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# Effect of Low Intensity Pulsed Electric Field on Ethanol Fermentation and Chemical Component Variation in a Winemaking Culture

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**Abstract** Electric polarity of working electrode and counter electrode was periodically switched at the intervals of 30 sec. Electric current generated by anodic and cathodic reaction of working electrode was reached to +30 and -12 mA in low intensity pulsed electric field (LIPEF). The yeast growth, ethanol production, and malate consumption in the initial cultivation time were more activated in the LIPEF than the conventional condition (CC). Polyphenol, total phenolic contents (TPC), and total flavonols (TF) were gradually decreased in all cultivation conditions during incubation for 2 weeks but antioxidation activity was not. TF was significantly lower in 3 and 4 V of LIPEF than CC and 2 V of LIPEF; however, the polyphenol, TPC, and antioxidation activity were a little influenced by the LIPEF. After ripening of the winemaking culture for 15 days, polyphenol, TPC, and TF were a little increased but the antioxidation activity was not.

**Keywords:** winemaking culture, low intensity pulsed electric field, *Saccharomyces bayanus*, ethanol fermentation, antioxidation activity

## Introduction

In an effort to improve the wine quality, some physical techniques, such as flash evaporation and cooling time controls, have been applied to the preparation of grape must in the winemaking process; however, the physical techniques can not be a critical factor affecting yeast growth and fermentation metabolism (1). Typically, wine quality has been largely dependent on grape species and yeast physiology (2,3), which are natural factors incapable of artificially regulating. The oxidation-reduction potential, dissolved oxygen and bacterial contamination in the winemaking cultures may be controlled by modification or alteration of environmental factors (4,5). Practically, the fermentation techniques to control yeast growth, fermentation rates, and chemical components have not yet been developed in the winemaking process. Sulfite has been applied to the winemaking process in order to control bacterial contamination (6) but may inhibit some yeast strains and can induce unwanted chemical reaction in the winemaking culture (7,8). The grape must is naturally infected with various bacteria and wild yeasts during harvesting and extraction (9). The wild yeast that naturally contaminates the grape must is difficult to control, but may be partially controlled by the inoculation of specific yeast strain, allowing the inoculum itself to become the predominant (10). Once ethanol fermentation has begun, the oxidation-reduction potential of the winemaking culture may be maintained at the lower level than 0 V (V. Ag/AgCl) because the dissolved oxygen can be purged from the winemaking culture by $CO_2$ generated by yeast (11). $CO_2$ is toxic to bacterial cells, especially the species depending on respiratory metabolism (12). Accordingly, the ethanol and $CO_2$ produced by yeast may inhibit bacterial contaminants, but do not influence yeast physiology.

All kinds of winemaking yeasts produce ethanol by a common metabolic pathway (EMP) under anoxic conditions without exception (13) but can catalyze various sugars, organic acids, and even ethanol by mitochondrial metabolism under aerobic conditions (14). The critical dissolve oxygen of oxidation potential for activation of mitochondrial metabolism and ethanol fermentation may be difficult to induce in the winemaking culture by the existing fermentation technology (15). Aeration or agitation is the general techniques employed in typical bioreactors to induce oxidation potential or aerobic condition. However, neither aeration nor agitation can induce the critical oxidation potential to optimize the metabolism of the winemaking yeast (16).

In this study, the low intensity pulsed electric field (LIPEF) effects on yeast growth, ethanol production, malate consumption, and variation of chemical components including polyphenol, total polyphenol, total flavonols (TF), and antioxidation activity were estimated as a part of efforts to improve the wine quality and development of new winemaking process.

## Materials and Methods

**Chemicals** All chemicals and a platinum wire used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) except medium ingredients.

**Electrochemical bioreactors** An electrochemical bioreactor was designed to induce a LIPEF in a winemaking culture

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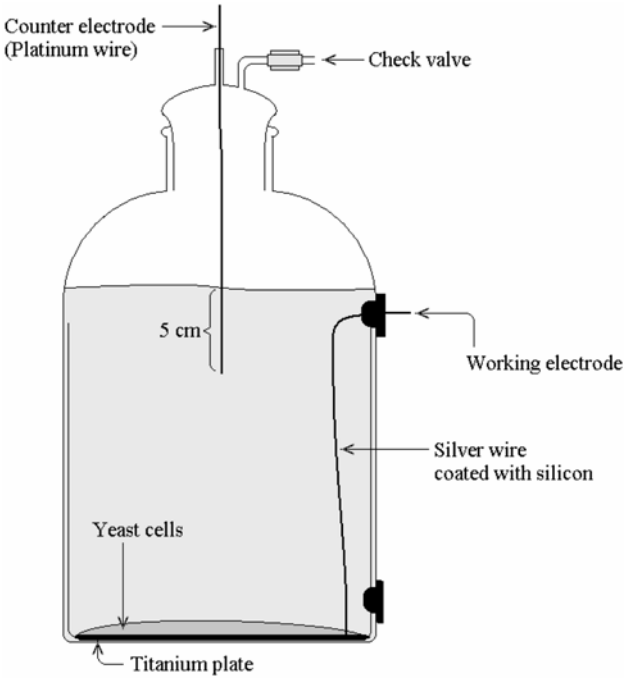


**Fig. 1. Schematic structure of an electrochemical bioreactor.** Designed to induce the pulsed electric field, in which *Saccharomyces bayanus* was cultivated with grape must.

as shown in Fig. 1. A titanium plate (thickness 0.5 mm, diameter 120 mm, VWR, Chicago, IL, USA) and platinum wire (length 50 mm, diameter 0.5 mm, Sigma-Aldrich) were used as the working electrode and counter electrode, respectively. Two, 3, or 4 V of direct current (DC) electricity was charged to electrodes and electrode polarity of working and counter electrode was exchanged at the intervals of 30 sec, by which the anodic (oxidation) and cathodic (reduction) reaction was alternately generated on working electrode. Electric current was not generated in 1 V of LIPEF but the color of winemaking culture was significantly changed in 5 V of LIPEF. On the basis of these results, the 2, 3, and 4 V of electric intensity were selected. The reactor volume was adjusted to 2,000 mL and the headspace pressure was adjusted to approximately 1.05 atm with a check valve.

**Cultivation and ripening condition** The winemaking culture was incubated at 25°C and ripening temperature was adjusted to 20°C.

**Measurement of electric pulse** The electric pulse generated at the working electrode was measured with an ampere-voltage meter, which was connected with a data acquisition system controlled by a personal computer.

**Yeast strain** The yeast strain used in this study was *Saccharomyces bayanus* (EC-1118), which was purchased from a professional wine yeast producer (Lallemand Inc., Montreal, ON, Canada). The inoculum size and process were consistent with the supplier's specifications.

**Grape must** Grape must was presented by a winemaking company (Muju, Jeonnam, Korea), of which glucose and malate contents were analyzed with high performance liquid chromatography (HPLC) prior to use. Other chemical components were spectrophotometrically analyzed. In order to produce 12-13% of ethanol, the glucose concentration was adjusted to a final 1.5 M by the direct addition of glucose powder to the grape must.

**Analysis of ethanol, glucose, and malate** Ethanol, glucose, and malate were analyzed via an HPLC apparatus (YoungLin, Seoul, Korea) equipped with an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, CA, USA) and a refractive index detector (YoungLin). Samples were prepared from yeast culture via filtration with a membrane filter (pore size 0.22-μM, Satorius, Hannover, Germany) and injected directly into the HPLC injector. Concentration of target materials was determined by comparison with retention time and peak area of the chromatograms of standard materials.

**Analysis of polyphenols** Polyphenols were determined according to Purssion blue spectrophotometric method (17). Winemaking culture was filtrated with a membrane filter (pore size 0.22-μM) and diluted with pure water in the range of standard curve. A 3.0 mL of 0.1 M $FeCl_3$ in 0.1 M HCl were added to 1 mL of the filtrate followed immediately by timed addition of 3.0 mL of freshly prepared 0.008 M $K_3Fe(CN)_6$. The absorbance was measured on a spectrophotometer (UV-1601; Shimadzu, Tokyo, Japan) at 720 nm after 10 min from the addition of reagents. A standard curve was prepared for expressing the results as tannic acid equivalent, i.e., amount of tannic acid (mg/L) which gave a color intensity equivalent to the given by polyphenols after correction for blank.

**Analysis of total phenolic contents (TPC)** TPC was determined using the Folin-Ciocalteu colorimetric method (18). The filtrated winemaking culture with a membrane filter (pore size 0.22-μm) was properly diluted with pure water in the range of standard curve. In a 10 mL-conical tube, 6.4 mL distilled water, 0.1 mL filtrate, and 0.5 mL Folin-Ciocalteu reagent (1:1 with water) were added and mixed. After exactly 1 min, 3.0 mL of $Na_2CO_3$ (10 g/100 mL) was added, and the mixture allowed to stand at room temperature in the dark for 2 hr. The absorbance was read at 765 nm, and the total polyphenols concentration was calculated from a calibration curve developed using 10-100 mg/L of gallic acid.

**Analysis of total flavonols (TF)** TF contents were determined using the slightly modified *p*-dimethylamino-cinnamalde-hyde (DMACA) method (19). The filtrated winemaking culture with membrane filter (0.22-μm pore) was properly diluted with pure water in range of standard curve. A 0.2 mL of filtrate was introduced into a 10-mL conical tube and added 3 mL DMACA solution (0.1% in 1 mol/L of HCl in methanol). The mixture was vortexed and allowed to react at room temperature for 10 min. The absorbance at 640 nm was read against a blank prepared following the sample procedure as above but without DMACA. The concentration of TF was estimated from a calibration curve developed using 6.25-200 mg/L of catechin.

**Antioxidant activity** Antioxidation activity was determined by the metal chelating capacity (20). Filtrated and diluted winemaking culture (3.8 mL) was mixed 50 μL of 10 mM $FeCl_3 \cdot 4H_2O$ and incubated at room temperature for 30 min. A 150 μL of 5 mM Ferrozine was added to the mixture and mildly shaken. After 10 min, the $Fe^{2+}$ was monitored by measuring the formation of ferrous ion-ferrozine complex at 562 nm. The concentration of $Fe^{2+}$ reduced from $Fe^{3+}$ by antioxidant was estimated from a calibration curve developed using ferrous sulfate and ferrozine.

**HPLC analysis of test wines** After fermentation was finished, the winemaking cultures were ripened at 20°C for 15 days, which are specified as the test wines. Grape must, the test wines and French wines were analyzed with HPLC equipped with $C_{18}$ column (Pinnacle II Amino 5 μm and 250×4.6 mm, Restek, Bellefonte, PA, USA). Catechin, tannic acid, and malate were analyzed to compare the chromatographic patterns under same condition. Acetonitrile and water (75:25) mixture was used as an elution buffer, of which flow rates was adjusted to 0.6 mL/min. Wave length of UV detector was adjusted to 210 nm and column temperature was adjusted to 35°C. All samples were filtrated with 0.22-μm membrane filter before injection.

**Statistical analysis** All tests were carried out in triplicate and the results were presented as mean±standard deviation (SD).

## Results and Discussion

**Effect of LIPEF on redox reaction** Reciprocal exchange of the working electrode polarity induced current variation in the winemaking culture. At the second that electric potential of the working electrode was switched from −3 to 3 V, the current was positively increased from −4 to 32 mA and then gradually dropped to 0.5 mA for 30 sec, and at the second that the potential was switched from 3 to −3 V, the current was negatively increased from 0.5 to −12 mA and then gradually dropped to −4 mA for 30 sec (data not shown). Whenever the electrode polarity was switched, the current either getting positively or negatively increased at the regular current level; this is effective to generate electric oxidation reaction from the working electrode with the temporary electrode polarization. The electrode polarization can be generated by an electrical double layer formed at the interface between the aqueous surrounding electrode and the electrode with a different potential from the aqueous one (21). The electrical double layer can be disrupted by the PEF but not by fixed electric field (FEF). The LIFEF induced electrode polarization, by which the current is gradually decreased to the lower than 0.5 mA at 3 V of electric intensity. Theoretically, 3.45, 14.88, and 21.82 mL of oxygen are generated from working electrode at the 2, 3, and 4 V of LIPEF, respectively, for 1 hr. The maximal current (32 mA) generated in 3 V of LIPEF was approximately 64 times higher than that (0.5 mA) generated by 3 V of LIFEF. Some of experimental data present a possibility that the oxidation potential or oxygen may detrimentally influence the carbon metabolism of lactic acid bacteria (22), but simultaneously functions as an essential factor for yeast growth (23,24).

Generally, the PEF is generated by higher than 1 kV/cm of electric intensity and induce irreversible structural changes of cytoplasmic membrane, resulting in pore formation and loss of the selective permeability properties of the membrane (25-27). Both Gram (+) and Gram (-) bacteria were known to be inactivated in higher than 20 kV/cm of PEF (28). The high intensity PEF causes cell electroporation, whereas, the lower than 0.4 V/cm of LIPEF may not damage microorganisms themselves and change even physicochemical condition around microorganisms. Theoretically, the electric field strength generated by LIPEF may be limitedly influence electrode to induce electrolysis of water or oxidation-reduction reaction of some chemicals. Practically, yeast cells growing on working electrode located at the bottom may be directly influenced by the redox reaction or oxygen generated from working electrode by the LIPEF. Meanwhile, the floated bacterial cells in the winemaking culture may be indirectly influenced by the redox reaction or oxygen generated from counter electrode by LIPEF. The oxygen is toxic for bacteria but activator for fatty acid synthesis for yeast. The LIPEF is useful to constantly generate electrochemical redox reaction without electrode polarization. The electric current is proportional to the oxidation or reduction reaction between working and counter electrode; however, the redox reaction has to be limited around electrode because the electric pulse time is not enough to distribute electrical charge through the winemaking culture between working and counter electrode. In the fixed electric field, however, the electrical charges may be transferred from anode to cathode or cathode to anode. Distribution of positive and negative electrical charges into cathode and anode may be a cause to gradually decrease the electrochemical redox reaction and finally stop.

**Effect of LIPEF on winemaking culture and wine** The ethanol production and malate consumption in 3 V of LIPEF were not significantly different from those in 4 V LIPEF, but on the other hand were significantly higher than those in 2 V of LIPEF as shown in Table 1 and Fig. 2. This is a clue that the optimal PEF for activation of yeast physiology may be generated from the electric intensity between 2 and 3 V. The ethanol production was reached to

**Table 1. Effect of pulsed electric field (PEF) charged to the winemaking cultures on malate consumption**

| Incubation time (day) | Malate variation (g/L) | | | |
|---|---|---|---|---|
| | Conventional condition | 2 V PEF | 3 V PEF | 4 V PEF |
| 0 | 13.52±1.48 | 13.58±1.39 | 13.54±1.24 | 13.56±1.41 |
| 8 | 10.65±2.02 | 6.93±1.41 | 5.22±0.91 | 5.21±1.08 |
| 15 | 9.67±0.88 | 3.48±0.46 | 1.61±0.32 | 1.65±0.47 |

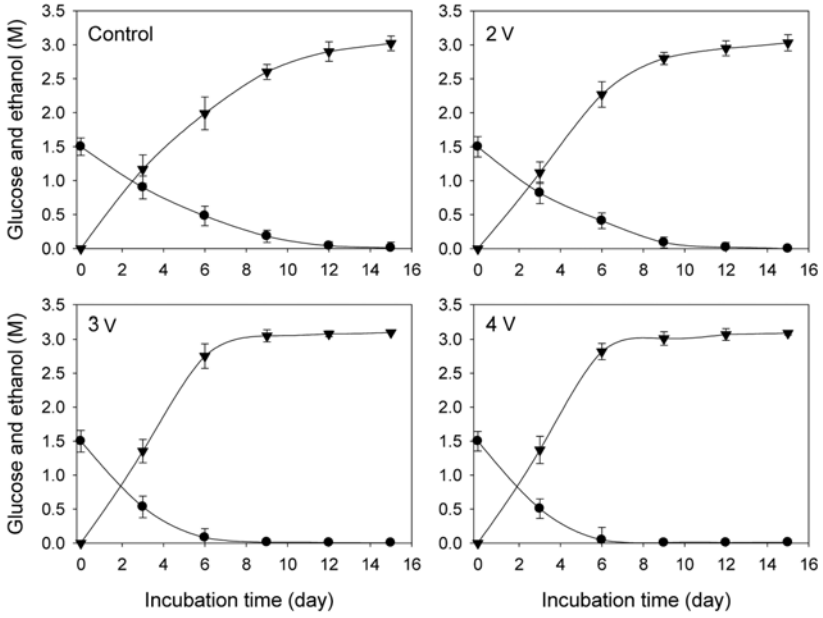


**Fig. 2. Effect of pulsed electric field (PEF) on ethanol production (▼) in coupling with the consumption of glucose (●) by *Saccharomyces bayanus*.**

maximal value (3 M) at the 10th and 8th day of incubation time in 2 and 3 V of LIPEF, respectively. These differences may be caused by the difference of oxygen generated from the working electrode or oxidation potential charged to working electrode. Rosenfeld *et al*. (29) reported that addition of oxygen to a lipid-depleted medium resulted in the synthesis of sterols and unsaturated fatty acids, by which cell viability and fermentation rates were increased and fermentation period was shortened. Other studies have demonstrated that the fatty acids and molecular oxygen are required for lipid biosynthesis, plasma membrane integrity, and the maintenance of ethanol production rates (30). The LIPEF may be a fermentation technique for activation of yeast growth, ethanol production, and malate consumption based on the results from this research and reported by other researchers; however, it remains unclear as to what potential intensity is the optimum for the yeast physiology activation, as well as the manner in which the chemical components such as polyphenol, TPC, and TF are not or minimally decreased. Aeration or agitation of the winemaking culture may be a typical method to induce the oxygenic condition but causes the oxidation potential to be erratically fluctuated (31). Practically, the ethanol production by *S. bayanus* was decreased by 15 and 25% as the result of shaking at 80 and 120 strokes, respectively. Precise and regular control of oxygen concentration or oxidation potential in the winemaking culture may be a critical technique, which can be accomplished by control of electric current generated in the LIPEF.

In the winemaking culture, shortening of fermentation period, increasing of ethanol production, and malate consumption are required to be balanced with maintenance of the chemical components. Concentration of the chemical components may depend principally upon the grape species but may be altered by the yeast metabolism and cultivation condition (32). As shown in Table 2, 3, 4, and 5, polyphenol, TPC, and TF dissolved in the winemaking culture was gradually decreased but antioxidation activity was gradually increased in proportion to the incubation time. After ripening at 20°C for 15 days, TF was significantly decreased but polyphenols, TPC, and antioxidation activity were a little increased. Generally, the chemical components

**Table 2. Effect of pulsed electric field (PEF) charged to winemaking cultures on polyphenol concentration**

| Incubation time (day) | Polyphenol concentration (mg/L) | | | |
|---|---|---|---|---|
| | Conventional condition | 2 V PEF | 3 V PEF | 4 V PEF |
| 0 | 55.46±2.1 | 55.53±1.4 | 55.36±2.4 | 55.56±0.9 |
| 1 | 42.38±0.8 | 42.79±1.5 | 42.84±1.5 | 44.15±1.2 |
| 3 | 44.66±1.4 | 43.32±1.2 | 46.49±2.6 | 45.84±1.6 |
| 5 | 43.58±2.1 | 45.52±0.3 | 42.62±0.6 | 39.95±3.3 |
| 7 | 41.39±1.7 | 44.50±1.2 | 41.38±0.9 | 38.88±2.6 |
| 9 | 40.72±2.4 | 43.72±2.2 | 37.55±3.8 | 36.74±4.2 |
| 12 | 35.77±1.3 | 39.70±2.3 | 35.37±4.1 | 31.86±3.8 |
| 15 | 30.78±2.6 | 31.17±2.1 | 28.66±4.2 | 26.43±1.8 |
| After ripening for 15 days | 37.23±1.3 | 37.83±0.9 | 34.88±1.1 | 33.10±1.4 |

**Table 3. Effect of pulsed electric field (PEF) charged to the winemaking cultures on TPC**

| Incubation time (day) | Total phenolic contents (mg/L) | | | |
|---|---|---|---|---|
| | Conventional condition | 2 V PEF | 3 V PEF | 4 V PEF |
| 0 | 925.87±35.5 | 925.87±32.9 | 925.87±22.5 | 925.87±38.0 |
| 1 | 733.42±82.8 | 744.86±66.9 | 880.94±84.1 | 745.74±69.2 |
| 3 | 643.94±46.6 | 698.67±77.7 | 678.68±61.3 | 734.47±64.5 |
| 5 | 726.49±55.1 | 719.73±32.4 | 726.49±49.2 | 732.38±52.7 |
| 7 | 650.55±26.4 | 548.35±31.7 | 546.26±30.3 | 529.58±41.2 |
| 9 | 545.58±48.6 | 450.58±43.5 | 448.59±51.1 | 433.85±38.2 |
| 12 | 520.41±29.5 | 454.95±22.8 | 438.73±43.9 | 424.46±47.8 |
| 15 | 444.36±41.7 | 448.61±35.9 | 430.33±42.1 | 416.54±29.7 |
| After ripening for 15 days | 458.65±30.2 | 459.80±24.4 | 434.49±22.9 | 445.56±18.3 |

**Table 4. Effect of pulsed electric field (PEF) charged to the winemaking cultures on total flavonols**

| Incubation time (day) | Total flavonols (mg/L) | | | |
|---|---|---|---|---|
| | Conventional condition | 2 V PEF | 3 V PEF | 4 V PEF |
| 0 | 35.38±2.8 | 35.42±4.2 | 35.40±4.6 | 35.48±3.9 |
| 1 | 33.48±2.2 | 31.16±3.2 | 31.67±4.1 | 26.25±2.2 |
| 3 | 24.92±3.6 | 24.65±2.8 | 22.85±3.2 | 21.35±2.5 |
| 5 | 24.25±1.7 | 23.39±2.6 | 21.29±3.6 | 20.96±2.8 |
| 7 | 23.48±2.1 | 22.52±2.6 | 20.44±2.8 | 19.01±2.1 |
| 9 | 22.67±3.6 | 21.54±3.1 | 17.48±1.6 | 16.84±0.6 |
| 12 | 20.48±3.3 | 19.61±3.3 | 12.65±2.1 | 9.49±0.8 |
| 15 | 19.32±3.2 | 18.38±2.6 | 9.79±1.3 | 8.78±0.2 |
| After ripening for 15 days | 11.84±1.5 | 11.85±2.1 | 7.93±1.4 | 6.83±0.4 |

**Table 5. Effect of pulsed electric field (PEF) charged to the winemaking cultures on antioxidant activity**

| Incubation time (day) | Antioxidant (mM $Fe^{2+}$) | | | |
|---|---|---|---|---|
| | Conventional condition | 2 V PEF | 3 V PEF | 4 V PEF |
| 0 | 1.280±0.1 | 1.280±0.1 | 1.280±0.1 | 1.280±0.1 |
| 1 | 1.125±0.2 | 1.169±0.2 | 1.136±0.2 | 1.129±0.2 |
| 3 | 1.167±0.2 | 1.323±0.3 | 1.211±0.2 | 1.297±0.3 |
| 5 | 1.349±0.1 | 1.455±0.4 | 1.517±0.3 | 1.418±0.3 |
| 7 | 1.423±0.2 | 1.594±0.1 | 1.606±0.2 | 1.781±0.2 |
| 9 | 1.884±0.3 | 1.993±0.1 | 1.776±0.3 | 1.886±0.2 |
| 12 | 1.913±0.3 | 2.133±0.3 | 1.859±0.1 | 1.827±0.1 |
| 15 | 1.968±0.2 | 2.421±0.2 | 1.933±0.2 | 1.845±0.3 |
| After ripening for 15 days | 1.981±0.2 | 2.465±0.1 | 1.924±0.1 | 1.877±0.1 |

and antioxidation activity of the test wines produced in 2 V of LIPEF were a little higher than those produced in other conditions.

Various chemical components contained in the winemaking culture or the test wines are originated from grape (grape must); however, which may be altered by the biochemical reactions during fermentation and ripening based on the HPLC chromatogram. Standard compounds of catechin, tannic acid, and malate were selectively separated and detected by HPLC, which was compared with the wine-specific and grape must-specific compounds as shown in Fig. 3. Some wine-specific peaks around 5.2 min of retention time were commonly observed in the chromatograms of the test wines in comparison with 2 French wines but not, instead, malate-specific peak was remarkably appeared in the chromatogram of grape must. The grape must-specific peaks observed in retention time from 7 to 21 min were selectively detected in the test wines produced in the conventional condition and 2 V of PEF, which may be a factor to discriminate from those produced in 3 and 4 V of LIPEF. The catechin or tannic acid-similar peaks found in the chromatograms for the test wines or French wines does not indicate that the chemical components of the test wines are catechin or tannic acid but shows that those may belong in a category of the wine-specific compounds.

In conclusion, a new technique may be not necessarily required for the traditional winemaking process that has been successfully succeeded for a long time; however, the negative factors influencing the wine quality and taste may be still remained and the subject to be studied. The sulfite addition for control of bacterial contaminant, the malolactic fermentation for convert of malate or citrate to lactate, and

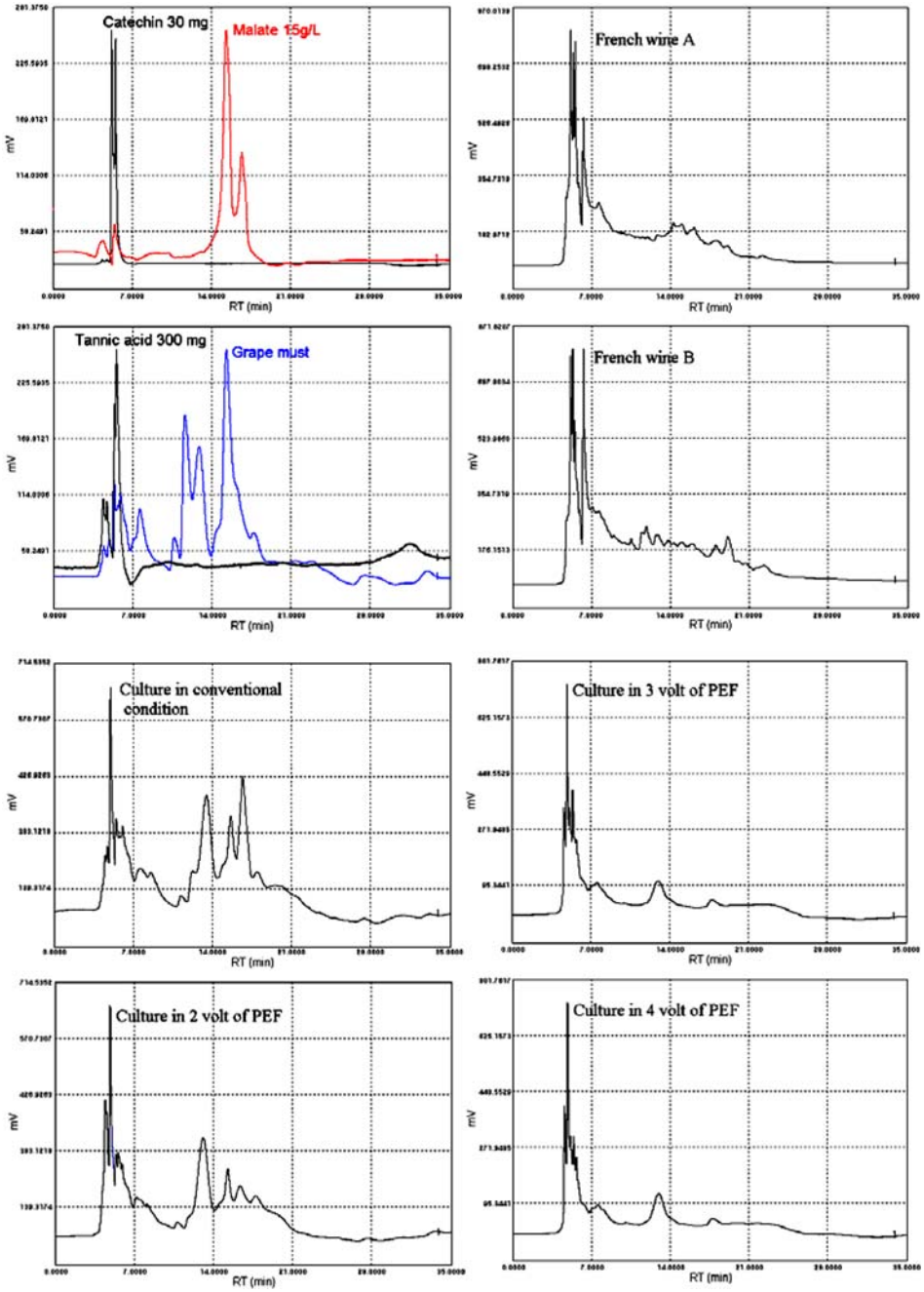


**Fig. 3. HPLC chromatograms of grape must and test wines produced in the conventional condition (CC), 2, 3, and 4 V of pulsed electric field (PEF).** Malate, tannic acid, catechin, and French wines were analyzed to compare with the test wines.

the long-term incubation for complement of low fermentation rates may be the major factors to be studied. The chemical components contained in various wines also may be the subject of study because some of them may be negatively influence the wine taste and quality.

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