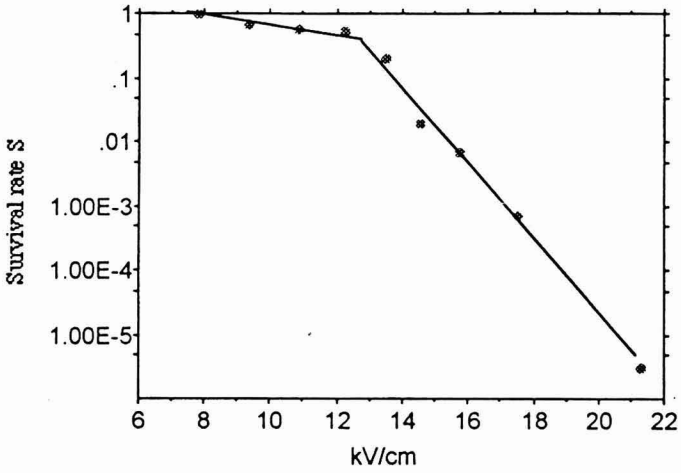

FIG. 10. RELATIONSHIP BETWEEN SURVIVING RATE OF *LACTOBACILLUS BREVIS* IN MILK AND 20 PULSES OF DIFFERENT ELECTRICAL FIELD STRENGTHS
(Sitzmann 1990)

Maxwell Patent

Dunn and Pearlman (1987) assigned patents to Maxwell Laboratories, Inc., San Diego, CA, describing pulsed electric field technology and equipment to preserve fluid foods such as dairy products, fruit juices, and fluid egg products. In the patented method, the foodstuff is treated with high voltage electrical field pulses between 12 and 25 kV/cm for 1 to 100 μs. The electrically treated food is maintained in a sterile package during and after the pulsed electric field treatment. By treating the food products with a pulsed electric field at pasteurization temperatures in the range of 63 to 75C obtained by heating the foodstuff with in line heat transfer, shelf-life extensions are greater than with pasteurization alone. Prompt cooling of the thermally and electrically pasteurized food in a refrigeration system to a temperature of 4C further improves the shelf-life.

The apparatus proposed by Dunn and Pearlman (1987) is a 2 cm $\times$ 10 cm chamber with two parallel stainless steel electrodes. The treated fluid is passed through a hole in one of the electrodes to completely fill the chamber. The electric pulsing apparatus comprises a high voltage power supply, two 400 kilohm resistors, two 50 megohm resistors, a bank of six 0.4 microfarads capacitors connected in parallel, a spark gap switch and a dump relay.

The Maxwell patent explained the microbial inactivation achieved by pulsed high electrical field treatment as dielectric breakdown of the cell membrane (Sale

and Hamilton 1968; Zimmermann *et al.* 1976a; Hülsheger *et al.* 1980, 1981, 1983; Jacob *et al.* 1981; Kinosita and Tsong 1977a,b).

Dunn and Pearlman (1987) treated orange juice, milk, yogurt and fluid eggs with pulsed high voltage electrical fields (Table 4). The orange juice contained naturally occurring microbial contaminants such as yeasts, molds and bacteria. The orange juice was treated with 35 pulses of 100 μs and an intensity of 33.6–35.7 kV. The temperature during treatment was 42–65C. Inactivation of the naturally occurring microorganisms was observed over five logs on the first day after treatment. The shelf-life was extended about one week beyond the normal three day shelf-life, and taste and odor of the orange juice was acceptable. Untreated orange juice exhibited an unacceptable taste and odor after four days.

Homogenized and pasteurized milk was inoculated with a concentration of 8.1×10^6 cell/ml *E. coli* prior to the pulsed electric field treatment. Milk was treated with 23 100-μs pulses of 28.6–42.8 kV. The temperature during treatment was not reported. The percentage inactivation of *E. coli* immediately after treatment was 99.1%. Milk inoculated with 3.8×10^4 cell/ml *Salmonella dublin* was treated with 40 100-μs pulses of 36.7 kV electric field. The temperature during treatment was 63C. Milk treated with pulsed electric fields was chilled and stored at 7–9C. After 192 h the bacteriological count was 400 milk bacteria per milliliter. Treated milk contained 10^7 milk bacteria per milliliter and zero *S. dublin*. The results suggested that the *S. dublin* were preferentially inactivated over the milk bacteria.

Pulsed electric field treatment was applied to a yogurt containing 6.5×10^8 cells of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* per milliliter or 4.7×10^4 cells of *Saccharomyces cerevisiae* per milliliter. The temperature during the electrical treatment reached 63C. The yogurt, after being treated with 20 100-μs pulses of 23–38 kV was cooled and stored at 4C. The lactic acid bacteria count of yogurt after the treatment was 6×10^6 cells/ml for untreated yogurt. After 92 h, the *S. cerevisiae* count from treated yogurt was reduced to 100 cells/ml compared to 6.8×10^4 cells/ml for untreated yogurt.

Bach Process

Bach (1975) patented an invention concerning a pasteurization process of uniform warming designed for preservation of semi-solid dairy products and other foodstuffs, including beverages and high fat nondairy products. Pasteurization is achieved by the use of ultra high frequency (UHF), 2,450 GHz, and high frequency (HF), 27,120 MHz, electromagnetic fields. The Bach method treats food and package together in one operation without using high temperatures. The food is heated as a result of the applied energies of the UHF and HF fields. The length of heating time necessary is reduced as the electromagnetic fields reach parts of

the food uniformly in a fraction of a second. The treatment requires a maximum of 90 s. The Bach process represents an attractive technology for prolonging the shelf-life of semi-solid dairy products (Anon. 1980).

CONCLUSIONS AND FUTURE WORK

Inactivation of microbial cells in liquids was effectively performed by pulsed high voltage electric field treatments. Pulsed high voltage electric fields produce a series of degradative changes in blood, algae, bacteria and yeast cells. The changes include electroporation and disruption of semipermeable membranes, leading to cell swelling and/or shrinking, and finally to the lysis of the cell. Bacteria, yeast and mold counts suspended in milk, yogurt, orange juice, and fluid eggs were reduced five log cycles by pulsed electric field treatment. Lipase from milk was also inactivated. Spores were not inactived by PEF. The effects of pulsed electric fields on the physical, chemical, microbial, enzyme activity and nutritional characteristics of foods needs to be better understood before food products are safely processed with pulsed high voltage electric fields (PEF).

It is expected, due to the nature of the microbial inactivation, that the energy input will not be too great and PEF will potentially be an energy efficient food process. To extrapolate laboratory research to industrial scale, it is important to address specific problems: (1) Synchronization of the food flow rate and the number and duration of the electric field pulses; (2) availability of power supply units and capacitor banks to warrant the feasibility of the process; and (3) minimizing the induced heat by refrigeration of the food and the electric discharging unit.

SYMBOLS

V_m = Membrane potential, volts
E_c = External field strength, volts/cm
a = Radius, μm
f = Shape factor
d = Diameter, μm
dT = Differential increase in temperature, C
V = Applied voltage, volts
dt = Pulse duration, μs
R = Resistance, ohms
V = Volume of the chamber, ml
c_p = Specific heat of the liquid dispersion, cal/g C
p = Specific mass of the solution, g/cm^3

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