

DIELECTRIC SPECTROSCOPY: A RAPID METHOD FOR THE DETERMINATION OF SOLVENT BIOCOMPATIBILITY DURING BIOTRANSFORMATIONS

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Dielectric spectroscopy provides a convenient means of determining the degree of intactness of biological cells. 4-terminal dielectric measurements of suspensions of *Saccharomyces cerevisiae* at 0.4 MHz show that, as with all other biological cells, these organisms possess a substantial β -dispersion. The additional of octanol to such suspensions causes a rapid decrease in the electrical capacitance of the suspension, which parallels the cellular viability as determined by methylene blue staining. The kinetics of cell death are determined in part by the rate of dissolution of the organic solvent in the aqueous phase. The toxicity of several organic solvents to *S. cerevisiae* is studied using this technique, and is found to be dependent upon the polarity of the solvent. The present method provides a simple and rapid means for assessing the biocompatibility of solvents used in biotransformations.

KEY WORDS Organic solvents, biotransformations, bioconversions, toxicity, dielectric spectroscopy.

INTRODUCTION

A pin struck at random into Beilstein would pierce a water-insoluble compound. Thus for rapid and efficient biotransformations of the overwhelming majority of xenobiotics, it is necessary to add organic solvents or cosolvents to the reaction mixture of interest (e.g. Butler, 1979; Lilly, 1982; Brink and Tramper, 1985; Fukui and Tanaka, 1985; Laane, Boeren and Vos 1985; Lilly and Woodley, 1985; Luisi, 1985; Luisi & Laane, 1986; Laane, 1987; Aldercreutz and Mattiasson, 1987; Halling, 1987; Deetz and Rozzell, 1988; Luisi *et al.*, 1988). Similarly, because of the toxicity of certain fermentation products to the cells which produce them, there is a continuing interest in the use of extractive solvents or reagents in biotechnology (e.g. Playne and Smith, 1983; Mattiasson and Larsson, 1985). Finally, there is a substantial interest in the ability of organic solvents to act as a matrix of low water activity for the performance of bioconversions in a direction opposite to that in which they would go in aqueous media (Zaks & Klibanov, 1985; Morihara, 1987; Semenov *et al.*, 1987).

The major problem with the use of organic solvents during bioconversions is that many organic solvents can act as protein denaturants (e.g. Tanford, 1970;

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Schellman, 1978) and are cytotoxic (Tanford, 1980). The toxicity, to a variety of cells, of medium- and long-chain alcohols has been widely studied, mainly because of their prevalence in the environment and (in some cases) as drugs of abuse (see Browning, 1965; Sax and Lewis, 1987 and references therein). Of course the toxicity of any given solvent depends on the cell type and physiological state, so that, whilst some general truths are emerging (e.g. Brink and Tramper, 1985; Laane, 1985), it is not possible in any given case to design or choose the best organic (co)solvent for a bioconversion of interest. Thus it would be particularly desirable to devise a means by which one might rapidly screen a range of such solvents for their biocompatibility.

The chief site of cytotoxic action of organic solvents is the cytoplasmic membrane of cells (Tanford, 1980), due to the hydrophobicity or amphipathicity of such molecules and their ability to partition into, and to dissolve, such membranes (Seeman, 1972). Thus a screen based on the assessment of membrane damage is indicated.

Dielectric spectroscopy is a technique which, at the appropriate frequencies, provides a signal that is relatively specific for the intactness of biological membranes (Grant, Sheppard and South, 1978; Pethig, 1979; Harris & Kell, 1985; Kell & Harris, 1985a,b; Kell 1987a,b; Pethig & Kell, 1987; Kell, 1988). This is because the so-called β -dispersion (Schwan 1957), which occurs at low radio-frequencies, is caused overwhelmingly by the charging of the cell membrane capacitance and may be observed as the macroscopic capacitance (at such frequencies) of cell suspensions. Destroying the integrity of cellular membranes, e.g. with detergents (Asami, Hanai and Koizumi, 1977), should thus lead to a decrease in capacitance which will thus reflect the ability of any such added molecule to destroy the intactness of cellular membranes and thereby to be cytotoxic. Since dielectric spectroscopy is a perfectly non-invasive technique (Kell 1987a,b, Harris *et al.* 1987), it occurred to us that it might prove suitable for devising a rapid, convenient and on-line assay for the biocompatibility or otherwise of solvents of interest in bioconversions.

In the present article we show, using *Saccharomyces cerevisiae* as a model organism of some interest in biocatalysis (e.g. Vanmiddlesworth and Sih, 1987) and of course in fermentation technology generally (Trivedi and Jacobson, 1986), (a) that dielectric spectroscopy does indeed provide a convenient means for monitoring the rate and extent of cell non-viability induced by organic solvents; (b) that the dielectric permittivity of a given cell suspension is well correlated with the viability of the suspension as judged by the methylene blue staining technique; and (c) that the toxicity of organic solvents to biological cells depends strongly on the dielectric permittivity (polarity) of such solvents.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from the Sigma Chemical Company (Poole, Dorset) or BDH Chemicals (Poole, Dorset) and were of analytical grade. All water used was doubly-distilled in an all-glass apparatus.

Preparation of Cells

S. cerevisiae was obtained locally in the form of a cake. Cell suspensions were prepared by dispersing the yeast cake (approx 75 g) in buffer (450 ml containing 180 mM sorbitol, 20 mM HEPES, 10 mM KCl, pH = 7.5 with 3 M NaOH). The cells were centrifuged for 10 minutes at $2000 \times g$, washed four times in the same buffer and resuspended as a stock solution by homogenising the washed-cell pellet with 50 ml of the same medium. The conductivity and permittivity of cells so prepared changed by less than 10% in periods as long as 24 hours, indicating that negligible "natural" or significant leakage of cell constituents was occurring.

Cell Viability

This was determined using methylene blue, according to the method of Gurr (1965; see also Jones, 1987). Three stock solutions were prepared: (1) methylene blue (0.23 g) in distilled water (20 ml); (2) KH_2PO_4 (2.722 g) in distilled water (100 ml); (3) Na_2HPO_4 (0.284 g) in distilled water (10 ml). A working solution was prepared by mixing 2 ml of (1), 99.7 ml of (2) and 0.25 ml of (3). At least 0.02 ml yeast suspension was mixed with 0.8 ml suspension medium plus 0.08 ml of methylene blue, and put on a microscope slide and examined at a magnification of 1000. Using this method, "dead" cells appear blue.

Dielectric Measurements

All dielectric measurements were made using a 4-terminal dielectric spectrometer, the BUGMETER, obtained from Aber Instruments, Aberystwyth Science Park, Cefn Llan, ABERYSTWYTH SY23 3AH, Dyfed, U.K. The operation of this instrument has been described by Harris *et al.* (1987), and for present purposes it is only worth stressing that the use of a 4-terminal instrument means that artefacts due to electrode polarisation are avoided (see Schwan and Ferris, 1968, Kell 1987b). The electrode used had a standard 25 mm o.d. fermentation-type probe geometry and was constructed of an epoxy resin through which passed 4 parallel stainless steel electrodes. The cell constant of this electrode arrangement was obtained using a solution of known specific conductivity (10 mM KCl) and the following equations:

$$\sigma' = G(d/A) \quad (1)$$

and

$$\epsilon' = C(d/A\epsilon_0) \quad (2)$$

where ϵ_0 is the permittivity of free space with a numerical value equal to $8.854 \cdot 10^{-12}$ F/m; σ' and ϵ' are respectively the conductivity and permittivity. The factor (d/A) is the cell constant and has dimension length^{-1} . In the present arrangement, the cell constant took the value $0.78(5) \text{ cm}^{-1}$. The electrode was mounted horizontally in the side of a plastic beaker in which all measurements were made, and baseline scans of capacitance *vs* frequency indicated that such electrodes were essentially non-polarisable under the conditions used.

All dielectric measurements were carried out at room temperature ($\approx 20^\circ\text{C}$) using a frequency of 0.4 MHz and a time constant of 1s. Typically, cells were

added from the stock suspension to 220 ml of the same buffer with which they had been washed, until the capacitance measured had attained a value of 25 pF. This required approximately 25 ml stock suspension. The reaction mixtures were stirred vigorously (500 rpm) using a magnetic follower. Small volumes of the solvent of interest were added, and the capacitance and conductance of the suspension followed. Samples were taken at periodic intervals for the estimation of cell viability.

RESULTS AND DISCUSSION

To ensure that the measured capacitance was linear with the cell concentration for a given set of conditions, a calibration curve was obtained (Figure 1). It may be seen (Figure 1) that the linearity is essentially perfect in the region from 0 to 55 pF considered here.

Figure 2A shows the time-dependent capacitance and conductance of a yeast cell suspension prepared as described above, before and after the addition of octanol (1 ml). Octanol was added in two ways. In the first method (curve (b)) octanol (1 ml) was injected straight into the stirred cell suspension. Curve (b) shows that there was an initial increase in the capacitance of the suspension. This was due to the fact that organic solvents will intercalate into the membrane structure, increasing its volume (Seeman, 1972) and thus (Schwan, 1957; Kell & Harris, 1985a,b; Harris *et al.*, 1987) the permittivity observed at frequencies

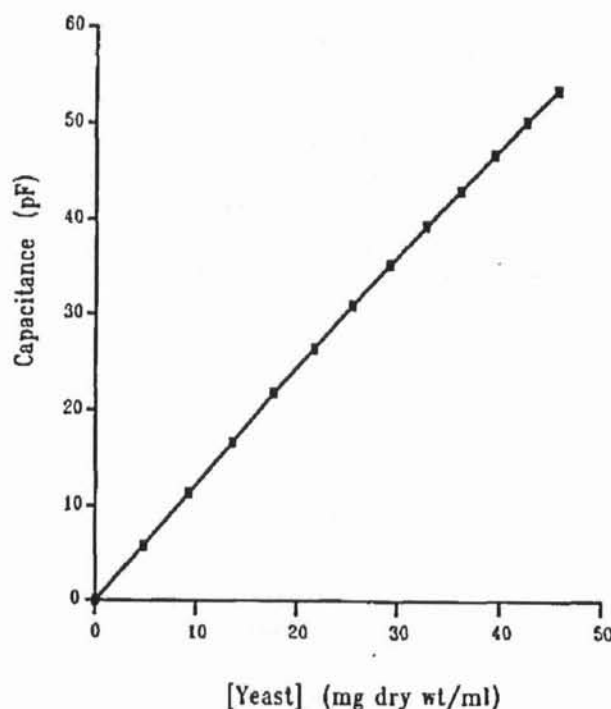


Figure 1 Relationship between the capacitance of a suspension of *S. cerevisiae* and the concentration of cells. Cells were prepared and measurements were performed using the Bugmeter as described in the text, at a frequency of 0.4 MHz. The suspending medium contained 180 mM sorbitol, 20 mM HEPES, 10 mM KCl, pH = 7.5 with 3 M NaOH and the conductivity of the medium was ca. 1.6 mS/cm.

"low" with respect to the characteristic frequency of the β -dispersion. This initial rise in the capacitance of the suspension was followed by a rapid decrease of the capacitance and a concomitant increase of the conductance of the cell suspension with time. These changes correlated closely with the measured cell viability (Figure 2B and see also Figure 3). In particular, the cell viability as judged by the methylene blue technique is well correlated with the decreased values of

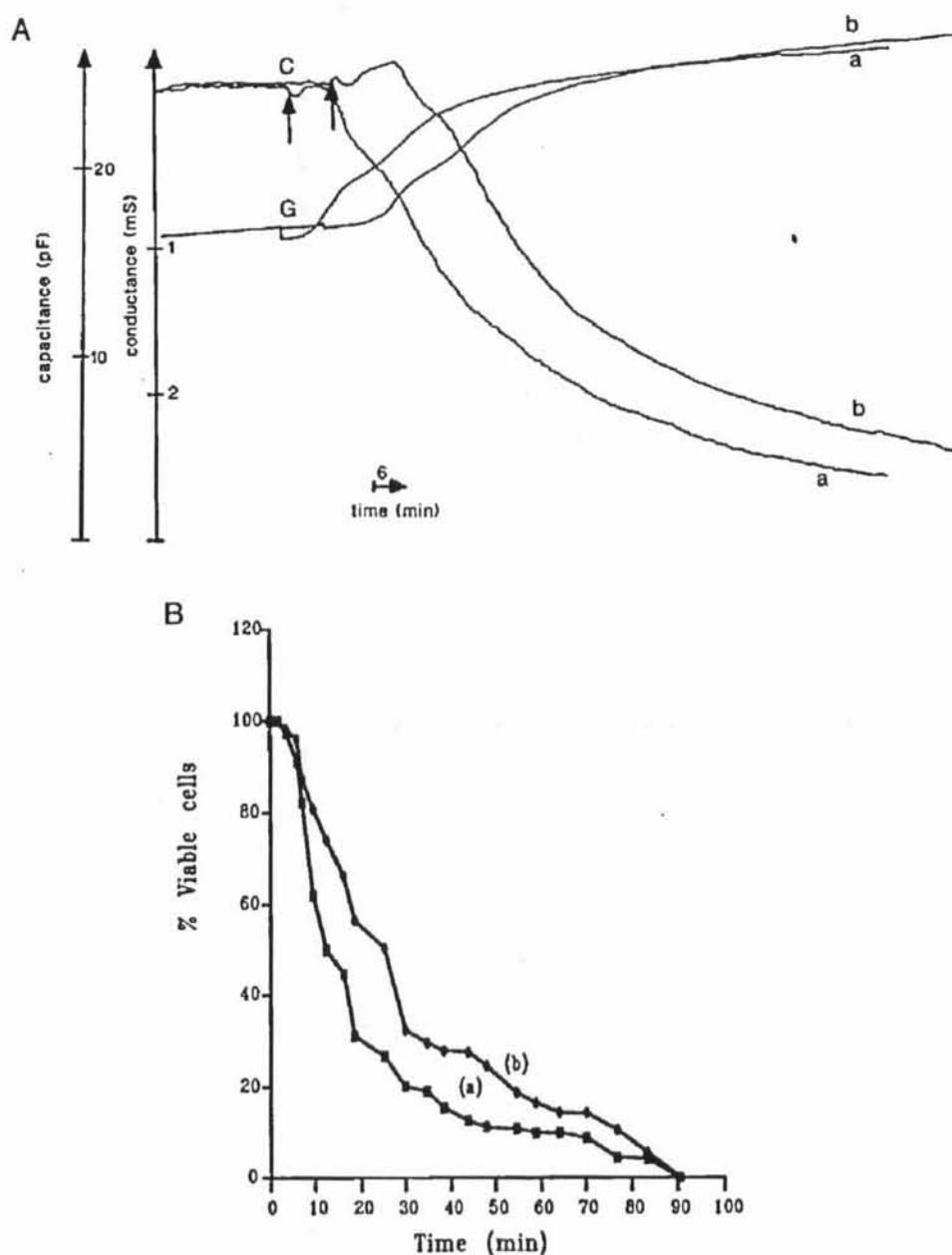


Figure 2 Effect of octanol on the dielectric properties and viability of suspensions of *S. cerevisiae*. Measurements were carried out as described in Materials and Methods and in the text. A. Dielectric properties. At the arrow, 1 ml octanol was added to the suspension, either together with 4 ml ethanol (a) or alone (b), and the capacitance (C) and conductance (G) recorded. B. Viability. Cell viability was determined by taking samples from the experiment in A at the times indicated, time zero being taken as the time at which the octanol was added.

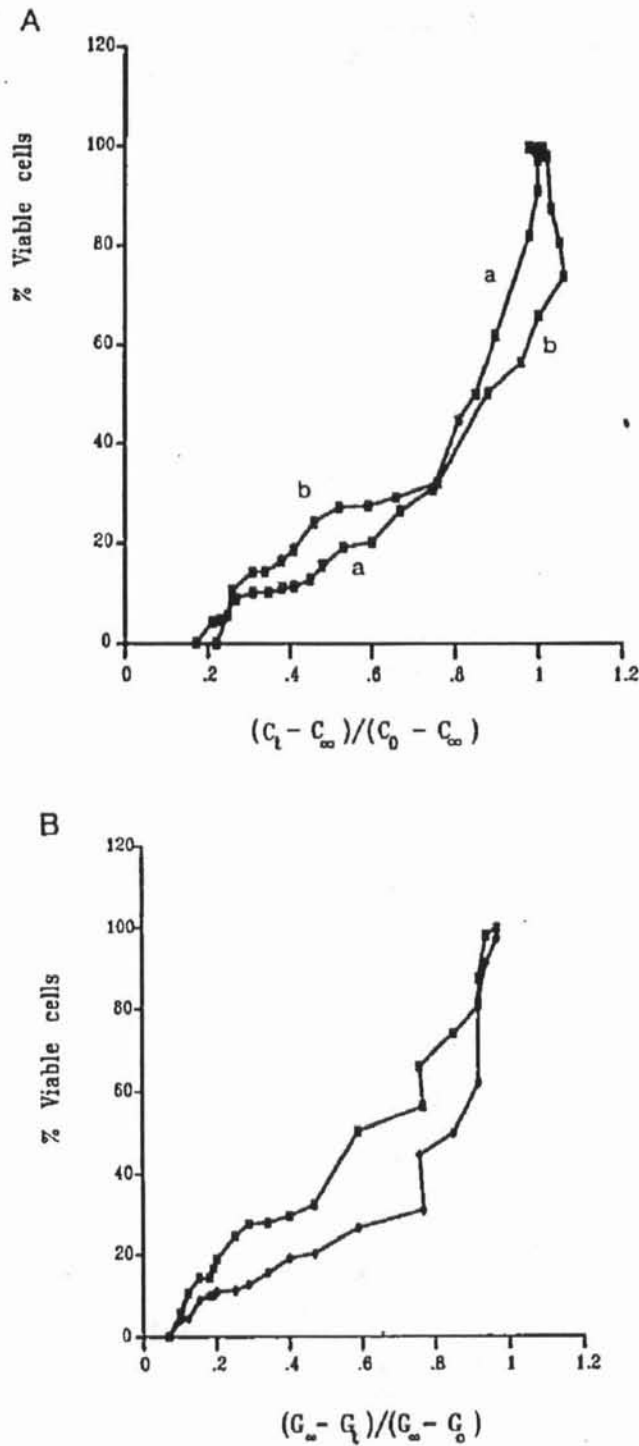


Figure 3 Relationship between the dielectric properties of a suspension of *S. cerevisiae* and its viability. The data are taken from the experiment displayed in Figure 2. Curves (a) and (b) have the same meanings as in Figure 2. A. Normalised capacitance versus cell viability. B. Normalised conductance versus cell viability.

capacitance. It may be observed (Figure 2A) that octanol is extremely toxic to *S. cerevisiae*, in that 25 min following its addition to the cell suspension, 50% of the cells yeast were non-viable and after 95 min all cells had been killed. Thus the measurement of the dielectric behaviour of cell suspensions provides a rapid, convenient and accurate measurement of the viability of a cell suspension.

To establish the degree to which the kinetics of cell death were controlled by the rate at which octanol could dissolve in the aqueous phase and thence pass into the cellular membrane, octanol:ethanol mixtures were also used (control experiments showing that ethanol alone at the concentrations used was without effect). Qualitatively similar results were obtained (Figure 2A, curve (a)) when octanol (1 ml) mixed with ethanol (4 ml) was added to a cell suspension similar to that represented by curve (b). A small difference between the two curves was observed in that the rate at which octanol exerted its toxic action was stimulated by ethanol; after 25 min 73% of the cells were dead, and after 77 min all the cells were dead.

Figure 4 shows the dependence of cell viability on the concentration of octanol at different times following its addition to a cell suspension of the type displayed in Figure 2. It may be seen from the figure that whilst amounts of octanol of 1 ml or greater are rather toxic, lower amounts (less than 1 ml) do not greatly influence the cell viability. This indicates that a certain ("threshold") amount of octanol *per cell* is necessary to cause an irreversible breakdown of the barrier function of the cytoplasmic membrane.

Figure 5 shows the relative toxicities to *S. cerevisiae* of 2 concentrations of seven different hydrophobic organic compounds, as judged by the same means as described above. Whilst all measurements were carried out on-line, the data displayed are those obtained 30 min after the solvents were added. Of these solvents, the most toxic was octanol, in that (Figure 5) after 30 min octanol (1 ml)

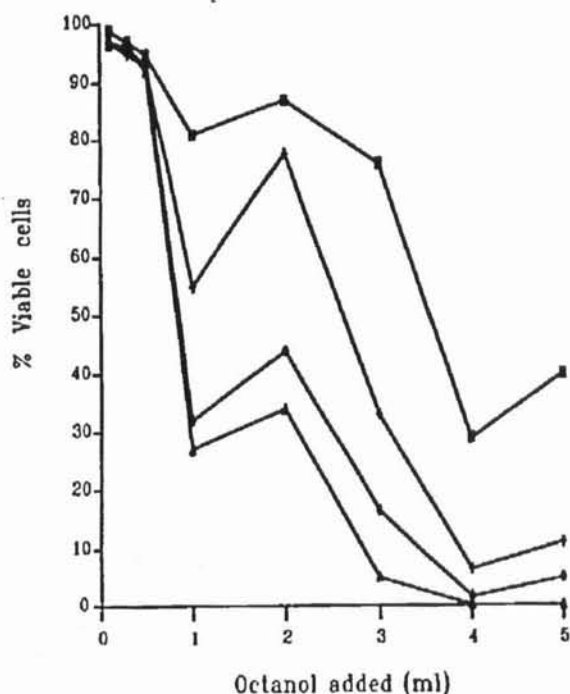


Figure 4 Dependence of cell viability on the concentration of octanol at different times following its addition to a cell suspension of the type displayed in Figure 2. Experiments were performed as described in the legend to Figure 2, except that the concentration of octanol was varied as indicated. Cell viabilities were determined in samples taken 10 minutes (■), 20 minutes (+), 30 minutes (◆) and 40 minutes (▲) following the addition of octanol.

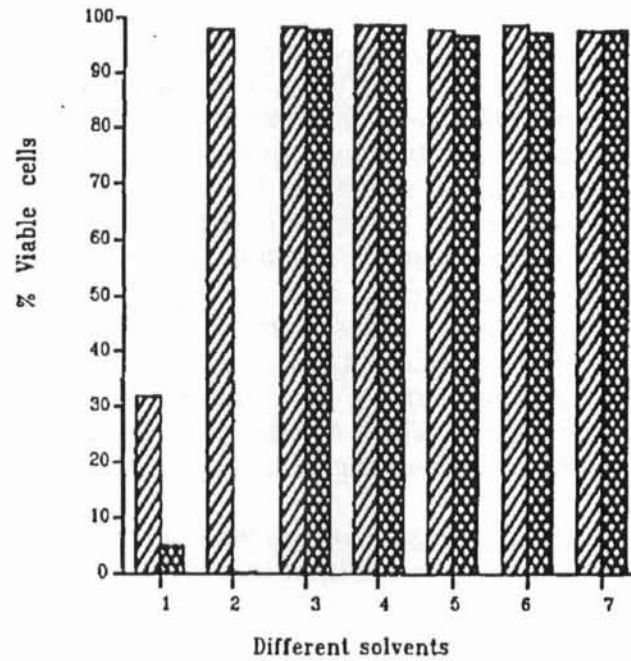


Figure 5 Toxicity to *S. cerevisiae* of different organic compounds. Measurements of cell viability using the dielectric method and the methylene blue technique were made as described in the legend to Figure 2. Either 1 ml (left-hand portion) or 5 ml (right-hand portion) of the substance of interest were added. 1: octan-1-ol, 2: hexan-1-ol, 3: butan-1-ol, 4: ethanol, 5: iso-octane (2,2,4-trimethyl pentane), 6: sodium tetraphenyl borate (10 mM), 7: butan-2-ol.

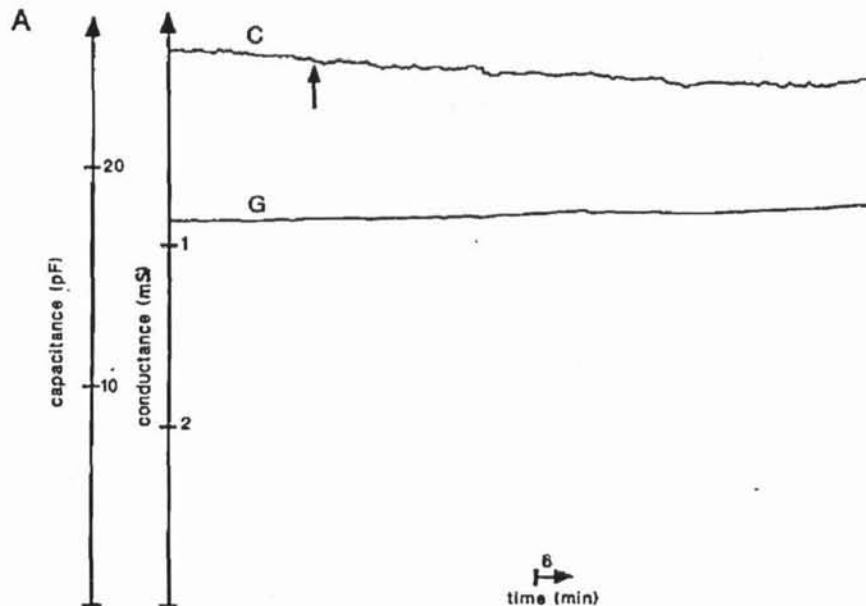


Figure 6 Effect of iso-octane on the dielectric behaviour and viability of *S. cerevisiae*. Measurements were made as described in the legend to Figures 2 and 4. A. Dielectric behaviour. At the arrow, 5 ml iso-octane were added. B. Viability versus normalised capacitance. C. Viability versus normalised conductance.

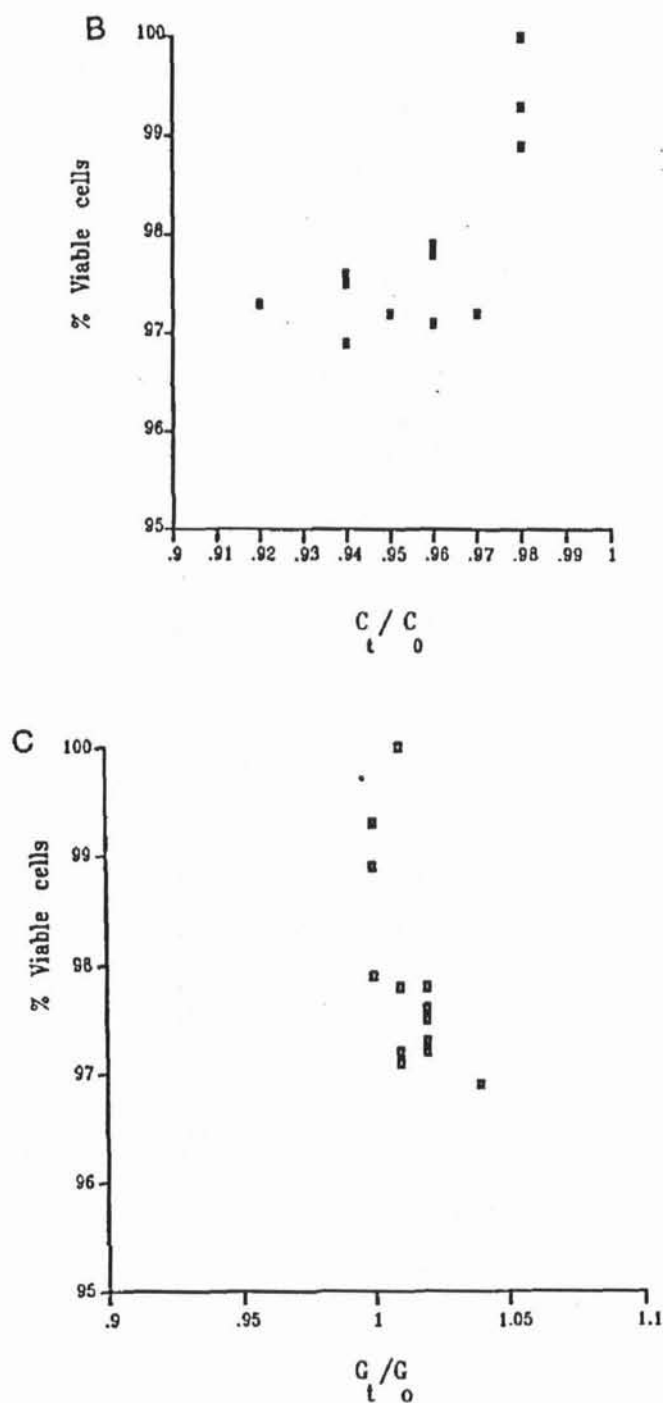


Figure 6. (contd.)

kills nearly 70% of the cells, while the same quantity of other solvents causes essentially no change in cell viability. In general, the data obtained give weight to the views of Brink and Tramper (1985) and of Laane (1985) that the most biocompatible solvents are those of the lowest polarity.

Iso-octane was one of the organic solvents investigated and it was of interest to determine whether the good correlation between capacitance measurements and

cell viability as judged by methylene blue staining held when cells were challenged with a relatively non-toxic organic solvent. The relevant data are given in Figure 6, where it may be observed that there is still an excellent correspondence between the (in)ability of a solvent to diminish the capacitance of a cell suspension and its ability to affect cell viability judged with methylene blue staining. In fact, iso-octane was sufficiently non-toxic that the cell viability did not decrease below 98% of the control value when monitored for periods of 2 hours, nor when its concentration was doubled.

In conclusion, the present dielectric approach provides a novel and convenient means by which to screen solvents (and indeed substrates) for their biocompatibility. Since dielectric measurements are simply realised in flowing streams (e.g. Alder, Fielden and Clark 1984), the automation of this method and its extension to flow-injection systems (Ruzicka and Hansen, 1981) are straightforward.

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