Evaluation and optimization of the derivatization reaction conditions of glyphosate and aminomethylphosphonic acid with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate using reversed-phase liquid chromatography

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Evaluation and optimization of the derivatization reaction conditions of glyphosate and aminomethylphosphonic acid with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate using reversed-phase liquid chromatography

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List of non-conventional abbreviations used:

AMPA	Aminomethylphosphonic Acid
AMQ	6-aminoquinoline
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl Carbamate
FMOC	9-fluorenylmethylchloroformate
GLP	Glyphosate
IARC	International Agency for Research on Cancer
NHS	N-hydroxysuccinimide
OPA	*o*-phatalaldehyde
TFA	Trifluoroacetic Acid

Keywords: Aminomethylphosphonic Acid, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, Derivatization, Glyphosate, Reversed-phase liquid chromatography

Abstract

Due to the polar and ionic characteristics of glyphosate and its main metabolite, aminomethylphosphonic acid, a derivatization reaction is required before performing liquid-chromatographic determination of these compounds. In this study, reaction conditions using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as the derivatization reagent are assessed. A two-level full-factorial design is applied here to optimize the derivatization time (ranging from 0.5 to 20 min) and temperature (from 24 to 55ºC). It is found that neither of these two variables have a significant effect on the derivatization process and that the reaction is quantitatively achieved in a few seconds at room temperature (24ºC). The results obtained indicate that derivatization reaction with ~~AQC~~ 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate is achieved in milder conditions, with a faster kinetic reaction, than those required with the most conventional derivatization reagents used today ~~(FMOC and OPA)~~, and the derivatives are more stable. It has been found that the most important parameter affecting the chromatographic separation is the pH of the mobile phase, as it has a significant effect on the retention time of the hydrolyzed excess of reagent. When ammonium acetate is used in the mobile phase, buffered solutions at pH around 5.0 are required.

1. Introduction

Glyphosate (N-phosphonomethyl-glycine; GLP) is a broad-spectrum systemic herbicide widely used as a crop desiccant. It is one of the most commonly used herbicides in agriculture due to the development of glyphosate-resistant genetically modified crop varieties [1,2] and it is also widely applied for urban and residential weed control [3]. The global glyphosate market is projected to reach USD 12.54 billion by 2024, with a compound annual growth rate of 6.8% over the forecast period, from 2019 to 2024 [4].

GLP is considered to be non-persistent in the environment as it degrades by microbial organisms in water and soil to form the primary metabolite product, aminomethylphosphonic acid (AMPA). However, due to its intensive use worldwide, its impact on the environment is increasingly significant and it is regularly found in surface waters. The half-life time of GLP depends upon the environmental conditions (e.g., temperature, water depth, macrophytes, and sediment ratio) and can range between 2 and 215 days in soils and between 2 and 90 days in waters [3,5,6]. Both compounds are usually analyzed together as it is uncommon to detect GLP without the presence of AMPA [3].

Another point of interest is the toxicity and carcinogenicity of GLP. Since its introduction, most regulatory assessments have established that GLP has a relatively low toxicity in mammals [7]. However, in March 2015, the International Agency for Research on Cancer (IARC) listed glyphosate as “probably carcinogenic to humans” (group 2A), a category that “is used when there is limited evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals” [8]. Therefore, GLP carcinogenicity is controversial. One review evaluating fourteen carcinogenicity studies in rats and mice reached the conclusion that glyphosate is not of concern with regards to its carcinogenic potential in humans [9]. After the IARC

evaluation, the European Union (EU) conducted a detailed assessment of all available information and concluded that glyphosate is unlikely to pose a carcinogenic hazard to humans and the evidence does not support classification with regard to its carcinogenic potential according to Regulation No 1272/2008 [10]. Therefore, GLP is currently approved in the EU and can be used as an active substance in plant protection products until December 2022. This controversy has raised a social debate that has increased the interest in the analysis of GLP and AMPA in environmental samples.

There are different measurement methods for the determination of GLP and AMPA, which have been recently reviewed by Valle et al. [11]. Due to the polar and ionic characteristics of GLP and AMPA, liquid chromatographic techniques with derivatization of the target compounds are the most suitable. Another review found that 99% of the liquid chromatographic methods used for the determination of these compounds were performed with derivatized species [12]. The two most common derivatization reagents used are 9-fluorenylmethylchloroformate (FMOC) and *o*-phatalaldehyde (OPA), with around 75% of the applications using one of these two derivatization reagents [12].

The derivatization reaction with OPA is fast (<1 min) but the derivatives are unstable after a few minutes. Therefore, this reagent is usually applied as a post-column derivatization, after chromatographic separation of the target compounds with a strong cation-exchange column [13,14]. OPA presents other significant limitations since it is sensitive to air oxidation, degrades over time, and should be prepared fresh for optimum sensitivity or stored under an inert gas to maintain its activity for one week [14,15]. Moreover, this reagent only reacts with primary amines and GLP is a secondary amine and so an intermediate step must be applied before derivatization using an oxidizing solution (e.g., hypochlorite solution) to oxidize GLP to glycine [13,14].

Since the study of Moye and Boning [16], FMOC has become the most common derivatization reagent for HPLC determination of GLP and AMPA [12,17,18]. This derivatization reaction is slower than with OPA and different reaction times have been proposed, ranging from 10 min [19] to overnight [20]. Pinto et al. [21] performed a two-level factorial experimental design to assess the significance of different variables during the derivatization reaction and found that the variable derivatization time did not have a significant effect, suggesting a reaction time of 10 min. However, other studies found optimum reaction times of 30 min [18,22,23] and 60 min [24]. Once the pre-fixed derivatization time is achieved, the solution must be acidified to stop the reaction and to obtain stable derivatives for some days [20-22,25-27]. Due to the stability of FMOC derivatives, this reagent is applied as a pre-column derivatization reaction. Another challenge with the use of FMOC is the formation of derivatization by-products, such as FMOC-OH [18,22,24,25], which are formed by hydrolysis and decarboxylation of the excess of reagent. These by-products have strong fluorescence at the same excitation and emission wavelengths of the derivatives, which may complicate the chromatographic determination. For this reason, and to decrease matrix effects, the removal of interferents, by liquid-liquid extraction [17,28] or solid phase extraction [18], are recommended to maintain a good specificity of the method.

Recently, another derivatization reagent usually applied for amino acid determination has been proposed as a rapid and simple alternative to conventional OPA and FMOC derivatization procedures [29,30]. In this case, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was applied as the derivatizing reagent with very promising results (Figure 1). This reagent reacts with both primary and secondary amines, usually in a simple and fast way, yielding stable and fluorescent derivatives. It has the advantage that the excess of reagent is rapidly hydrolyzed to 6-aminoquinoline (AMQ), which

does not need to be removed before analysis given that AMQ has a different emission spectrum to the derivatized amines, allowing for selective detection in the presence of a large excess of AMQ [31]. Unfortunately, the first study evaluating AQC for GLP and AMPA derivatization did not study the derivatization reaction and the authors directly applied the commercial indications of the supplier of the reagent for the analysis of amino acids [29]. Given this, we have performed the optimization of the derivatization reaction conditions with AQC using a two-factorial experimental design, combined with liquid chromatography with ultraviolet detection for the separation and detection of the analytes.

2. Materials and Methods

2.1. Reagents and Solutions

GLP and AMPA (analytical-grade) were purchased from Sigma-Aldrich (Germany). Stock solutions containing either one or the two analytes at 200 $mg \cdot L^{-1}$ in 0.1 M HCl were prepared. Acidification with HCl to pH 1 has been found to be necessary in the case of environmental samples since multivalent cations form stable complexes with GLP and AMPA, which are not derivatized [11,20,25-27,32,33]. All standard and working solutions were stored at 4ºC in polypropylene material given that GLP binds to active sites on glass when it is not derivatized [22,24,27]. Working solutions were freshly prepared before use.

The AQC derivatization reagent (AccQ·Tag™ derivatization kit) was purchased from Waters Corporation (USA). Methanol for HPLC was from Fisher Chemical (Fisher Scientific, UK). Deionized water was from a Milli·Q Ultrapure water system (Millipore Iberica, Spain). Ammonium acetate for HPLC LiChropur was obtained from Merck (Germany). Trifluoroacetic acid (TFA, 99%) was from Fluka (Germany).

Mobile phases were a mixture of (A) an aqueous buffer solution containing 50 mM ammonium acetate, adding TFA until pH=5.0, and (B) methanol. All mobile phase solutions were filtered through 0.45 µm filters (Whatman, Germany) and degassed before use.

2.2. Instrumentation

The chromatographic experiments were performed using two HPLC systems: a SpectraSYSTEM (Thermo Scientific, USA) liquid chromatograph and an 1120 Infinity LC Compact system (Agilent Technologies). UV detection was performed at 254 nm. Two reversed-phase columns were evaluated for the separation of the derivatives: (i) a conventional C18 reversed-phase column: Kromasil 100-C18 (200 mm x 4.6 mm, 5 µm, 100 Å pore size, surface area 320 $m^2 \cdot g$, 19% carbon load) (Teknokroma, Spain), and (ii) an ethyl-bridged column: Gemini - NX C18 110A (250 mm x 4.6 mm, 5 µm, 110 Å pore size, surface area 375 $m^2 \cdot g$, 14% carbon load) (Phenomenex, USA). All columns were protected with their corresponding C18 guard columns (10 mm x 3.2 mm for the Kromasil column, and 4 mm x 3 mm for the Gemini column). The void volume was experimentally determined for each column, including guard column, and was 2.1 ml for the Gemini-NX column and 1.7 ml for the Kromasil 100 column.

After assessing different HPLC separation conditions, the following gradient was chosen for this study: 10% B for 3 min, 10-50% B in 8 min, 50-100% B in 3 min, 100% B for 4 min, 100-10% B in 3 min and re-equilibration for 4 minutes. Injection volume was 10 µl and flow rate was set at 1.0 $ml \cdot min^{-1}$. Oven column temperature was maintained at 40ºC.

2.3. Derivatization procedure and experimental design

171 The method involves derivatization with the AccQ·Tag™ kit. Therefore, derivatization
172 of GLP and AMPA was first conducted according to the manufacturer's instructions for
173 amino acid analysis. Briefly, 10 µL of a stock mixture was mixed with 70 µL of borate
174 buffer and 20 µL of AQC (AccQ·Tag reagent, previously dissolved in 1.0 mL of
175 diluent) [34]. The derivatization reaction was performed for 10 min at 55ºC in a dry
176 heating block.

177 Thereafter, the derivatization time and temperature were modified and assessed to find
178 the optimum derivatization conditions for GLP and AMPA. For this reason, a two-level
179 full factorial design (2^k, where k denotes the number of factors) was applied to evaluate
180 the effect of these two variables. This factorial design permits the study of the effect of
181 each variable and the presence of interactions between the variables. The reaction time
182 (variable t) was studied in the range from 0.5 to 20 min, and the temperature (variable
183 T) was evaluated in the range from room temperature (24ºC, as the laboratory was
184 thermostated at this temperature) to 55ºC. The central point (axial point, 10 min, 40ºC)
185 was also measured and considered as an experiment. All experimental points were
186 performed in triplicate to assess the precision, and all the experiments were performed
187 in a random order. Significance was set at 0.05.

188 2.4. Quality control

189 Method blank controls were prepared following the same derivatization procedure as for
190 samples, using HCl 0.1 M as the blank matrix. Duplicate standards were used as control
191 samples to confirm the stability of the results obtained.

192 Before proceeding with the HPLC procedure, a standard was analyzed sequentially until
193 stable retention times were obtained for GLP and AMPA (<0.1 min variation in
194 retention times in three consecutive injections). Once the stability of the system was
195 obtained, a method blank was injected to confirm that no system contamination took

196 place. After each 5-6 samples, a method blank and a quality control were analyzed to
197 confirm the stability of the system.

198 2.5. Validation study

199 The linearity of the derivatization method was assessed by analyzing six standards in
200 the 1-150 $mg \cdot l^{-1}$ range. Repeatability and inter-day precision were determined applying
201 a nested design [35,36], evaluating three replicates daily at a low concentration (10
202 $mg \cdot l^{-1}$) and three at a high concentration (150 $mg \cdot l^{-1}$) on three consecutive days. The
203 limit of detection (LOD) was determined by analyzing six independent replicates of
204 blanks spiked at 5-10 $mg \cdot l^{-1}$, taking the standard deviation (SD) as SD_{blank}. The
205 $3.3SD_{blank}$ criterion was then applied [37].

206 **3. Results and Discussion**

207 3.1. Evaluation of the HPLC conditions

208 For the assessment of the liquid chromatographic conditions, derivatization was
209 performed according to the supplier's recommendations for amino acid analysis (10 min
210 at 50ºC). As was also described for the separation of amino acids derivatized with AQC
211 [38], the Gemini NX column (ethyl-bridged column) gave lower retention times and
212 reduced back pressure than a conventional C18 column due to its greater surface area
213 and carbon loading. For both columns, it was observed that injection volumes of 20 µl
214 at room temperature resulted in excessive sample loading and AMPA showed a split
215 peak (Figure 2a). The increase of the column oven temperature to 40ºC helped to reduce
216 the peak-widths but AMPA still gave peak splitting. The use of an oven temperature of
217 40ºC and an injection volume of 10 µl solved this problem with the ethyl-bridged
218 column (Figure 2b). However, the AMPA peak still gave excessive fronting ~~tailing~~ with
219 the conventional C18 column. It was necessary to reduce the injection volume to 5 µl to

obtain adequate peak shapes with this column, which led to a large decrease in the sensitivity for GLP. Therefore, the Gemini column was chosen as being the most appropriate one for this study, with the column thermostated at 40ºC and injecting 10 µl.

Previous studies using AQC as a derivatization reagent for amino acids have shown that the retention time of the hydrolyzed excess of reagent (AMQ) is highly dependent on the pH of the mobile phase [31,38-42], and variations in the retention time as large as 7 min were obtained with differences of only 0.35 units in the pH of the mobile phase [38]. We have evaluated the effect of the pH on the separation of GLP, AMPA, and the excess of reagent AMQ with the ammonium acetate mobile phase used in this study. This salt resulted in a solution with neutral pH and, in these conditions, peak-shapes for GLP and AMPA, and the resolutions obtained, were adequate. However, this solution is not a buffer and its pH is highly labile, which means that changes in the pH of the injected solutions will modify the pH of the mobile phase, possibly having significant effects on chromatographic separations. Moreover, if mass spectrometry detection is required, ammonium acetate solutions electro-sprayed in positive ion mode are likely to undergo acidification as they are not buffered [39]. For these reasons, the addition of modifiers to obtain a buffered mobile phase was evaluated. Ammonium acetate provides buffering around pH 4.75 (the pK_a of acetic acid) and pH 9.24 (the pK_a of ammonium), and both pH ranges were evaluated (Figure 3), with the addition of TFA to reach pH around 4.8 and ammonium to obtain pH values around 9.2. The results showed that retention time and peak shape for GLP were not affected at the different pH values evaluated. It was confirmed that retention time for the excess of hydrolyzed reagent increased significantly with the increase in the pH of the mobile phase (Figure 3a) [38], and the retention time for AMQ at pH=9.2 (adjusted with ammonium) was >18 min (Figure 3b), which resulted in an excessive total analysis time. Moreover, it was

observed that the AMPA peak was split and deformed at this pH (Figure 3b), making these conditions inadequate for its quantification.

When the 50 mM ammonium acetate solution was buffered around pH 4.76 with the addition of TFA, good chromatographic peaks and resolutions for GLP and AMPA were obtained, and the retention time for AMQ was decreased to <14 min. Depending on the gradient applied for the separation, a co-elution between AMQ and AMPA were obtained at pH 4.5-4.7 (Figure 3a). The increase of the pH to 5.0 was enough to avoid any co-elution between these two compounds and the AMQ peak was always baseline separated after the AMPA peak (Figure 2b). For this reason, pH=5.0 (ammonium acetate 50 mM with addition of TFA until reaching the target pH) was chosen for the buffered solution A.

The evaluation of different flow rates ranging between 0.6 and 1.2 $ml \cdot min^{-1}$ showed that there were no significant differences at any of the flow rates evaluated. The number of theoretical plates and resolution values obtained increased slightly when the flow rate was decreased, but the parameters obtained were appropriate at all flows evaluated. Therefore, the flow rate was set at 1 $ml \cdot min^{-1}$, also taking into account the total analysis time and the back pressure generated.

3.2. Optimization of the derivatization reaction

Cohen and Michaud demonstrated the ability of AQC to derivatize amino acids [31]. This reagent has the advantage that it reacts quickly with primary and secondary amines, and the excess of reagent is also rapidly hydrolyzed (<1 min) to yield AMQ, N-hydroxysuccinimide (NHS) and carbon dioxide. In a preliminary study [29], this reagent has shown promising results for the pre-column derivatization and analysis of GLP and AMPA, but the reaction conditions suggested by the supplier for the derivatization of amino acids were applied without a study into the effect of the

derivatization time and temperature. As was found in the preliminary study of Cohen and Michaud [31], AQC usually reacts with primary and secondary amines in just a few seconds. In the case of amino acids, a longer reaction time (10 min) is required due to the fact that tyrosine needs a temperature of 50ºC for 10 min to obtain a stable derivative.

In the present study, an experiment domain was defined to evaluate the influence of derivatization time and temperature on the yield of derivatized compounds. A two-level full factorial design was used to study not only each variable individually, but also the existence ~~presence~~ of interactions between the two variables. Table 1 summarizes the results obtained and the significances (p-values) are given. As can be seen, neither of the two variables evaluated were significant (p>0.15) in the range evaluated for the two target analytes. Despite a slight curvature and an interaction between time and temperature being observed for AMPA, the calculations revealed that it was not significant (p=0.162).

The stability of the derivatized compounds stored at 4ºC was evaluated daily for seven consecutive days and no significant differences in the peak areas measured were observed for either analyte (p>0.35). In conclusion, it is seen that the derivatization reaction is achieved in a few seconds at 24ºC, yielding stable compounds for at least one week. This finding shows that the reaction conditions for AQC are significantly simpler than those of ~~clearly simplifies the reaction conditions required for AQC when compared with~~ FMOC and OPA.

3.3. Validation

The efficiency of the conditioning procedure and the stability of the chromatographic column was evaluated from the retention time of controls over a five day period (n=3

each day). Residual standard deviations (RSD) <0.3% were obtained for GLP and <0.5% for AMPA.

The quality parameters obtained for the selected AQC derivatization/HPLC-UV procedure are shown in Table 2. Linearity was confirmed from the evaluation of the residual distribution, and determination coefficients (R^2) were always >0.999. Statistical evaluation of the results showed that intercept values were always non-significantly different from zero. Repeatability (RSD<8% at the low level and <4% at the high level) and inter-day precision (RSD<9% at the low level and <5% at the high level) were considered acceptable. No target compounds were detected in the blank controls evaluated.

The LODs of the method obtained (7-8 $mg \cdot l^{-1}$, applying the $3.3SD_{blank}$ criterion [37]) were of the same order as those obtained for the analysis of derivatized amino acids with AQC and also using HPLC-UV [38]. Table 2 also gives the LODs obtained applying the signal-to-noise (S/N) ratio because this criterion is widely applied in chromatography. However, the S/N is an instrumental LOD and method limits should only be derived from instrumental limits when the analytical procedure does not contribute significantly to variability and bias of analytical results [43]. The results indicate that the derivatization reaction introduces significant variability to the results that must be taken into account. The limits obtained with UV detection are too high ~~excessive~~ for the analysis of waters since the European quality standards for drinking water set a parametric value of 0.1 $\mu g \cdot l^{-1}$ for pesticides [44], a value that is many times exceeded when analyzing surface waters [20,29,45]. A more sensitive detector, such as fluorescence or mass spectrometer, would be required for this application, although this has no effect on the optimized derivatization conditions found in the present study. In

this respect, the study of Caretta et al. [29] has already demonstrated that LODs in the range 0.05-0.2 $\mu g \cdot l^{-1}$ can be reached with a mass spectrometry detector.

4. Conclusions

It has been found that the use of the derivatization reagent AQC yields highly stable derivatives for glyphosate and AMPA with fast and simple reaction conditions (a few seconds at room temperature), which permits the reaction to be performed directly in the injection vial. The requirements for the derivatization reaction with this reagent present some advantages with respect to the most common derivatization reagents used for this determination (FMOC and OPA). Due to the high dependence of the retention time of the hydrolyzed excess of reagent on the pH of the mobile phase, it is important to use buffered mobile phases at pH 5.0 when using this derivatization reagent.

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Conflict of interest

The authors declare no conflict of interest

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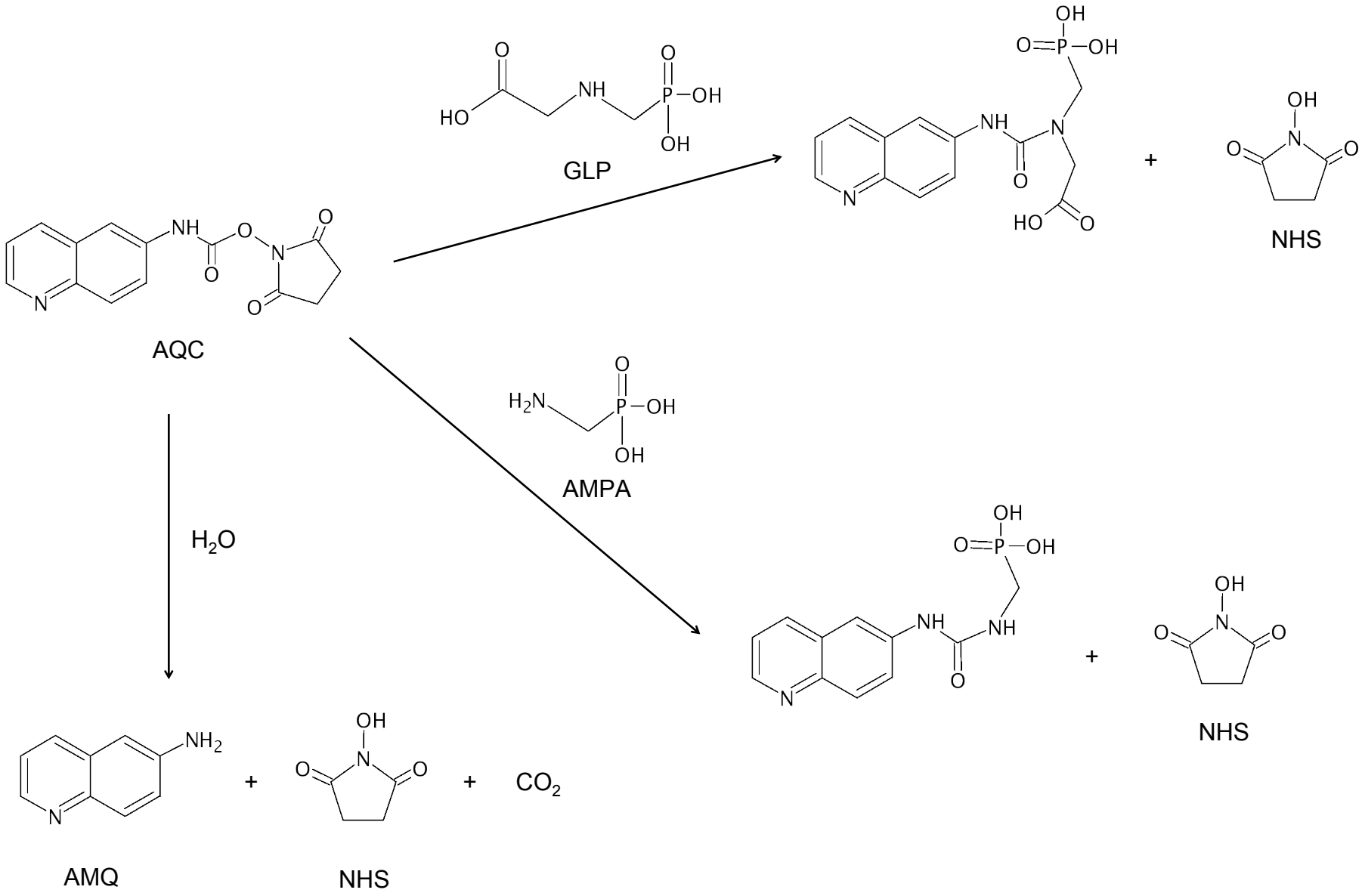
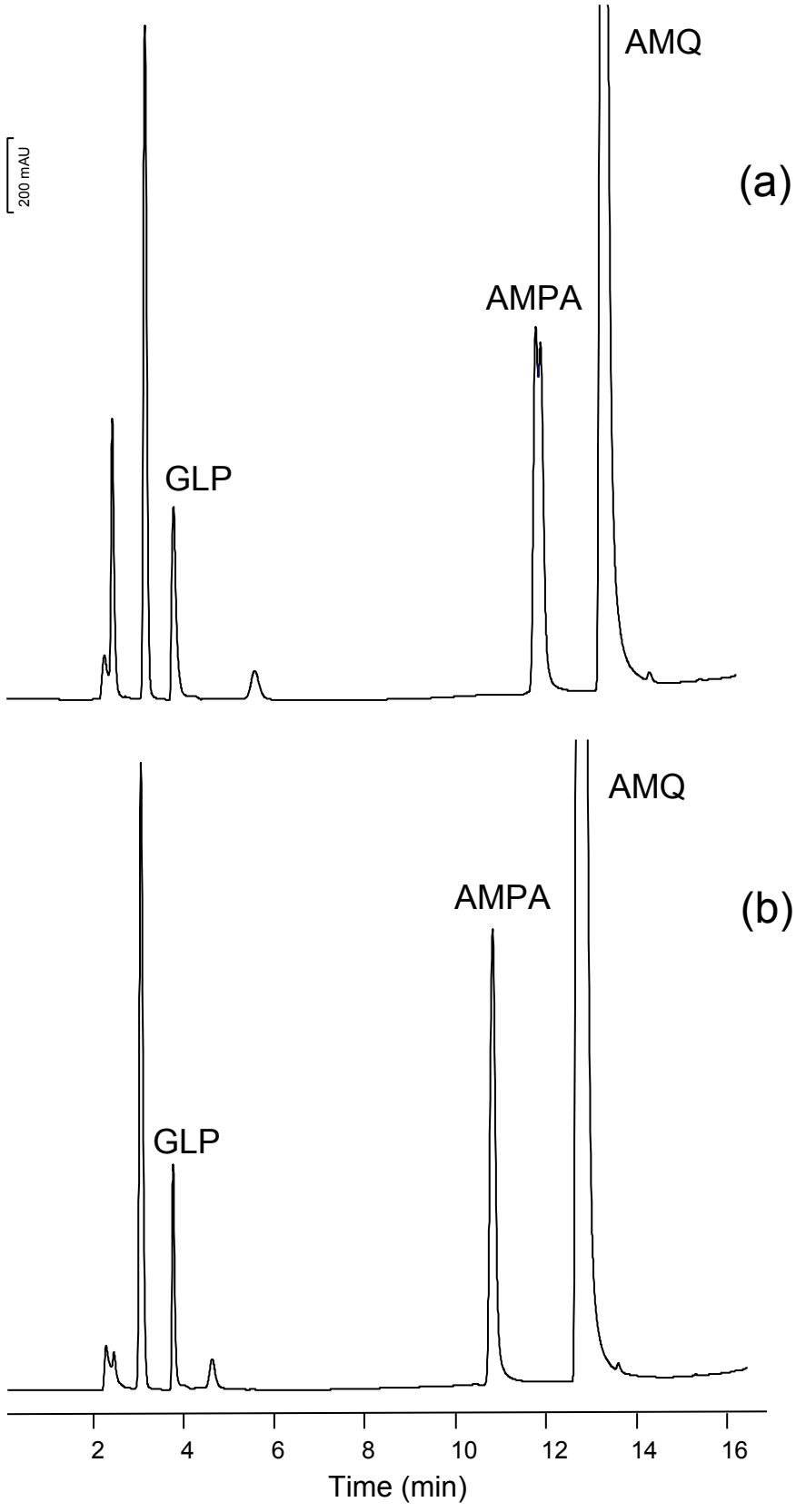
Figure captions

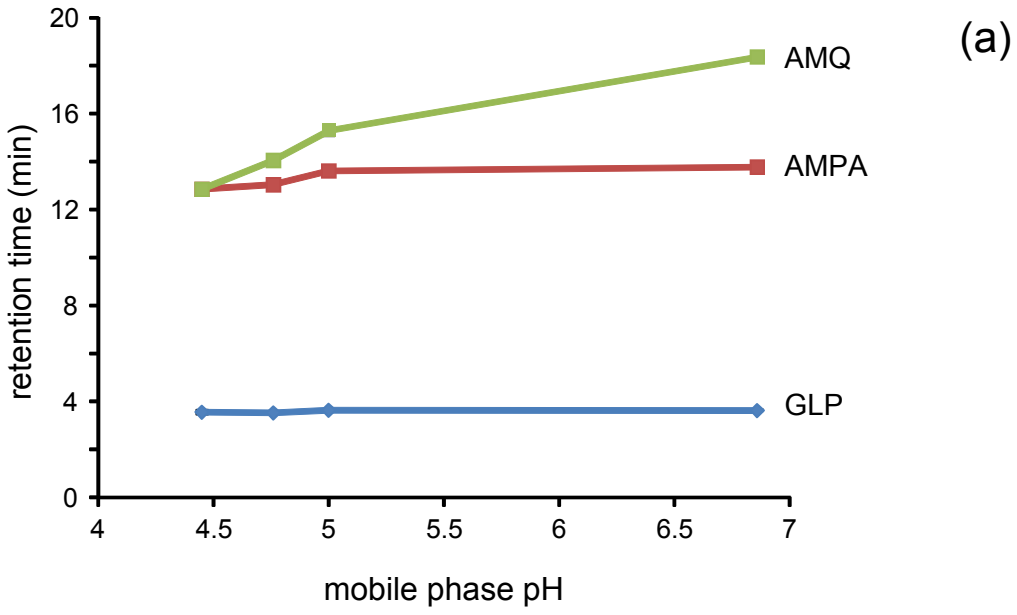
Figure 1. Derivatization reactions proposed for GLP and AMPA with AQC. Hydrolysis reaction of the excess of reagent to yield AMQ is also indicated. Experimental results confirm that quantitative reactions are achieved at room temperature in a few seconds in all cases.

Figure 2. Chromatograms obtained with the Gemini column in the analysis of a standard mixture containing GLP and AMPA (both at 100 $mg \cdot l^{-1}$). (a) 20 µl sample injection and column at room temperature; (b) 10 µl injection and column thermostated at 40ºC. Gradient conditions as described in section 2.2.

Figure 3. (a) Effect of the mobile phase A pH on the retention time for the derivatized compounds and the hydrolyzed excess of reagent (AMQ). Separation gradient applied: 10% B for 3 min, 10-50% B in 12 min, 50-100% B in 2 min, 100% B for 4 min, 100-10% B in 3 min and re-equilibration for 4 minutes. (b) Chromatogram of a standard with mobile phase A buffered at pH=9.2. Separation gradient: 10% B for 5 min, 10-70% B in 20 min, 70-100% B in 2 min, 100% B for 3 min, 100-10% B in 3 min and re-equilibration for 4 minutes.


GLP
AQC
AMPA
NHS
NHS
H_2O
AMQ
NHS
CO_2


AMQ
200 mAU
(a)
AMPA
GLP
AMQ
AMPA
(b)
GLP
2
4
6
8
10
12
14
16
Time (min)


(a)
AMQ
AMPA
GLP
retention time (min)
mobile phase pH
0
4
8
12
16
20
4
4.5
5
5.5
6
6.5
7

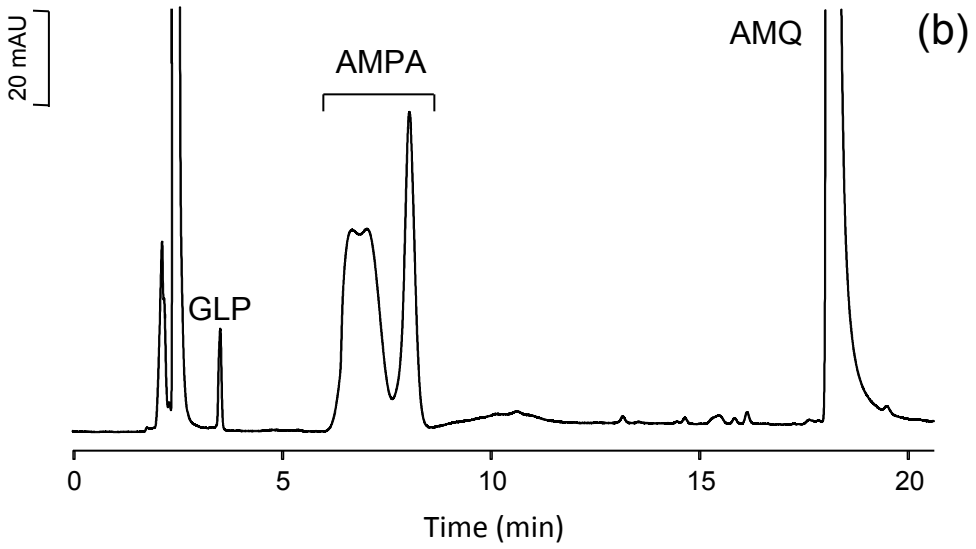

(b)
AMQ
AMPA
GLP
20 mAU
Time (min)
0
5
10
15
20

Table 1. Statistical results obtained for the experimental design, *p*-values are given for the main effects and interactions.

Variable	GLP	AMPA
temperature, T (ºC)	0.267	0.369
time, t (min)	0.442	0.585
time x temperature	0.825	0.162

Table 2. Quality parameters of the HPLC-UV method with the derivatized compounds. Calibration range: 1-150 $mg \cdot l^{-1}$.

	Calibration parameters			Precision [a] (RSD, %)				LOD ($mg \cdot l^{-1}$)	
	Slope (SD)	Intercept (SD)	R^2	Repeatability		Inter-day			
				Low	high	low	high	$3.3SD_{blank}$ [b]	S/N=3
GLP	5.53	8	0.9996	8.0	3.8	8.8	5.0	7	0.5
AMPA	22.0	56	0.9993	7.1	3.7	7.7	4.3	8	0.2

[a] Nested design (n=3, m=3)

[b] Analysis of seven independent fortified blanks