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Revisiting the fundamentals of *p*-nitrophenol analysis for its application in the quantification of lipases activity. A graphical update

Revisando los fundamentos del análisis de p-nitrofenol para su aplicación en la cuantificación de la actividad de lipasas. Una actualización gráfica.

Revisão dos fundamentos da análise do p-nitrofenol para sua aplicação na quantificação da atividade da lipase. Uma atualização gráfica.

Randall Syedd-León
randall.syedd.leon@una.ac.cr
Maestría en Manejo de Recursos Naturales
Universidad Estatal a Distancia
Mercedes de Montes de Oca, Costa Rica
Laboratorio de Investigación en Biorrefinería,
Escuela de Química.
Universidad Nacional.
Heredia, Costa Rica.
Orcid: http://orcid.org/0000-0002-6746-9298

Manuel Sandoval-Barrantes
manuel.sandoval.barrantes@una.ac.cr
Laboratorio de Investigación en Biorrefinería, Escuela de
Química.
Universidad Nacional.
Heredia, Costa Rica.
Orcid: http://orcid.org/0000-0002-2946-8016

Humberto Trimiño-Vásquez
humberto.trimino.vasquez@una.ac.cr
Laboratorio de Investigación en Biorrefinería, Escuela de
Química.
Universidad Nacional.
Heredia, Costa Rica.
Orcid: https://orcid.org/0000-0002-0373-2923

Luis Roberto Villegas-Peñaranda
luis.villegas.penaranda@una.ac.cr
Laboratorio de Investigación en Biorrefinería, Escuela de
Química.
Universidad Nacional.
Heredia, Costa Rica.
Orcid: https://orcid.org/0000-0002-3748-9554

Gerardo Rodríguez-Rodríguez
garodriguezr57@gmail.com
Laboratorio de Fitoquímica, Escuela de Química.
Universidad Nacional.
Heredia, Costa Rica.
Orcid: http://orcid.org/0000-0002-1224-0277

Abstract

p-Nitrophenol (*p*NP) is a widely used compound for analytical determinations of several esterases (EC. 3.1.1.X), including lipases (E.C. 3.1.1.3). Most enzymatic measurements employ *p*NP derivatives such as esters, which are broken down by enzymatic hydrolysis, releasing *p*NP that is quantified by its absorbance at 410 nm. Although this type of methods was developed a few decades ago, the spectrophotometric analysis of *p*NP requires analytical measurements of pH and temperature to achieve reliable determinations. The aim of this paper is to offer a graphical update of how pH and temperature affect the *p*-nitrophenol absorbance at different wavelengths in lipase emulsified media, due to its relevance for the quantitative determination of lipase activity using spectrophotometric methods. To highlight the importance of each variable involved in this analysis, we dissolved *p*NP in emulsified media (for lipase activity quantification) at several pH values from 4.00 to 11.00, and measured its absorbance in a range of 270 nm – 500 nm and at several temperatures from 25°C to 50°C. The absorption patterns of *p*NP under the established conditions were graphed in 3D plots. The constructed 3D plots showed that, regardless of the temperature, below pH 6.00, *p*NP predominantly absorbs at 317 nm, due to the greater abundance of its protonated form, which is completely predominant at pH 3.50 and below. On the other hand, at pH 10.0 and above, the major absorption occurs at about 401 nm, confirming that the equilibrium is completely shifted to the *p*NP anionic form. These results also indicate that close to neutral pH value *p*NP, it displays a temperature dependence effect, increasing absorbance to 410 nm at higher temperatures. Due to many analytical determinations of enzymatic activities, the release of *p*NP is carried around pH 7.00. It is necessary to consider the determinant role of both pH and temperature over these measurements, how these variables must be strictly controlled, and how the calibration curves and blanks should take the reaction media pH and temperature into account.¨

Keywords: *p*-Nitrophenol; lipase; absorbance pH-dependence; temperature dependent dissociation; 3D pH-wavelength-absorbance spectra.

Resumen

El *p*-nitrofenol (*p*NP) es un compuesto ampliamente utilizado para la determinación analítica de esterasas (EC. 3.1.1.X), incluidas las lipasas (E.C. 3.1.1.3). La mayoría de las mediciones enzimáticas emplean sus derivados, tales como ésteres, que se descomponen por hidrólisis, liberando *p*NP que se cuantifica por su absorbancia a 410 nm. Aunque, este tipo de métodos se desarrolló hace algunas décadas, el análisis espectrofotométrico de *p*NP requiere mediciones precisas de pH y temperatura, para lograr determinaciones confiables. El objetivo de este trabajo es ofrecer una actualización gráfica de cómo el pH y la temperatura afectan la absorbancia de *p*-nitrofenol a diferentes longitudes de onda en medios emulsionados para ensayos con lipasas, debido a su relevancia para la determinación cuantitativa de la actividad de las lipasas bajo métodos espectrofotométricos. Para resaltar la importancia de cada variable en este análisis, se disolvió *p*NP en medios emulsionados (para la cuantificación de la actividad de la lipasa) a varios valores de pH de 4.00 a 11.00 y se midió su absorbancia en un rango de 270 nm a 500 nm a varias temperaturas en el rango de 25 °C a 50 °C. El comportamiento químico de *p*NP bajo estas condiciones se correlacionó mediante la construcción de gráficos tridimensionales. Como resultado, los gráficos 3D construidos experimentalmente, mostraron que, independientemente de la temperatura, por debajo de pH 6.00 el *p*NP absorbe principalmente, a 317 nm, debido a la mayor abundancia de su forma protonada que es

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completamente predominante a pH 3.50 y inferiores. Por otro lado, a pH 10.0 y superiores, el equilibrio se desplaza completamente a la forma aniónica pNP, que absorbe a 410 nm. Nuestros datos confirman que en valores de pH cercano a la neutralidad, el pNP muestra un efecto de dependencia a la temperatura, aumentando la absorbancia a 410 nm a temperaturas más altas. Debido a muchas determinaciones cuantitativas de las actividades enzimáticas, el pNP se libera en un medio de reacción de alrededor de pH 7.00. Es necesario recordar el papel determinante del pH y la temperatura sobre estas mediciones y cómo estas variables deben ser estrictamente consideradas.

Palabras clave: p-nitrofenol, lipasa; absorbancia dependiente del pH; disociación dependiente de la temperatura; espectro 3D de absorbancia; pH; longitud de onda

Resumo

O p-nitrofenol (pNP) é um composto amplamente utilizado para a determinação analítica de esterases (EC. 3.1.1.X), incluindo lipases (E.C. 3.1.1.3). A maioria das medições enzimáticas utiliza seus derivados, como ésteres, que se decompõem por hidrólise, liberando pNP quantificado por sua absorvência a 410 nm. Embora esses tipos de métodos tenham sido desenvolvidos há algumas décadas, a análise espectrofotométrica de pNP requer medições precisas de pH e temperatura para obter determinações confiáveis. O objetivo deste trabalho é oferecer uma atualização gráfica de como o pH e a temperatura afetam a absorvência do p-nitrofenol em diferentes longitudes de onda em meios emulsionados para ensaios de lipase, devido a sua relevância para a determinação quantitativa da atividade das lipases sob métodos espectrofotométricos. Para destacar a importância de cada variável nesta análise, o pNP foi dissolvido em meio emulsionado (para quantificação da atividade da lipase) em vários valores de pH de 4,00 a 11,00 e sua absorvência foi medida na faixa de 270 nm a 500 nm a várias temperaturas na faixa de 25 °C a 50 °C. O comportamento químico do pNP nessas condições foi correlacionado mediante a construção de gráficos tridimensionais. Como resultado, os gráficos 3D construídos experimentalmente mostraram que, independentemente da temperatura, abaixo de pH 6,00, o pNP absorve principalmente a 317 nm, devido à maior abundância de sua forma protonada, que é completamente predominante em pH 3,50 e inferiores. Por outro lado, o pH 10,0 e superiores, o equilíbrio muda completamente para a forma aniônica pNP, que absorve a 410 nm. Nossos dados confirmam que em valores de pH próximos à neutralidade, o pNP mostra um efeito de dependência da temperatura, aumentando a absorvência a 410 nm em temperaturas mais altas. Em virtude de muitas determinações quantitativas de atividades enzimáticas, o pNP é liberado em um meio de reação de cerca de pH 7,00. É necessário lembrar o papel determinante do pH e da temperatura nessas medições e como essas variáveis devem ser estritamente consideradas.

Palavras-chaves: p-nitrofenol; lipase; absorvência dependente do pH; dissociação dependente da temperatura; espectro de absorvência 3D; pH; longitude de onda
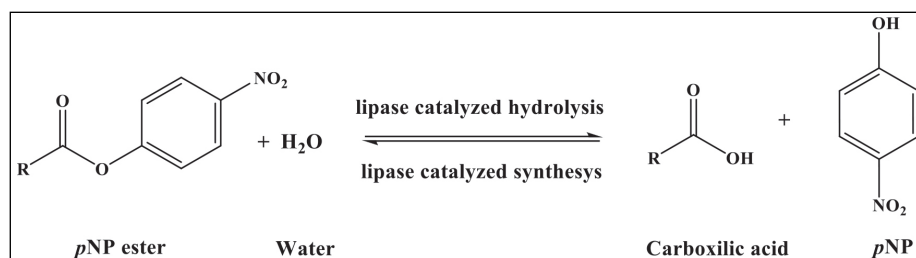
Introduction

Esterases (acyl hydrolases, EC. 3.1.1.X) and more specifically lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are a widely researched group of enzymes, due to their application as green catalysts in the regioselective transesterification, hydrolysis or synthesis of acyl groups aiming to prepare fine chemicals and pharmaceuticals (Daiha, Angeli, de Oliveira, & Almeida, 2015; Kapoor & Gupta, 2012; Kumar, Dhar, Kanwar, & Arora, 2016; Liu, Zheng, Zhang, & Zheng, 2012; Lopes, Fraga, Fleuri, & Macedo, 2011).

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Analytical determinations of lipase activity are usually carried on using p-nitrophenol (pNP) esters as substrates, such as p-nitrophe-

Scheme 1. Lipase catalyzed synthesis or hydrolysis of pNP ester.

nyl butyrate, p-nitrophenyl laurate, and p-nitrophenyl palmitate. The reaction is generally carried out in emulsified media. In one of the classical methods of lipase activity determination (Winkler & Stuckmann, 1979), the hydrophobic ester p-nitrophenyl palmitate is dissolved in isopropanol and then mixed with the buffer of choice, also containing gum arabic and sodium deoxycholate as stabilizers. In this method, due to the excess of water, the equilibrium is shifted toward the hydrolysis reaction. Since then, several modifications to improve the method have been published (Hernández-García, García-García, & García-Carmona, 2017; Mayordomo, Randez-Gil, & Prieto, 2000; Pratama, Helianti, Suryani, & Wahyuntari, 2017).
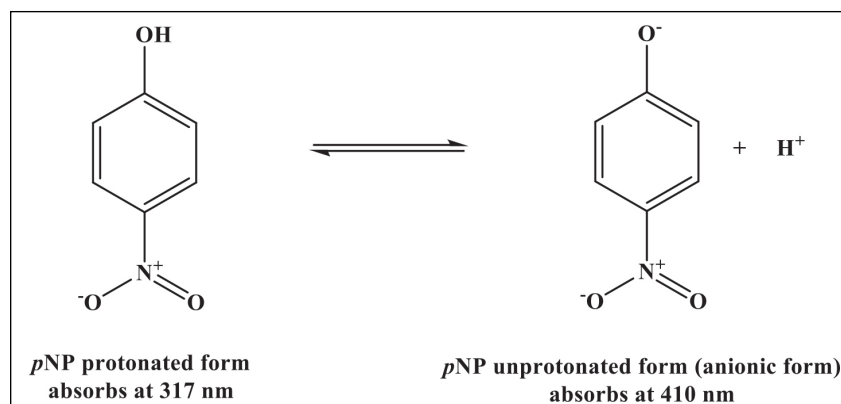
After the enzymatic hydrolysis, pNP, as well as the carboxylic acid forming the original pNP ester, are released to the emulsion (Scheme 1) and the released pNP is quantified spectrophotometrically measuring its absorption at 405-420 nm (Alcantara et al., 2014; Farnet et al., 2010; Stoytcheva, Montero, Zlatev, A. Leon, & Gochev, 2012).
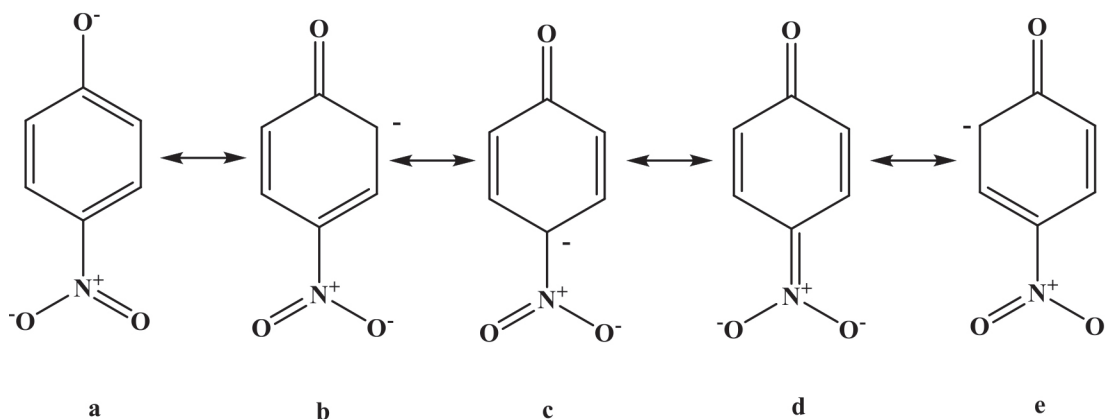
In alkaline conditions, the phenol group releases one proton to the media to form a phenolate base (see Scheme 2). Once formed, the phenolate base undergoes a resonance equilibrium (see Scheme 3). The unprotonated resonant forms absorb in the range from 400 to 420 nm (Biggs, 1954).

The first study describing the dependence of pNP absorbance on the pH of the solution (Biggs, 1954) found that there are two absorption maxima: the first one, in the 310-320 nm range occurs at low pH and is due to the presence of a completely protonated form, while the second one at around 420 nm appears at alkaline pH, due to the presence of a completely deprotonated form. The currently accepted value for the pKa for the pNP dissociation in water at 25°C (see Scheme 2) was established as 7.15 (Serjeant & Dempsey, 1979).

Scheme 2. Acid dissociation equilibrium of pNP.

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Scheme 3. Contributors to the resonance hybrid from the anionic form of pNP.

Since dissociation constants are pH-dependent and may also be affected by temperature, it is necessary to access how pH and temperature may affect pNP determination in aqueous emulsions. On this regard, Prof. Colas group warned in 1999 that spectrophotometric determinations using aqueous solutions of pNP derivatives may be affected by both temperature and pH, and pointed out the importance of considering these effects on the absorbance of the released pNP (Fourage, Helbert, Nicolet, & Colas, 1999). In the present work, the pNP dissociation concept is revisited, by showing the interrelationship of pH, temperature and pNP absorbance in emulsified media, by means of three-dimensional plots. This work aims to offer a graphical update of the most fundamental concepts in the quantification of pNP emulsions for esterase and lipase researchers.

Based on widely used methods to prepare p-nitrophenyl palmitate emulsions (Gupta, Rathi, & Gupta, 2002; Orlando Beys Silva, Mitidieri, Schrank, & Vainstein, 2005; Palacios, Busto, & Ortega, 2014), a traditional emulsifying media for p-nitrophenyl palmitate was selected for this study. In this paper, the effects of pH and temperature in the absorption spectra of pNP dissolved in an assay mixture for the determination of lipase are discussed.

Materials and methods

Materials: p-nitrophenol (pNP), gum arabic, triton X-100, isopropanol, and sodium dibasic phosphate were purchased from Sigma Aldrich. Other chemical reagents were of analytical grade.

Solutions A. Buffered lipase assay emulsions: All Solutions A (buffer solutions) were freshly prepared by dissolving 550 mg of gum arabic in 1 L of the 50 mM buffer (for each corresponding pH) and mixing to achieve a complete solution. These solutions were 0.55 g/L gum arabic and 50 mM in the corresponding buffer.

Thirteen buffer solutions were prepared for this work: five 50 mM citric acid/disodium phosphate buffer solutions with pH of 4.00, 4.50, 5.00, 5.50 and 6.00; five 50 mM disodium phosphate buffer solutions with pH of 6.50, 7.00, 7.50, 8.00; and 8.50 and three 50 mM sodium carbonate buffer solutions with pH of 9.00, 10.00 and 11.00.

Solution B. Detergent solution: 1 volume of Triton X-100 was dissolved with 4 volumes of isopropanol and thoroughly mixed using a Vortex mixer.

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Buffered *p*NP preparations: To achieve the final emulsion, 20 mL of Solution A (buffered lipase assay emulsion) were mixed with 2.00 mL detergent solution. This final emulsion was used as the solvent to prepare a 2.40 mM *p*NP emulsion (13 pH values from 4.0 to 11.0 were tested).

Analytical procedure: Aliquots amounting to 15 μL of *p*NP emulsion were placed in microplate wells, then 285 μL of the respective buffer was added to each well and the plate was stirred for mixing. The final *p*NP concentration in each well was 0.120 mM. All solutions and emulsions were freshly prepared prior to their use, pH in the microplate was assumed as the same value of the added buffer.

UV–visible spectrophotometric determinations were recorded on a Synergy™ HT Multi-Detection Microplate Reader. The pH values were measured at 25°C using a HI 2211 pH meter (Hanna Instruments).

At each pH value, three *p*NP samples were prepared as described above and absorption spectra ranging from 270 nm to 500 nm, with a 2 nm variation per reading, were run for each microplate well. Surfactant solutions without *p*NP were also analyzed to study their effect in the absorbance of this analyte.

At each pH, samples in microplates were progressively heated to reach each of 6 different temperatures: 25°C, 30°C, 35°C, 40°C, 45°C and 50°C. For each temperature, the mixture was previously incubated at the required temperature for 5 minutes before the spectrophotometric measurement. Overall errors in curves were less than 0.3%.

Absorbance blanks were performed at different pHs and temperatures with only surfactant emulsion.

Graphical analysis: Data obtained from spectrophotometric measurements were analyzed in three dimensional plots correlating three of the following variables: temperature, pH, wavelength, and absorbance. Plots were made using Origin Pro 5.0 software (OriginLab, Massachusetts, USA).

Results and discussion

As shown in Figure 1, the absorbance maxima at 35°C and different pHs for the surfactant emulsifier were around 290 nm.

Also, in Figure 2, it can be seen that at pH 5 and different temperatures, the absorbance maxima for the surfactant emulsifier were also around 290 nm. The fact that these absorbance maxima, at different temperature and pH conditions, do not appreciably change, can be considered as evidence of the surfactant emulsifier stability under these reaction conditions. These results showed that the emulsifying medium employed in lipase measurements does
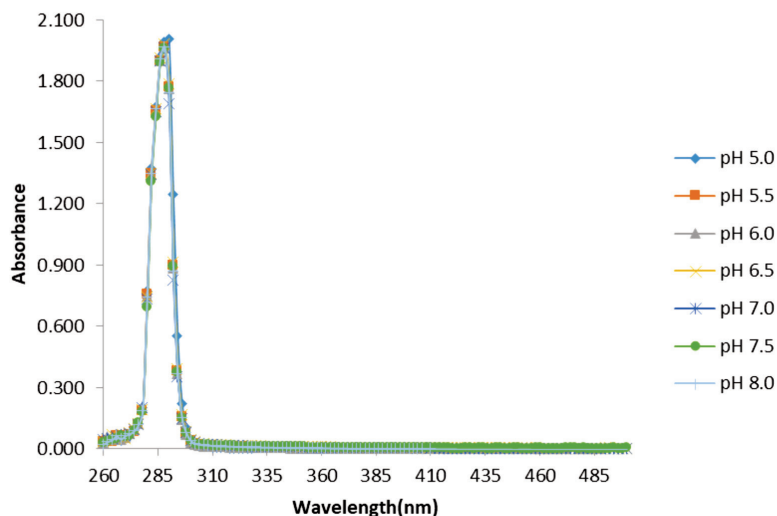

Figure 1. Absorbance spectrum from surfactant solutions at 35°C and different pH values. *Source*: own research.
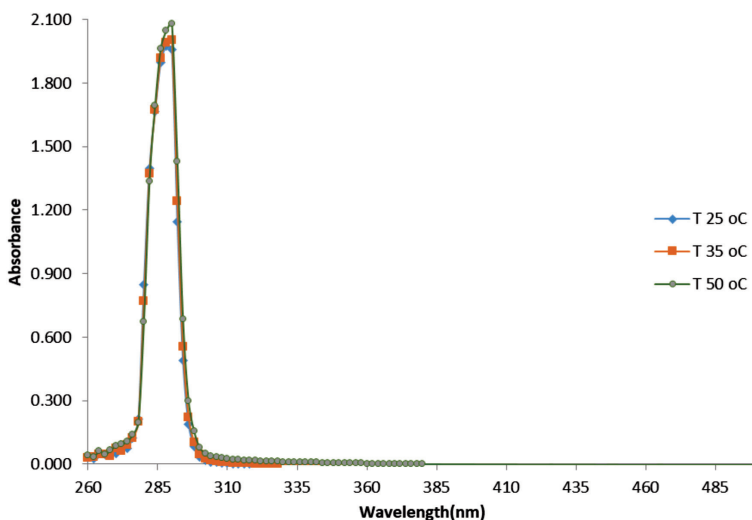
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Figure 2. Absorbance spectrum from surfactant solutions at pH 5.00 and different temperatures. *Source*: own research.

At pH 6.00 and lower, there is a very low concentration of unprotonated yellow form of pNP, making it difficult to measure spectrophotometrically at 410 nm. Therefore, it would be more convenient to measure, at pH 6.00 and below, the UV absorbance at 317 nm as it has been reported in scientific papers related to the inorganic chemistry field (Shen, Sun, Zhou, Li, & Yeung, 2014; Tang *et al*., 2011; Zhang *et al*., 2013). However, this wavelength is not normally reported in lipase and biocatalysts research papers, probably due to the fact that it is close to absorbance maxima of the pNP esters. In the case of p-nitrophenyl palmitate the absorbance maximum, according to our experimental
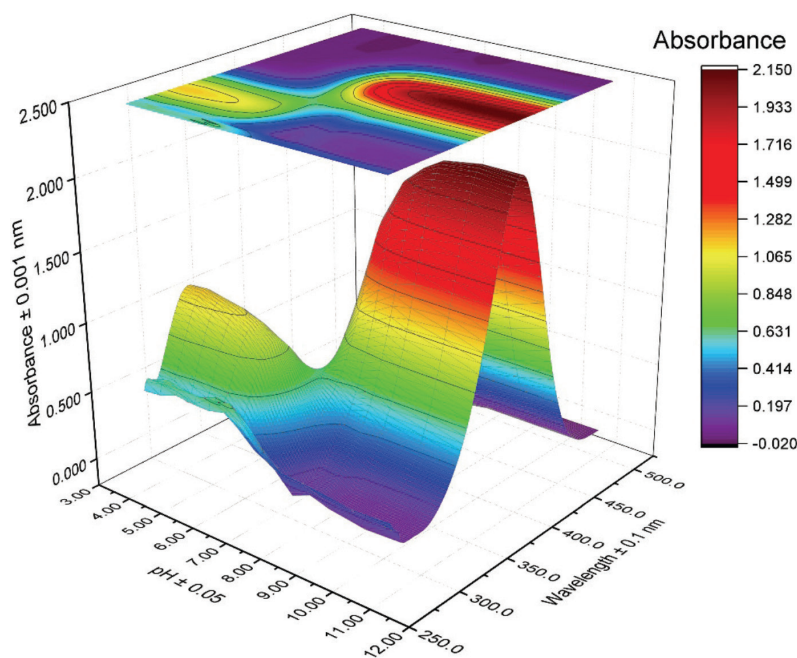
not interfere with the analysis of pNP in the different pH and temperatures tested, which can. Thus, triton X-100, isopropanol and gum arabic act as proper emulsifying agents for these types of substrates, due to their capacity to stabilize pNP esters in aqueous emulsions, their stability at different pHs and temperature conditions, and their null effect on pNP spectrophotometric quantification.

As shown in Figure 3, at pH 6.00 and lower, the pNP absorbance maxima are in the 310 nm to 320 nm band, due to the relative abundance of the protonated form of this substance. On the other hand, at alkaline pH 7.00 and higher, due to the abundance of anionic form, the pNP absorbance maxima are in the 400 nm to 420 nm range. (Fourage *et al*., 1999).

Figure 3. pH effect over pNP absorption spectra at 25°C. *Source*: own research.

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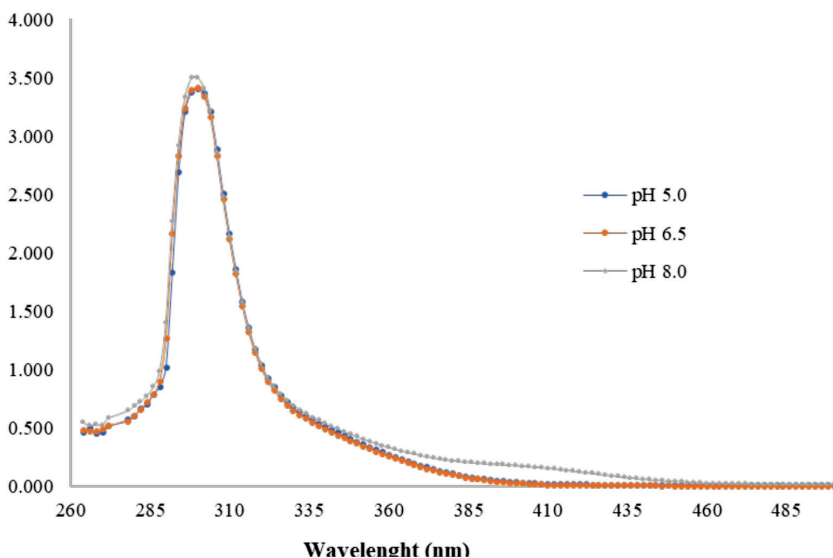
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Figure 4. Absorbance spectra of *p*-nitrophenyl palmitate (*p*NPP) at 25 °C and three different pH values in a surfactant solution. *Source*: own research.

Thus, the increase in absorbance with temperature seems not to be related to interconversion of protonated and deprotonated forms; but to a variation in absorbance of the deprotonated one. A possible explanation for this effect is that the resonant forms have different absorptivity and the higher temperatures shift the resonance equilibrium toward the forms with higher absorptivity. Possibly, the least thermodynamically stable resonance form of the deprotonated *p*NP, which might be the ketone form (**d,** in Scheme 3), is the most unstable at low temperatures. The instability of this contributor of the resonance hybrid may be attributed to two main factors, 1) it is the resonance
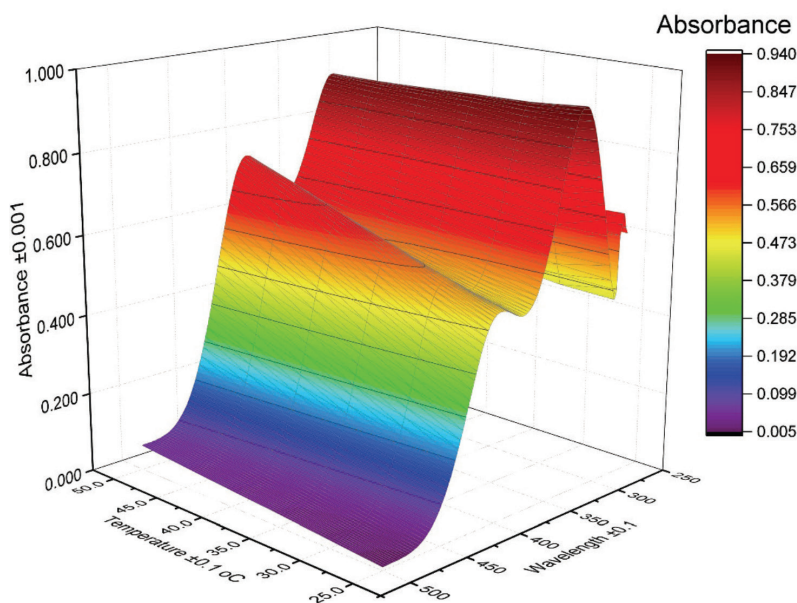
data, is at 300 nm but is has appreciable absorption at 317 nm (see Figure 4).

The results hereby discussed indicate that the pH has a more decisive role than the temperature in the absorbance of *p*NP. However, an appreciable effect of temperature on the deprotonated form of the *p*NP has been found. At higher temperatures, the absorbance at 410 nm from anionic *p*NP was favored, while the absorbance at 317 nm of the protonated form of *p*NP did not change. In Figure 5, the above-described effect of temperature at pH 6.50 can be noticed.

Figure 5 raises an interesting concern since temperature affects the absorbance of the deprotonated form of the *p*NP but not of the protonated one.

Figure 5. Temperature effect over *p*NP absorption spectra at pH 6.50. *Source*: own research.

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contributor that most affects the benzene ring stability, since it generates a system of four pairs of π electrons on the ring, affecting aromaticity, a condition that could only be favored at high temperatures; 2) it places the double bond on nitrogen, an electronegative atom with a formal charge of +1.

In order to analyze the effect of temperature at a fixed pH value, two additional absorbance measurements were made in the range from 25 ° C to 50 ° C. One of them at pH 4.0 where there is a predominance of the protonated form of the pNP that absorbs at 317 nm and another at pH 8.0 where the anionic form of the pNP that absorbs at 410 nm predominates. As shown in Figure 6, it is possible to appreciate that under both pH conditions, where one of the two forms of the pNP predominates, the effect of temperature on the acid dissociation equilibrium of the pNP and therefore of its absorbance is barely appreciable.

Many analytical determinations using pNP release for enzyme-catalyzed reactions in aqueous solutions are based on reaction quenching by enzyme denaturation using alkaline reagents such as Na$_2$CO$_3$ or NaOH (Lam, Cortez, Nguyen, Kato, & Cheruzel, 2016; Sandoval et al., 2012). This is a useful alternative when

Figure 6. Temperature effect over pNP absorbance at **a)** pH 4.00, and **b)** pH 8.00. *Source*: own research.

the substrate is an alkaline stable compound due to reaching higher pNP dissociation (sodium carbonate solutions are about pH ~ 11,20 and the pNP equilibrium is completely shifted to the dissociated form). However, p-nitrophenyl esters are easily hydrolyzed in moderate alkaline environments. Therefore,
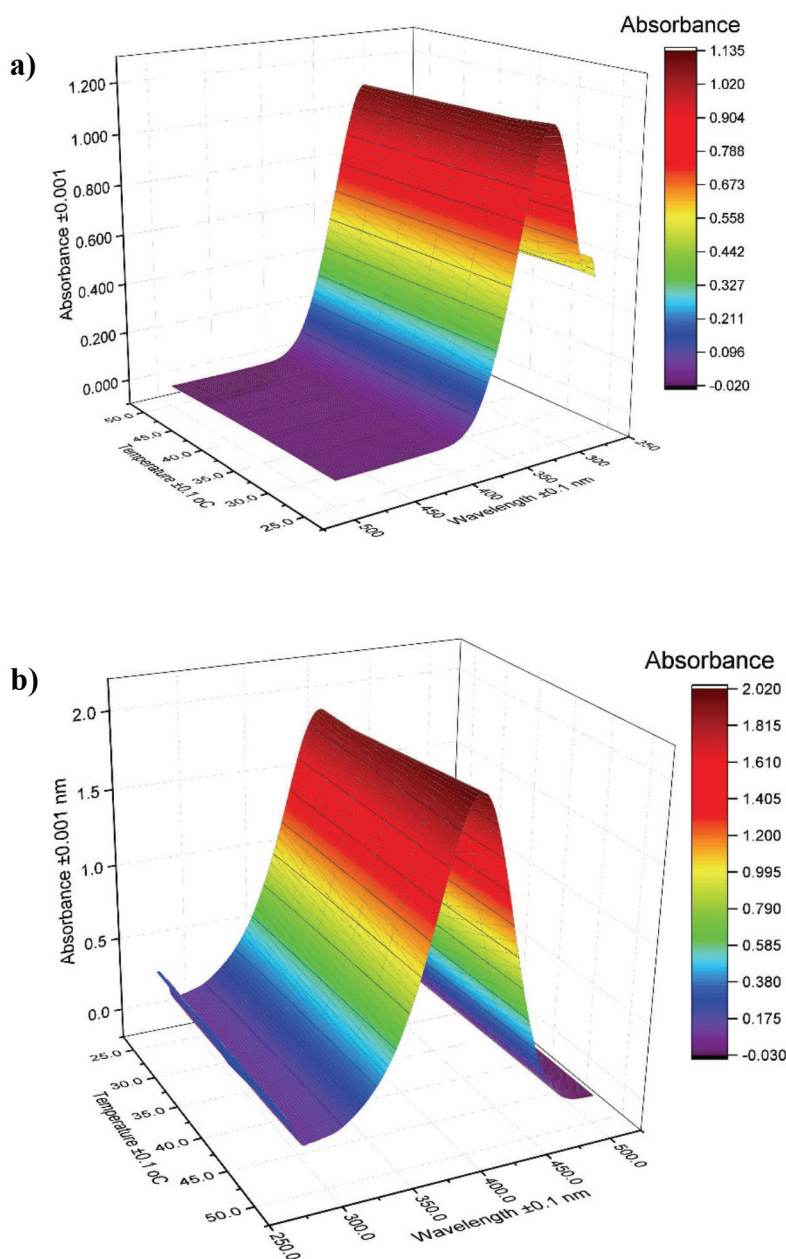
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it is not recommended to determine the real pNP amount released in the reaction due to enzymatic catalysis.

Figure 7 depicts the kinetics of the spontaneous hydrolysis of p-nytrophenyl palmitate (pNPP) in buffer solution at pH 8.00. As it can be seen, lower pH values did not present absorbance at 410 nm which means high stability of these pNPP below pH 7.00. On the other hand, under alkaline pH conditions, the substrate releases pNP as a result of its spontaneous hydrolysis.

Because of this, a good choice is the use of continuous determinations of the pNP in the spectrophotometer by continuous monitoring of the absorbance at different times, with no need of the alkaline quenching of the reaction (Sandoval et al., 2014), but keeping in mind that any analytical determination of pNP must require establishing the pNP absorptivity in the same pH, buffer and temperature conditions than the enzymatic assay. To this respect, a recent publication (Peng, Fu, Liu, & Lucia, 2016) proposed, as an alternative method, the use of the isosbestic point of p-nitrophenol at 347 nm, a stable wavelength, for the appropriate determination of this compound, since the absorbance of this chromophore remains without change regardless the pH. Another option for the accurate determination of the p-nitrophenol released by this reaction is the use of organic solvents (such as ethanol, acetone and other organic mixtures) as alternative quenchers, since they do not interfere in the analytical quantification of pNP and do not favor the hydrolysis of pNP esters (Lam et al., 2016; Palacios et al., 2014; Sandoval et al., 2012).

Conclusions

From the current study, the following conclusions are proposed:

Triton X-100, gum arabic and isopropanol are suitable emulsifying agents to stabilize aqueous suspensions of p-nitrophenyl palmitate. They have shown to be stable at different pH and temperature values, with absorbance ranges that do not overlap either the protonated or anionic forms of pNP and possess great capacity to stabilize pNP esters in aqueous emulsions.

According to experimental results, above pH 10.0 there is total deprotonation of pNP and under pH 3.5 there is total protonation of this compound. However, at pH values close to 7.0 there are varying amounts of both forms of pNP and, since many of the enzyme assays are carried out at pHs close
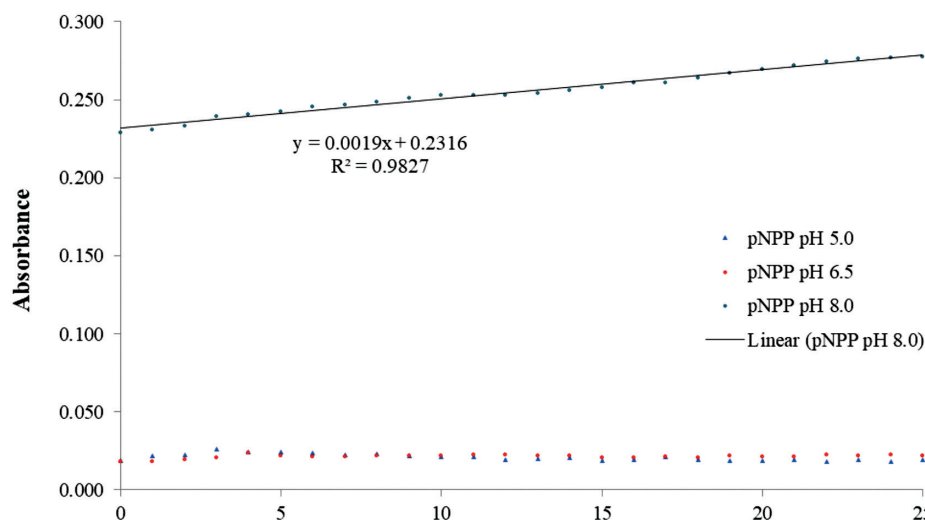

Figure 7. Spontaneous hydrolysis kinetics of pNPP in surfactant solution at 25°C and different pH. Source: own research.

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to neutral, it is important to include strict pH and temperature controls for assay methods that release this substance as a chromophore.

The use of 317 nm to perform measurements of the protonated form of the *p*NP can be considered, but it should be kept in mind that this wavelength is very close to the maximum absorbance of the *p*-nitrophenyl palmitate —which absorbs at 300 nm— thus requiring a complex mathematical design to blank out the varying substrate contribution.

At pH 6.5, it was found that temperature affects the absorbance of the deprotonated form but not of the protonated form of the *p*NP. This phenomenon could be associated with electronic aromatic phenomena that might involve the resonance hybrid contributors of deprotonated *p*NP.

The *p*-nitrophenol esters, particularly *p*-nitrophenyl palmitate, are susceptible to spontaneous hydrolysis under alkaline aqueous medium. This leads the researcher to consider the pH value and the use of appropriate blanks for this type of substrates.

Acknowledgments

References

Alcantara, A.; Pace, V.; Hoyos, P.; Sandoval, M.; Holzer, W. & Hernaiz, M. (2014). Chemoenzymatic Synthesis of Carbohydrates as Antidiabetic and Anticancer Drugs. *Current Topics in Medicinal Chemistry*, *14*(23), 2694-2711. doi: 10.2174/1568026614666141215151056

Biggs, A. I. (1954). A spectrophotometric determination of the dissociation constants of p-nitrophenol and papaverine. *Transactions of the Faraday Society*, *50*(0), 800. doi: https://doi.org/10.1039/tf9545000800_

Daiha, K. de G.; Angeli, R.; de Oliveira, S. D. & Almeida, R. V. (2015). Are Lipases Still Important Biocatalysts? A Study of Scientific Publications and Patents for Technological Forecasting. *PLOS ONE*, *10*(6), e0131624. doi: https://doi.org/10.1371/journal.pone.0131624

Farnet, A. M.; Qasemian, L.; Goujard, L.; Gil, G.; Guiral, D.; Ruaudel, F. & Ferre, E. (2010). A modified method based on p-nitrophenol assay to quantify hydrolysis activities of lipases in litters. *Soil Biology and Biochemistry*, *42*(2), 386–389. doi: https://doi.org/10.1016/J.SOILBIO.2009.11.015

Fourage, L.; Helbert, M.; Nicolet, P. & Colas, B. (1999). Temperature Dependence of the Ultraviolet–Visible Spectra of Ionized and Un-ionized Forms of Nitrophenol: consequence for the Determination of Enzymatic Activities Using Nitrophenyl Derivatives—A Warning. *Analytical Biochemistry*, *270*(1), 184–185. doi: https://doi.org/10.1006/ABIO.1999.4071

Gupta, N.; Rathi, P. & Gupta, R. (2002). Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Analytical Biochemistry*, *311*(1), 98–99. doi: https://doi.org/10.1016/S0003-2697(02)00379-2

Hernández-García, S.; García-García, M. I. & García-Carmona, F. (2017). An improved method to measure lipase activity in aqueous media. *Analytical Biochemistry*, *530*, 104–106. doi: https://doi.org/10.1016/J.AB.2017.05.012

Kapoor, M. & Gupta, M. N. (2012). Lipase promiscuity and its biochemical applications. *Process Biochemistry*, *47*(4), 555–569. doi: https://doi.org/10.1016/J.PROCBIO.2012.01.011

Kumar, A.; Dhar, K.; Kanwar, S. S. & Arora, P. K. (2016). Lipase catalysis in organic solvents: advantages and applications. *Biological Procedures Online*, *18*(2),1-11. doi: https://doi.org/10.1186/s12575-016-0033-2

Lam, Q.; Cortez, A.; Nguyen, T. T.; Kato, M. & Cheruzel, L. (2016). Chromogenic nitrophenolate-based substrates for light-driven hybrid P450 BM3 enzyme assay. *Journal of Inorganic Biochemistry*, *158*, 86–91. doi: https://doi.org/10.1016/J.JINORGBIO.2015.12.005

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Liu, Z.-Q.; Zheng, X.-B.; Zhang, S.-P. & Zheng, Y.-G. (2012). Cloning, expression and characterization of a lipase gene from the Candida antarctica ZJB09193 and its application in biosynthesis of vitamin A esters. *Microbiological Research*, *167*(8), 452–460. doi: https://doi.org/10.1016/J.MICRES.2011.12.004

Lopes, D. B.; Fraga, L. P.; Fleuri, L. F. & Macedo, G. A. (2011). Lipase and esterase: to what extent can this classification be applied accurately? *Ciência e Tecnologia de Alimentos*, *31*(3), 603–613. doi: https://doi.org/10.1590/S0101-20612011000300009

Mayordomo, I.; Randez-Gil, F. & Prieto, J. A. (2000). Isolation, Purification, and Characterization of a Cold-Active Lipase from Aspergillus nidulans. *Journal of Agricultural and Food Chemistry*, *48*(1), 105–109. doi: https://doi.org/10.1021/jf9903354

Orlando Beys Silva, W.; Mitidieri, S.; Schrank, A. & Vainstein, M. H. (2005). Production and extraction of an extracellular lipase from the entomopathogenic fungus Metarhizium anisopliae. *Process Biochemistry*, *40*(1), 321–326. doi: https://doi.org/10.1016/J.PROCBIO.2004.01.005

Palacios, D.; Busto, M. D. & Ortega, N. (2014). Study of a new spectrophotometric endpoint assay for lipase activity determination in aqueous media. *LWT - Food Science and Technology*, *55*(2), 536–542. doi: https://doi.org/10.1016/j.lwt.2013.10.027

Peng, Y.; Fu, S.; Liu, H. & Lucia, L. A. (2016). Accurately Determining Esterase Activity via the Isosbestic Point of p-Nitrophenol. *BioResources*, *11*(4), 10099-10111. doi: https://doi.org/10.15376/biores.11.4.10099-10111

Pratama, L.; Helianti, I.; Suryani, A. & Wahyuntari, B. (2017). Isolation, Characterization, and Production of Lipase from Indigenous Fungal for Enzymatic Interesterification Process. *Microbiology Indonesia*, *11*(2), 35-45. doi: https://doi.org/10.5454/MI.11.2.%P

Sandoval, M.; Hoyos, P.; Cortés, A.; Bavaro, T.; Terreni, M. & Hernáiz, M. J. (2014). Development of regioselective deacylation of peracetylated β- d -monosaccharides using lipase from Pseudomonas stutzeri under sustainable conditions. *RSC Adv.*, *4*(98), 55495–55502. doi: https://doi.org/10.1039/C4RA10401C

Sandoval, M.; Ferreras, E.; Pérez-Sánchez, M.; Berenguer, J.; Sinisterra, J. V. & Hernaiz, M. J. (2012). Screening of strains and recombinant enzymes from Thermus thermophilus for their use in disaccharide synthesis. *Journal of Molecular Catalysis B: Enzymatic*, *74*(3–4), 162–169. doi: https://doi.org/10.1016/J.MOLCATB.2011.09.012

Serjeant, EP.; Dempsey, B. (1979). Ionisation Constants of Organic Acids in Aqueous Solution. In: IUPAC Chemical Data Series No 23 (p. 989). New York, USA: Pergamon Press.

Shen, Y.-Y.; Sun, Y.; Zhou, L.-N.; Li, Y.-J. & Yeung, E. S. (2014). Synthesis of ultrathin PtPdBi nanowire and its enhanced catalytic activity towards p-nitrophenol reduction. *Journal of Materials Chemistry A*, *2*(9), 2977. doi: https://doi.org/10.1039/c3ta14502f

Stoytcheva, M.; Montero, G.; Zlatev, R.; A. Leon, J. & Gochev, V. (2012). Analytical Methods for Lipases Activity Determination: a Review. *Current Analytical Chemistry*, *8*(3), 400–407. doi: https://doi.org/10.2174/157341112801264879

Tang, J.; Tang, D.; Su, B.; Huang, J.; Qiu, B. & Chen, G. (2011). Enzyme-free electrochemical immunoassay with catalytic reduction of p-nitrophenol and recycling of p-aminophenol using gold nanoparticles-coated carbon nanotubes as nanocatalysts. *Biosensors and Bioelectronics*, *26*(7), 3219–3226. doi: https://doi.org/10.1016/J.BIOS.2010.12.029

Winkler, U. K. & Stuckmann, M. (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescens. *Journal of Bacteriology*, *138*(3), 663–670. doi: http://www.ncbi.nlm.nih.gov/pubmed/222724

Zhang, P.; Sui, Y.; Xiao, G.; Wang, Y.; Wang, C.; Liu, B. & Zou, B. (2013). Facile fabrication of faceted copper nanocrystals with high catalytic activity for p-nitrophenol reduction. *J. Mater. Chem. A*, *1*(5), 1632–1638. doi: https://doi.org/10.1039/C2TA00350C

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