Development of a flow injection method for monitoring cell membrane damage of wine lactic acid bacteria

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Abstract. A flow injection analysis (FIA) system was developed for the determination of phosphate efflux from wine lactic acid bacteria (*Oenococus oeni* and *Lactobacillus hilgardii*) as an indication of cell membrane damage. The system allowed the direct injection of the cell suspension, avoiding the filtration step, with minimum sample treatment and minimized reagent consumption. The developed system is characterized by a linear concentration zone between 3.23×10^{-5} and 4.84×10^{-4} mol L^{-1} PO_4^{3-} and repeatability better than 2.9%. Bacterial suspensions were exposed to a chemical stress with phenolic acids and injected in the FIA system at regular intervals. The extracellular concentration of phosphate was measured spectrophotometrically. The experimental results obtained indicate that hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic) induced faster phosphate leakage rates than hydroxybenzoic acids (vanillic and *p*-hydroxybenzoic) in both strains tested, which could be related to their higher lipophilic character.

Key words: Flow injection analysis; membrane damage; phenolic acids; phosphate efflux; wine lactic acid bacteria.

Most analytical procedures for cell culture monitoring involve time-consuming steps such as sample collection, sample filtration and centrifugation and in most cases slow chemical reactions. These are conditions that difficult the adaptation of these methods to real time analysis and process control. The infrequent sampling and the delay in obtaining information [1–3] are referred as a main drawback of off-line control techniques. These difficulties are even more pronounced when the fast response of microbial cells to induced stress conditions is to be monitored. Flow systems for these purposes can be attractive alternatives since they significantly reduce the time of analysis and facilitate the interfacing of the on-line sampling device with the analytical system incorporating a ''flow through'' stable and compact detector.

Over the years flow injection analysis (FIA) has been acknowledged as a powerful analytical tool for serial assays and also for the study of kinetics of chemical interactions. Nowadays, its scope is broadening into environmental research and most importantly into a tool for biotechnology and for the study of the chemistry of life [4].

Leakage of cellular metabolites like nucleotides, amino acids and inorganic ions, is a primary indication of membrane damage in bacteria that can be caused by membrane-active chemical agents. This leakage can also indicate a reversible disorganisation of the cytoplasmic membrane at lower concentrations of

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Table 1. Common methods used for monitoring the efflux of cellular components of bacteria following cell membrane damage

Methods	Instrumental technique	Concentration Range	Chemical/Physical stress agent	Microorganisms	References
Potassium leakage	flame photometry	0–0.70 mmol/mg dry weight	phenol, chlorophenol	*Escherichia coli*	[7]
	potentiometry	10^{-4}–10^{-2} M	cetrimide, sodium pyrithione, dichlorophen, fentichlor, benzisothiazolone, zinc pyrithione	*Escherichia coli, Pseudomonas aeruginosa*	[8]
		0.1–1.0 mM	antimicrobial peptides	*Escherichia coli, Staphylococcus aureus*	[9]
	AA spectrophotometry	0–0.61 mmol/mg dry weight	carbon dioxide	*Lactobacillus plantarum*	[10]
	ICP spectrophotometry	0–3 mg/L	chlorocresol, *m*-cresol	*Staphylococcus aureus*	[5]
Magnesium leakage	AA spectrophotometry	0–6.7 µmol/mg dry weight	carbon dioxide	*Lactobacillus plantarum*	[10]
Phosphate leakage	VIS spectrophotometry	0–3 mg/L	chlorocresol, *m*-cresol	*Staphylococcus aureus*	[5]
ATP leakage	bioluminescence	0–0.15 nmol/mg dry weight	phenol, chlorophenol	*Escherichia coli*	[7]
		0–0.08 mg/L	chlorocresol, *m*-cresol	*Staphylococcus aureus*	[5]
		N/R	clofazimine, CTAB, Nisin, Protegrin IB-367	*Staphylococcus aureus*	[11]
		N/R	pulse-electrical field	*Escherichia coli, Listeria innocua, Saccharomyces cerevisiae*	[12]
		N/R	clofazimine	*Staphylococcus aureus*	[13]
Leakage of UV-absorbing material	UV spectrophotometry	N/A	phenol, chlorophenol	*Escherichia coli*	[7]
			cetrimide, sodium pyrithione, dichlorophen, fentichlor, benzisothiazolone, zinc pyrithione	*Escherichia coli, Pseudomonas aeruginosa*	[8]
			ethanol	*Oenococcus oeni*	[14]
			carbon dioxide	*Lactobacillus plantarum*	[10]
			chitosan	*Escherichia coli, Staphylococcus aureus*	[15]
			clofazimine	*Staphylococcus aureus*	[13]
			clofazimine, CTAB, Nisin, protegrin IB-367	*Staphylococcus aureus, Escherichia coli*	[11]
			pulse-electrical field	*Escherichia coli, L. innocua, Saccharomyces cerevisiae*	[12]
β-galactosidase leakage	fluorescence	N/R	chitosan	*Escherichia coli, Staphylococcus aureus*	[15]
			clofazimine, CTAB, Nisin, Protegrin IB-367	*Staphylococcus aureus*	[11]

N/R Not reported; *N/A* not applicable.

the antibacterial agents [5]. The measurement of the efflux rates of potassium or phosphate ions (using potentiometric or photometric methods) has been used to monitor cell membrane damage and to compare the effects of different chemical compounds on the cellular membrane [6]. Table 1 describes the most common methods used to measure efflux of different cellular components as an indication of the loss of integrity of the bacterial membrane.

The more frequently employed spectrophotometric methods for the determination of orthophosphate ion are based on the reaction with molybdic acid, leading to the formation of molybdophosphoric acid, a yellow compound that has an absorption maximum at 350 nm. The reduced form of molybdophosphoric acid produces an intensely coloured blue complex of molybdophosphate (phosphomolybdenium blue) that presents an absorption maximum at 880 nm. The sensitivity of the method depends on a number of factors such as the type of reducing agent, acidity and temperature [16]. To further improve the sensitivity of this determination antimony has been used as a catalyser of the reaction [17, 18].

Lactic acid bacteria (LAB) represent a heterogeneous group of Gram-positive bacteria with a strictly fermentative carbohydrate metabolism which have been used for centuries in industrial applications such as the production of fermented food products [19]. Some LAB strains are able to thrive in wine causing important changes in its chemical composition. Most red wines (and some white wines) undergo a secondary fermentation, just after the alcoholic fermentation, called the malolactic fermentation during which LAB decarboxylate malic acid to lactic acid with a subsequent decrease of the total acidity of the wine [20, 21]. This process normally results in ''softer'' wines with improved aroma. On the other hand, if left uncontrolled, these bacteria can also produce undesirable changes to wine quality which ultimately will cause its deterioration. Two of the most studied species of wine LAB are *Lactobacillus hilgardii* and *Oenococcus oeni*. The first species is frequently associated with the deterioration of fortified wines [22] while the second is used as a malolactic starter culture.

Wine is naturally rich in phenolic compounds, which include hydroxycinnamic and hydroxybenzoic acids in total concentrations ranging from 100 to 200 mg L^{-1}, depending on the grape variety and vinification process [23]. These compounds are known to have bacteriostatic and fungistatic activity against a number of microorganisms, including wine LAB [24, 25]. Phenolic compounds can act both as protoplasmic poisons and as membrane-active agents [26] increasing the permeability of the cell membrane which may cause the loss of ability to maintain the chemiosmotic balance by the cell and ultimately may lead to cellular death.

The objective of this work was to develop a FIA system to monitor the leakage of phosphate from damaged cells exposed to a chemical stress with phenolic compounds. The effect of five phenolic acids on the cell membrane integrity of two strains of LAB (*Lactobacillus hilgardii* 5 and *Oenococcus oeni* VF) was studied.

Materials and methods

Reagents and solutions

All chemicals used were of analytical reagent grade, and deionised water with a specific conductance of less than 0.1 μS cm^{-1} was used throughout the entire work.

The stock phosphorous standard solution (1000 mg L^{-1} P–PO_4^{3-} corresponding to 3.236×10^{-2} mol L^{-1}) was prepared by dissolving 0.4365 g of potassium dihydrogen phosphate (VWR International, www.vwr.com) in 100 mL of water. Working phosphorous standard solutions in the range 6.45×10^{-5}–4.03×10^{-4} mol L^{-1} were prepared by rigorous dilution of the stock solution with water.

The ammonium molybdate reagent was prepared by dissolving 4.00 g of ammonium heptamolybdate-tetra-hydrate and 0.108 g of potassium antimony(III) oxide tartrate hemihydrate (VWR International) in water, afterwards 17.5 mL of a concentrated sulphuric acid solution (VWR International) was added and the resulting solution was diluted to 500 mL. The ascorbic acid (VWR International) solution was obtained by dissolving 20.0 g of the solid in 500 mL of water. These solutions were prepared weekly and were kept refrigerated when not in use.

Three hydroxycinnamic (*p*-coumaric, caffeic and ferulic) and two hydroxybenzoic acids (*p*-hydroxybenzoic and vanillic) acids were used in this work (Sigma-Aldrich, Germany www.sigmaaldrich.com). The phenolic acid solutions (0.122 mol L^{-1}), were prepared in pure (99.8% v/v) ethanol. These solutions were regularly prepared and kept refrigerated for a maximum of two days.

Bacterial strains and growth conditions

In this work, we used *Lact. hilgardii* 5, a strain isolated from Port wine by Couto and Hogg [22] from the ESBUCP collection (Porto, Portugal) and *O. oeni* VF (Viniflora Oenos), a commercial starter culture strain from Christian Hansen.

The liquid growth medium used was a 1:1 mixture of MRS (Lab M, Bury, UK, www.lab-m.com) and Tomato Juice (Difco, Detroit, USA, www.vgdusa.com/DIFCO.htm). Before sterilization (121 °C, 15 min), the medium pH was adjusted to a 4.5–4.6 with HCl (6 mol L^{-1}). After sterilization, 5% v/v of ethanol was added to the medium since this concentration level was found to stimulate the growth of both strains [24]. The cultures were incubated in aerobiosis during 72 hours, without agitation.

Instrumentation

The solutions were propelled by a Gilson (Villiers-le-Bel, France, www.gilson.com) Minipuls 3 peristaltic pump equipped with PVC Gilson propelling tubes. The manifold tubing was made of PTFE (1 mm internal diameter). Gilson end fittings and laboratory made Y and T-shaped confluences were used to link the different parts of the manifold.

An ATI Unicam (Cambridge, U.K. www.thermo.com/spectroscopy) 5625 UV/VIS spectrophotometer equipped with a Hellma (Mullheim/Baden, Germany www.hellma-worldwide.de) 178.713-QS flow cell (inner optical volume 80 µL) was used as detection system which was connected to a Kipp & Zonen (Delft, Holland, www.kippzonen.com) BD chart recorder.

An Ismatec (Zurich, Switzerland, www.ismatec.com) MINI-S/640 peristaltic pump and Metrohm (Herishau, Switzerland, www.metrohm.com) E649 magnetic mixer was incorporated into the system with the purpose of recirculation of the cell suspension during the sampling process.

A Thermo Nicolet Evolution 100 double-beam spectrophotometer was used to obtain the absorption spectra of the various solutions and cell suspensions.

Sample preparation

Bacterial cells were grown to late exponential phase (3–4 days) and centrifuged in a Hettich Zentrifugen (Tuttlingen, Germany, www.hettich-zentrifugen.de) – Rotina 35R (10 min, $5500 \times g$). The obtained biomass was washed with ultrapure water and centrifuged again. After the second centrifugation, the cell *pellet* was weighed in an analytical scale before being resuspended in ultrapure water (18 mL) and transferred to a 50 mL flask with a magnetic stirrer. The average wet weight of the obtained pellets ($n = 30$) was 0.130 ± 0.044 g and 0.270 ± 0.050 g, for *Lact. hilgardii* 5 and for *O. oeni* VF, respectively. The cell *pellet* was resuspended immediately before analysis, and the cell suspension was stirred and injected in the FIA system, to measure the intrinsic absorbance of the cell suspension. The resting cells suspension was continuously recirculated from the reaction vessel to the sampling loop. Afterwards, 2 mL of phenolic acid solution was added to the cell suspension, to induce chemical stress. After a controlled time interval (10 s) needed to homogenise the suspension, several injections were made during a time period of 5 minutes, to follow the concentration of phosphate in the extracellular medium. This procedure was maintained for all the phenolic acids and for both strains tested, in a way that the phosphate efflux curves can be comparable. Control assays were done using pure (99.8% v/v) ethanol.

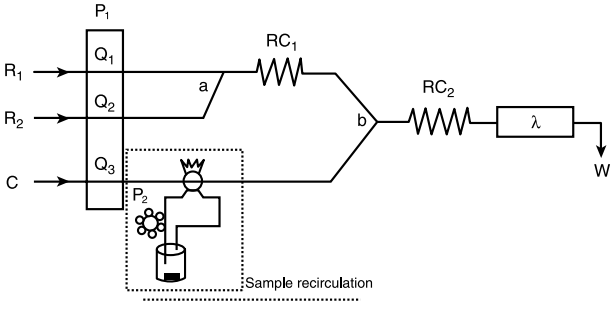

Fig. 1. Flow injection manifold developed for the determination of phosphate leakage of LAB exposed to phenolic acids with a sample recirculation system; P_1 and P_2 peristaltic pumps; R_1 molybdate reagent; R_2 ascorbic acid; C carrier; RC reaction coils (RC_1 100 cm; RC_2 100 cm); Q flow rates ($Q_1 = Q_2 = 1.9$ mL min^{-1}; $Q_3 = 4.0$ mL min^{-1}); W waste; a and b confluence points; λ UV/Vis spectrophotometer (880 nm)

Flow injection procedure

The flow injection system used is represented in Fig. 1. The flow injection procedure consisted in the inline mixing of ammonium molybdate solution (R_1) with ascorbic acid reducing reagent (R_2) in the first confluence (a). The sample (cell suspension under stress conditions) was injected in water carrier stream (C) and merged at confluence (b) with the previously formed reagent stream and allowed to react in RC_2 coil. Changes in absorbance were monitored spectrophotometrically at 880 nm.

Results and discussion

Study of the FIA system

System optimization was achieved by varying each parameter individually in order to optimize repeatability and sensitivity of the measurements. To prevent possible obstructions of the flow system, 1 mm inner-diameter tubes were used. This approach was successful as no blockage or sample carryover was observed along the work.

Since the colorimetric reaction used in this work has been widely studied in flow injection systems, and is an accepted reference method for the analysis of inorganic phosphate in waters and wastewaters, reagent concentrations were chosen based on the published literature [18]. The effect of the reducing reagent (ascorbic acid) concentration on the sensitivity was studied with the aim to compromise between sensitivity and reagent consumption. At 40 g L^{-1}, the sensitivity was satisfactory and this value was used in subsequent experiments.

The injection volume was set to 130 µL since this value allowed a sufficiently wide linear concentration range (around 0.3 to 5×10^{-4} mol L^{-1}) with good sensitivity. This reduced volume also allowed a fast refill of the loop and a lower dilution of the cell suspension.

A total flow rate of 7.8 mL min^{-1} was selected. Flow rates Q_1 and Q_2 were set to 1.9 mL min^{-1} and Q_3 was adjusted to 4.0 mL min^{-1}. This selection was made taking into account the sampling rate. It was found that increasing the total flow rate did not influence significantly the sensitivity of the determination.

The choice of the reactor length RC_2 was made according to the results obtained with tubes of 50, 100, 150 and 200 cm. Highest sensitivity was achieved

Table 2. Analytical characteristics of the developed system

Parameter	Values
Linear concentration zone, mol L^{-1}	3.23×10^{-5}–4.84×10^{-4}
Typical calibration[a]	Abs = 2112 (±215)C − 0.067 (±0.010)
C: concentration of PO_4^{3-}, mol L^{-1}	$r = 0.9996$ ($\pm 6 \times 10^{-4}$)
Repeatability (RSD) ($n = 10$)	1.4% (6.45×10^{-5} mol L^{-1})
	1.7% (2.42×10^{-4} mol L^{-1})
	1.6% (4.03×10^{-4} mol L^{-1})
Detection limit[b], mol L^{-1}	1.3×10^{-5}
Quantification limit[b], mol L^{-1}	4.3×10^{-5}
Determination frequency, h^{-1}	144
Reagent consumption per assay	
– Ammonium heptamolybdate	6.4 mg
– Potassium antimony(III) oxide tartrate	0.17 mg
– Sulphuric acid	30 µL
– Ascorbic acid	32 mg
Effluent production, mL min^{-1}	7.8

[a] Values between brackets are the standard deviations for calibration curve parameters, calibrations performed at 17 different working days; [b] Calculated according to IUPAC definition [27]. *RSD* Relative Standard Deviation or coefficient of variation at different concentration levels.

with the 100 cm reactor due to a compromise between dispersion and extension of the reaction.

Using the selected conditions the analytical characteristics of the developed system are summarised in Table 2.

Spectral interference studies

To study the interference of coloured phenolic acids (caffeic and ferulic acids) and bacterial cells on the spectrophotometric detection, a spectral scan was carried out between 340 and 900 nm. The obtained results suggest that the presence of caffeic or ferulic acids did not affect considerably the formation of the phosphomolybdenium blue complex (detected at 880 nm), comparatively to the control assay with ethanol (results not shown).

To quantify the effect of the phenolic acids on the extent of the colour reaction within the flow system, an additional interference study was carried out. Phenolic acids were mixed with phosphate standard solutions (2.42×10^{-4} mol L^{-1}) and injected in the FIA system. In all cases the phosphate standard solutions contained 0.0122 mol L^{-1} of phenolic acid. The results are summarized in Table 3.

The obtained results indicate that with the exception of the notorious interference from gallic acid the only phenolic acid that showed appreciable interference was caffeic acid, probably due to its intrinsic colour in solution. Gallic acid is known to react with heteropoly-phosphomolybdate [28] therefore this compound was excluded from the subsequent studies. In the case of coloured solutions (like caffeic acid solutions), the slight positive interference in the absorbance signal would not affect the final results as long as the absorbance signal remained within the limits of the linear calibration zone during the experiment (Fig. 2).

Table 3. Interference of the phenolic acids on the colour reaction

Compound	% Interference*
P-coumaric acid	−0.6
P-hydroxybenzoic acid	1.4
Ferulic acid	−0.8
Caffeic acid	11.6
Vanillic acid	1.5
Gallic acid	−51.9

* Results are expressed as relative differences from control, *i.e.* (concentration obtained with phosphate standard solution with added phenolic compound – concentration obtained in the control)/ concentration obtained in the control. The control was a phosphate standard solution (2.42×10^{-4} mol/L) with 10% (v/v) ethanol.

Under the established conditions the developed system was applied to the direct determination of phosphate release from *O. oeni* VF and *Lact. hilgardii* 5 in cell suspensions. The repeatability (RSD%, $n = 10$) for the direct injection of (unstressed) cell cultures

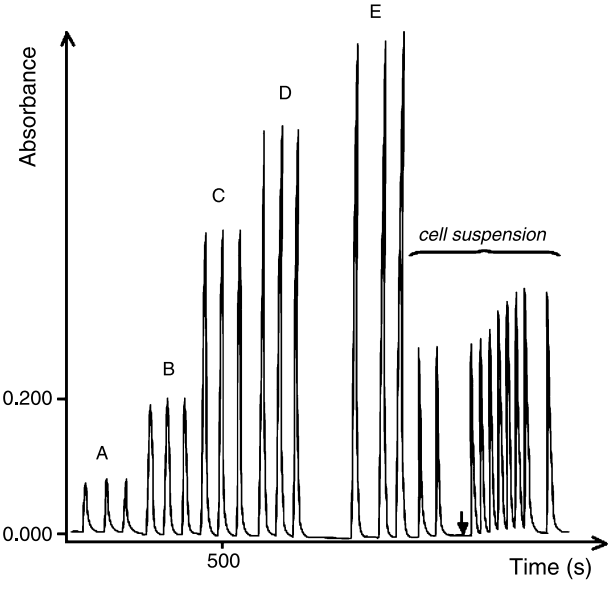

Fig. 2. Recorder output for the determination of phosphate efflux corresponding to injections of phosphate standards: (*A*) 6.45×10^{-5} (*B*) 1.29×10^{-4} (*C*) 2.42×10^{-4} (*D*) 3.23×10^{-4} (*E*) 4.03×10^{-4} mol L^{-1}; and sample: *O. oeni* VF; arrow indicates the time of addition of caffeic acid

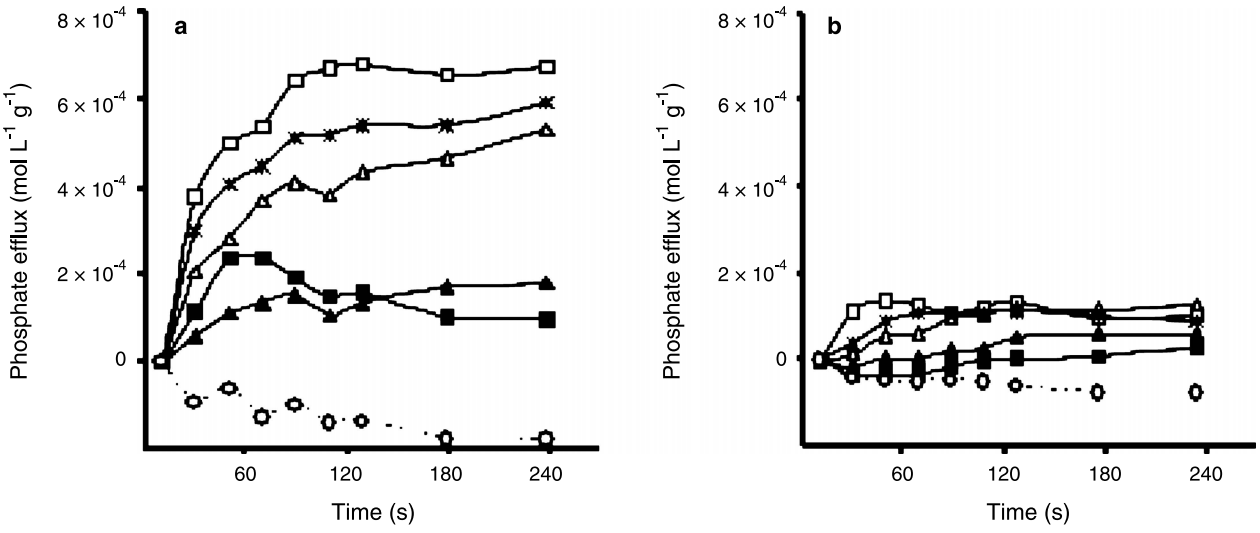

Fig. 3. Phosphate efflux of *Lact. hilgardii* 5 (a) and *O. oeni* VF (b) suspensions after chemical stress with phenolic acids at 0.0122 mol L^{-1}. (□) *p*-Coumaric acid, (■) *p*-hidroxybenzoic acid, (∗) caffeic acid, (▲) vanillic acid, (△) ferulic acid, (○) control (ethanol). Results are expressed as variation of phosphate concentration per wet weight of cellular *pellet*

was 1.2 and 2.9% for *O. oeni* VF and *Lact. hilgardii* 5 suspensions, respectively.

Influence of the phenolic acid concentration on the phosphate efflux from wine LAB

The phosphate efflux from *O. oeni* VF caused by different concentrations of *p*-coumaric acid (in the range of 3.05×10^{-3} to 1.22×10^{-2} mol L^{-1}) was also studied. The phosphate efflux corresponds to the change in the phosphate ion concentration in the liquid phase (on cell mass basis) relative to the initial phosphate concentration registered before the addition of the stress inducing phenolic acid. The obtained results indicate that increasing concentrations of *p*-coumaric acid caused an increase in phosphate efflux, although, over 0.0915 mol L^{-1} no observable difference was found.

Influence of phenolic acids on the phosphate efflux of O. oeni VF and Lact. hilgardii 5

All the phenolic acids studied in this experiment caused leakage of phosphates in both bacteria (Fig. 3), at the tested concentration levels (0.0122 mol L^{-1}). *p*-Coumaric, caffeic and ferulic acids had the highest effects of all tested phenolic acids in both strains. This effect was noticeable after a period of contact of a few seconds. Results obtained indicate that hydroxycinnamic acids have a stronger effect than hydroxybenzoic acids on cell permeabilization. These results agree with previously published data obtained in growth and inactivation experiments performed with the same strains [24, 29].

The different kinetics of phosphate efflux caused by phenolic acids could be related to the difference in their lipophilic character since hydroxycinnamic acids are more lipophilic than hydroxybenzoic acids [30].

Conclusions

The FIA system developed in this work proved to be a fast and reliable alternative method for comparing the effect of different compounds, such as phenolic acids, on the cellular membrane integrity of wine lactic acid bacteria. The proposed approach might have an application in the food or pharmaceutical industry to test the effects of different antimicrobial compounds on the membrane permeability of bacteria.

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