POLAROGRAPHIC DETERMINATION OF α-KETOACIDS IN WINE, AFTER DERIVATIZATION WITH o-PHENYLENEDIAMINE

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Abstract

A method for the determination of α-ketoacids was developed, involving derivatization with o-phenylenediamine and the differential-pulse polarographic determination of the resulting hydroxyquinoxalines. The method has a detection limit of about 5×10^{-7} M, a concentration that is lower than the usual concentrations of the α-ketoacids in fermented products; it is also possible to distinguish glyoxylic acid from pyruvic acid and α-ketoglutaric acid, with no need for a separation procedure.

Using the method developed in the direct analysis of a white wine, practically no glyoxylic was found and a total concentration of 3×10^{-5} M was obtained for the whole of pyruvic acid and α-ketoglutaric acid.

Introduction

α-ketoacids are acids that have a carbonyl group adjacent to a carboxylic group. α-ketoacids exist as intermediary compounds in many essential metabolic procedures, like glycolysis, the Krebs cycle, transaminations involved in the metabolism of all the aminoacids and several other metabolic ways[1].

In clinical analysis, the determination of α-ketoacids is used in the investigation and in the diagnosis of deficiencies in enzymes, specially those involved in the metabolism of aminoacids[2]. The number of applications in food analysis is limited; in wines, for instance, the presence of α-ketoacids can interfere with the operation of adding sulphite due to their tendency to produce stable compounds with sulphite ion[3], with a consequent decrease in the concentration of free sulphite.

The amount and type of α-ketoacids present in wine change during the operation of fermentation, depending on some vinification conditions, as temperature, aerating and pH[4]. Another important aspect is the possibility of correlating the maturing of grapes at the harvest with the levels of pyruvic acid and α-ketoglutaric acid found during the fermentation. In fact, rotten grapes have a lowering effect on the level of thyamine and, as this compound is a vital co-factor for the normal activity of the enzymes pyruvate carboxylase, pyruvate dehydrogenase and α-ketoglutaric dehydrogenase, the activity of these enzymes is lowered, with the consequent accumulation of the respective α-ketoacids.

Generally, the methodologies used in the determination α-ketoacids are different in clinic analysis and in food analysis. In the analytical method more frequently used in clinical analysis the α-ketoacids are derivatized with o-phenylenediamine (OPDA) and the hydroxyquinoxalines formed are separated and determined using HPLC with fluorimetric detection[5]. This procedure is quite appropriate, as the derivatization reaction is selective for α-ketoacids and can be performed in aqueous solution at room temperature. In the case of food analysis, as it is generally sufficient to know the overall amount of the main α-ketoacids, the use of enzymatic methods is preferably used[4].

In this work, a polarographic method for the determination of α-ketoacids was developed, also involving a previous derivatization with OPDA. Studies were conducted using pyruvic, α-ketoglutaric and glyoxylic acid, as in the case of foods these are the more important α-ketoacids[3].

The possibility of polarographic differentiation between the hydroxyquinoxalines derived from the three α-ketoacids was tried, with the objective of their simultaneous determination with no need for a separation procedure. This goal was partly accomplished, as the polarographic peak obtained with derivatized glyoxylic acid is perfectly distinguishable from the other two.The method was applied directly to wine samples, with success, as the interference of the matrix was very small.

Experimental

Instrumentation

Polarographic determinations were made by differential-pulse polarography using a Metrohm system consisting on a 646 VA-Processor and a 647 VA-Stand. A Metrohm multi-mode mercury electrode (MME) was employed as working electrode, a platinum bar and a silver/silver chloride (3M KCl) were employed as counter and reference electrode, respectively.

A Hitachi U-2000 model double beam spectrophotometer and a Shimadzu RF 3001PC model spectrofluorometer were used for the spectrometric and spectrofluorimetric measurements.

Reagents and solutions

All chemicals used were of analytical grade. Desionized and distilled water was used for preparation of solutions. Stock solutions of sodium pyruvate, sodium α-ketoglutarate and glyoxilic acid were prepared from the commercial products obtained from Aldrich. The 0.2% o-phenylenediamine derivatization solution in 0.05M HCl was prepared fresh daily.

Procedures

The recommended procedure for the determination of α-ketoacids in wine by polarography is as follows:

1. 10mL of 0.2% OFDA derivatization solution and 0.5mL of wine sample were added to a 25 mL volumetric flask;
2. after 30min of reaction, the pH was adjusted to 9 by addition of 5mL of 0.2M NH_4Cl solution and 5mL of 0.2M NaHO solution; the volume of the flask was completed with water.
3. the solution was transferred to the polarographic cell.

The differential-pulse polarographic determinations were performed in deoxygenated solutions (by bubbling nitrogen for 10 min) using the following polarographic conditions for the potential scan: tdrop=0.6s; pulse amplitude=-50mV; scan rate=10mV/s. The quantification was made using the standard addition method.

Results and Discussion

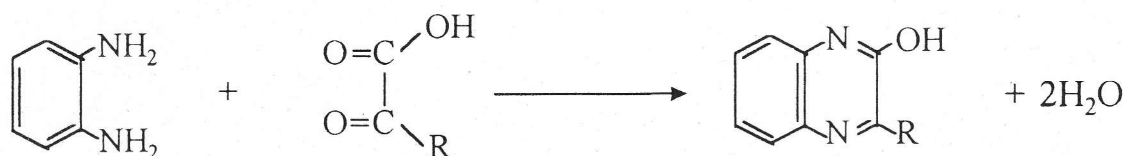
The work consisted in the development of a voltammetric method of analysis of α-ketoacids, using differential-pulse polarography, and in its application on the determination of those compounds in wines. The development of the polarographic method was divided in two parts, one involving the study of the derivatizing reaction of the α-ketoacids with OPDA and the other consisting in the

optimization of the polarographic conditions for the analysis of the resulting hydroxyquinoxalines (Figure 1).

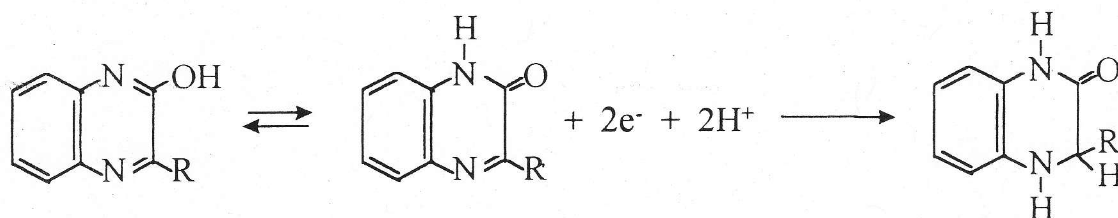
Derivatization reaction

The derivatization reaction of α-ketoacids with OPDA was studied, with the objective of trying to obtain, simultaneously, a high percentage of conversion and a short reaction time. The reaction was performed at room temperature and in the dark, due to the low stability of OPDA solutions at high temperatures and to light. The influence of the reaction pH and of the amount of the derivatizing agent were studied. Only in acid medium the extension of the reaction is significative, with a maximum yield for a pH between 1 and 2 (Figure 2). Concerning the derivatizing agent, it was found that there is no increase in the conversion for OPDA concentrations higher than 0.05%; although the time reaction is still decreasing for higher concentrations, the concentration of OPDA was limited to 0.2%, because the interference of its degradation products becomes significative for higher concentrations.

a) derivatization reaction[6]



b) polarographic analysis[7]



R	α-ketoacid	Derivatization product
H	glyoxylic acid	2 - hydroxyquinoxaline
CH_3	pyruvic acid	2-hydroxy-3-methylquinoxaline
CH_2CH_2COOH	α-ketoglutaric acid	3-carboxyethyl-2-hydroxyquinoxaline

Figure 1. Schematic representation of the analytical procedure used in the determination of α-ketoacids.

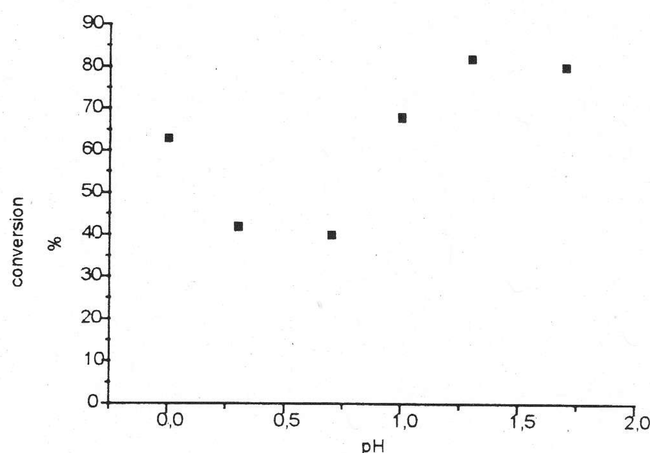


Figure 2. Influence of reaction pH on the extension of the derivatization of a solution $3x10^{-6}$ M in pyruvic acid. Concentration of OPDA = 0.05 %

In Table 1 we can see the conditions that were adopted for the derivatization of the three α-ketoacids. The degree of conversion was calculated comparing the polarographic signals obtained for the derivatized α-ketoacids with those obtained for the corresponding hydroxyquinoxalines. For concentrations of the α-ketoacids betwwen 10^{-5} M and 10^{-6} M, the variation on the degree of conversion was not significant. Reaction time was defined as the time needed for the stabilization of the signal of the derivatized α-ketoacid. The degree of conversion for the derivatization of α-ketoglutaric acid was not determined, because we could not obtain a standard of 3-carboxymethyl-2-hydroxyquinoxaline. This was not a problem, as the variation of the polarographic signal with concentration was linear; in fact, in analytical terms, the knowledge of the degree of conversion is not needed, as far as its value remains constant for the concentration range considered. The evaluation was made using the standard addition method.

In the case of the derivatization of pyruvic acid, some comparative studies using spectrophotometric and fluorimetric[8] detection were also made, and the results were similar to the polarographic ones. Polarography proved even to be advantageous, due to its relative insensitivity to OPDA instability, a problem that affects seriously the other two techniques.

α-ketoacids	derivatization medium	derivatizing agent (%)	reaction time	degree of conversion
glyoxylic acid	HCl 0.05 M	0.02	20 min.	100 %
α-ketoglutaric acid	HCl 0.05 M	0.02	25 min.	----------
pyruvic acid	HCl 0.05 M	0.02	25 min.	80 %

Table 1. Conditions developed for the derivatization of α-ketoacids

Polarographic method of analysis

In the polarographic analysis of the three derivatized α-ketoacids, a linear relationship between current intensity and concentration was only obtained for a pH equal or higher than 9; nevertheless, in this region sensitivity decreases markedly with pH increase. The results obtained with 2-hydroxy-3-methylquinoxaline (derivatized pyruvic acid) can be seen in Figure 3. The lack of linearity in acid or neutral medium can be attributed, probably, to distortions due to strong adsorption of the compounds at the surface of the mercury electrode[9].

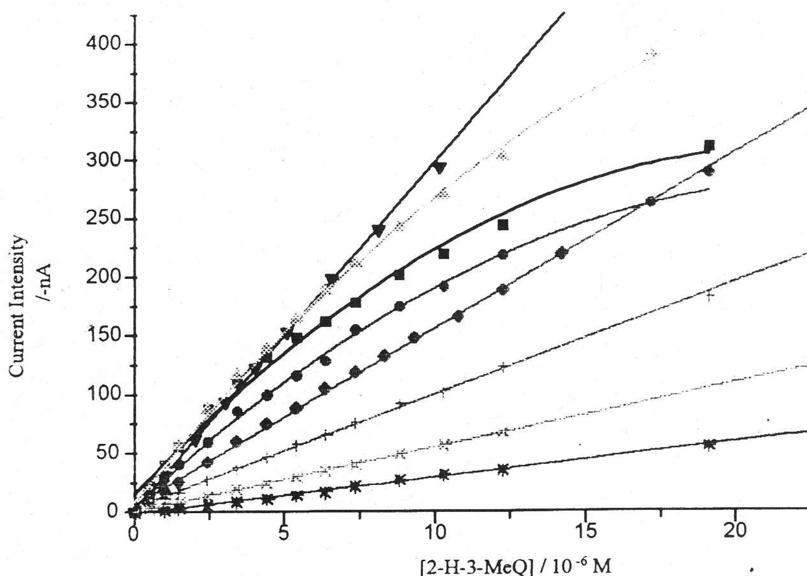


Figure 3. Calibration curves for 2-hydroxy-3-methylquinoxaline at different pH values, in the presence of OPDA 0.05%. pH: ■ - 1.0; ● - 4.5; ▲ - 7.0; ▼ - 9.0; ◆- 9.5; + - 10.0; × - 10.5; + - 12.0.

Concerning the simultaneous determination of the three α-ketoacids, we were not able to distinguish between pyruvic acid and α-ketoglutaric acid, due to the similarity of the polarographic peaks of the corresponding hydroxyquinoxalines. Nevertheless, glyoxylic acid can be distinguished from the other two α-ketoacids, as the peak potential of its derivatization product is different (Figure 4); it is important to note that this difference does not change with pH. Polarographic analysis was performed in pH = 9 ammonia buffer solution, allowing a detection limit of about $5x10^{-7}$ M.

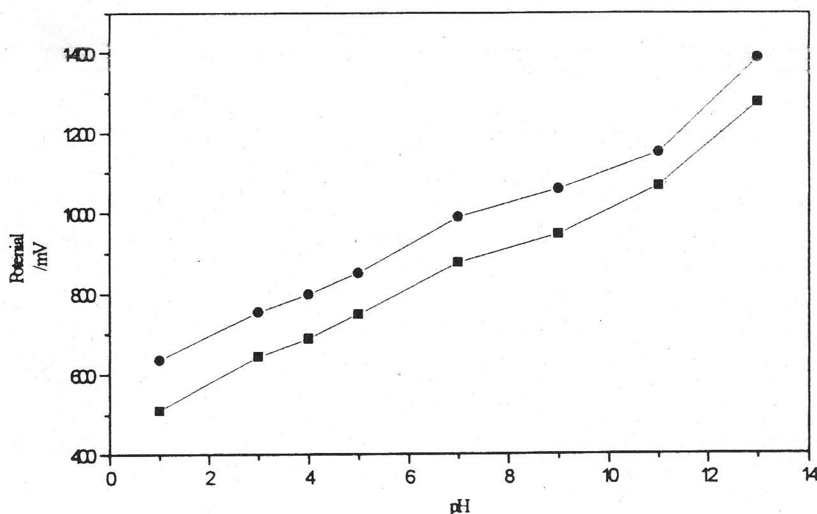


Figure 4. Influence of pH in peak potential, in the differential-pulse polarographic analysis of the hydroxyquinoxalines formed by derivatization with OPDA of: ● - pyruvic acid; ■ - glyoxylic acid.

Determination of α-ketoacids in wine

One of the main advantages of the polarographic method when compared with the spectrophotometric or the fluorimetric is the absence of matrix interferences, as we can see in Figure 5.a; the interference of OPDA is also quite small (Figure 5.b). The potentials of the peaks obtained after derivatization (Figure 5.c) suggest that the second peak must be due to derivatized pyruvic or α-ketoglutaric acids, with the first peak being attributed to derivatization products of other α-dicarbonyl compounds, like α-diketones or α-ketoaldehydes, that are easily reduced[10]. With a convenient addition of standards, it was possible to test these suppositions (Figure 5.d). In fact, it was confirmed that the first peak must be due to derivatized α- ketoaldehydes, like, for instance, methylglyoxal, and that the second peak must correspond to derivatized pyruvic acid or α-ketoglutaric acid or to a mixture of both, with an overall concentration of about $3x10^{-5}$ M; it is also clear from the figure that the amount of glyoxylic acid in the wine is negligible.

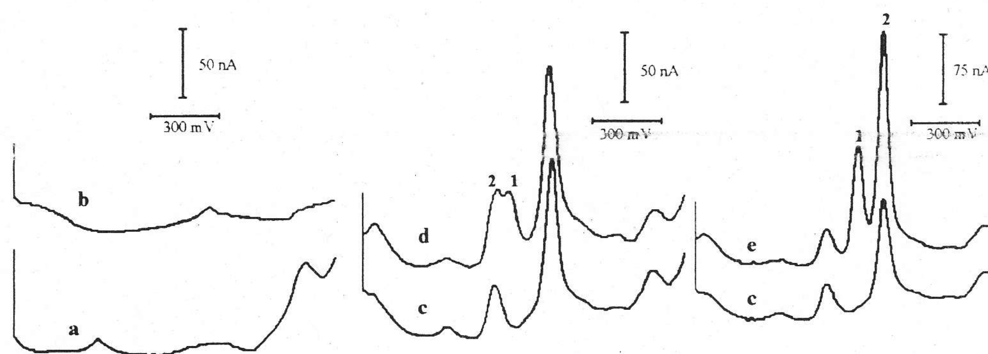


Figure 5. Identification and quantitation of α-dicarbonyl compounds in a sample of white wine, using the method of standard additions. Derivatization with 0.02% OPDA in HCl 0.05 M and polarografic determination at pH 9; initial potential = -200 mV. **5.a** - wine with no addition of OPDA; **5b** - OPDA solution with no addition of wine; **5.c** - wine with addition of OPDA solution; **5d** - addition of 2 standards: 1 - 2,3-dimethylquinoxaline (derivatized diacetyl) $4.2x10^{-6}$ M; 2 - 2-methylquinoxaline (derivatized methylglyoxal) $1.5x10^{-6}$ M; **5e** - addition of 2 standards: 1 - 2-hydroxyquinoxaline (derivatized glyoxylic acid) $6.0x10^{-6}$ M; 2 - 2-hydroxy-3-methylquinoxaline (derivatized pyruvic acid) $5.1x10^{-6}$ M.

Conclusions

α-ketoacids can be easily determined by differential-pulse polarography, after being converted into the corresponding hydroxyquinoxalines by reaction with o-phenylenediamine.

The detection limit of the polarographic method ($5x10^{-7}$ M) is well below the normal concentration of the compounds in fermented products.

Glyoxilic acid can be determined in the presence of pyruvic acid or α-ketoglutaric acid, with no need for any separation procedure.

Whenever it is possible and desirable to establish a correlation between the quality of a product and the nature and amount of α-ketoacids present in the product, the method developed can be very useful as a preliminary form of characterization, due to its simplicity.

Acknowledgments

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