FIA DETERMINATION OF GLYCINE IN THE PRESENCE OF ALANINE AND GLUTAMIC ACID IN A PHARMACEUTICAL FORMULATION BASED ON THE USE OF A COPPER CARBONATE SOLID-PHASE REACTOR AND FIRST-DERIVATIVE SPECTROPHOTOMETRIC DETECTION

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Abstract

An FI-procedure for the determination of glycine in the presence of alanine and glutamic acid is proposed. The procedure uses a heterogeneous continuous flow system provided with a solid-phase-reactor of copper carbonate immobilised in a polyester resin matrix. The amino-acids are forced to pass through the bed-reactor and the Cu(II)-amino-acid complexes formed are determined on the basis of the first derivative and the zero-crossing approach. The wavelength for monitoring glycine was 235 nm at which zero-crossings of alanine and glutamic acid are very close and the amplitude $(dA/d\lambda)_{235\ nm}$ are negligible and presents a compensated (plus-minus) contribution for both amino-acids. The procedure was applied to the determination of glycine in a pharmaceutical formulation containing a ternary mixture of the three amino-acids.

Key-words: Glycine, FIA, first-derivative spectrophotometry, solid-phase reactor,Cu(II)-amino acid complex.

Introduction

Derivative spectrophotometry is an effective means of resolving mixtures of substances with partly or fully overlapped spectra. This effectiveness is particularly interesting when combining it with the expeditiousness, reproducibility and sample throughput of FIA manifolds. The accuracy of the results depends on the wavelength range and spectral derivative order used.

Derivative UV-visible absorption spectrophotometry has been demonstrated to be over last few years a spectacular technique to solve some kind of problems [1]. Problems especially in pharmaceutical analysis [2-4], which are usually mixtures of two or three components showing different UV-visible spectra, as well as in clinical [5,6], environmental [7], organic [8] and inorganic

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analysis employing diverse chromogenic reagents [9,10]. Derivative spectophotometry has been also widely used of binary and ternary mixtures of amino acids. Special attention has been focused on the resolution of mixtures of phenylalanine, tyrosine and tryptophan due to the weak absorption of these aromatics in the near ultraviolet relatively free of strongly UV-absorption caused by accompanying compounds. In this sense mixtures of these amino acids have been studied either to the simultaneous determination in mixtures [11-15] or to identify aromatic residues contained in polypeptides and proteins [16-19].

Since when derivatized the maxima and minima of an original function take zero values, and inflections are converted into maxima or minima, respectively, the derivative curves are much more structured than the original spectra. The zero-crossing approach is based on the measurement of the amplitude of the derivative spectrum (first derivative or odd order) of a mixture at the wavelength for which the amplitude of one of the components is zero (zero-crossing). Performing this operation for the other component one can obtain an analytical signal related to one component and free of spectral interferences.

However, the resolution of mixtures of non aromatic amino acids has been scarcely treated probably due to several problems which are difficult to overcome, namely, scarce selectivity working in the UV region near 200 nm and strongly overlapped spectra (the organic groups in the molecules do not introduce dramatic changes in the shape of the spectra). These problems can even increase by using a derivatization reaction, where the modified spectra are mainly related to the chromogenic reagent and thus further potential interference is added.

In this paper we proposed a flow injection procedure for the determination of glycine in the presence of alanine and glutamic acid. The procedure is based on the formation of Cu(II)-amino acid complexes by forcing the non aromatic amino acids to pass through a copper carbonate solid phase reactor placed in an FI-assembly. The determination of glycine is made on the basis of the first derivative and the zero-crossing approach. This system overcomes the problems of selectivity, eliminates the excess of reagent reaching the detector, produces a displacement of the maximum of spectra to a visible region and also enhances the not significant spectral differences between amino acids. The method is applied to the pharmaceutical analysis.

Experimental

Reagents

All reagents were prepared with analytical grade-chemical and deionized water. Glycine (u.c.b), L-alanine (u.c.b) and L-glutamic acid (u.c.b) were prepared in a $Na_2B_4O_7 \cdot 10$ H_2O-NaOH buffer at pH 9.5 containing 50 cm^{-3} of 0.025 mol dm^{-3} $Na_2B_4O_7 \cdot 10$ H_2O (Panreac) and 8.8 cm^{-3} of 0.1 mol dm^{-3} NaOH (Panreac) per 100 ml. This buffer was employed as carrier solution.

The solid reactor was prepared with AL-

100-A polyester resin solution (Reposa) containing low-molecular-weight polyester chains, octoate of cobalt as activating agent of the reaction and methyl ethyl ketone as catalyst (AZKO). $CuCO_3Cu(OH)_2 \cdot 2\ H_2O$ was used as an immobilised complexing reagent for the present purpose.

Preparation of sample

The contents of seven capsules of TEBETANE (ELFAR-DRAG.SA.) were powdered in a grinder. An appropriated amount of powder was precisely weighed, dissolved in water, filtered and diluted to 250 cm^{-3} with water. This solution was used for preparing ternary mixtures of amino-acids buffered at pH 9.5 as described previously.

Apparatus and FIA assembly

A single-channel continuous flow assembly (Figure 1.a) was used where a copper carbonate reactor was set between a Rheodyne Model 5041 sample injector and a Perkin Elmer Lambda 16 spectrophotometer provided with a flow cell Hellma 18 µl inner volume. The flow system was made of PTFE tubing of 0.8 mm and 1.5 i.d. for the manifold and reaction column, respectively. A Gilson Minipuls 2 peristaltic pump was used for pumping the reagents.

Preparation of the bed reactor

A bed-reactor was prepared according to the procedure formerly published (20,21). A suitable amount of $CuCO_3Cu(OH)_2 \cdot 2\ H_2O$ was added to the polyester resin solution (the ratio reagent/resin was 1.5:1 (w/w)). The mixture was homogenised by manual stirring and then ethyl methyl ketone was added to the mixture and stirring was continued until the polymer became too rigid. The solid was dried for 2-3 hours at room temperature, then broken with a hammer and grinder in a coffe mill; particles between 300-400 µm were selected by sieving. The bed reactor was prepared by introducing the particles by means of a mini-funnel into a PTFE tubing 1.5 mm i.d. and 20 cm length.

FIA-outputs recording

Maximum FIA-outputs were obtained af-

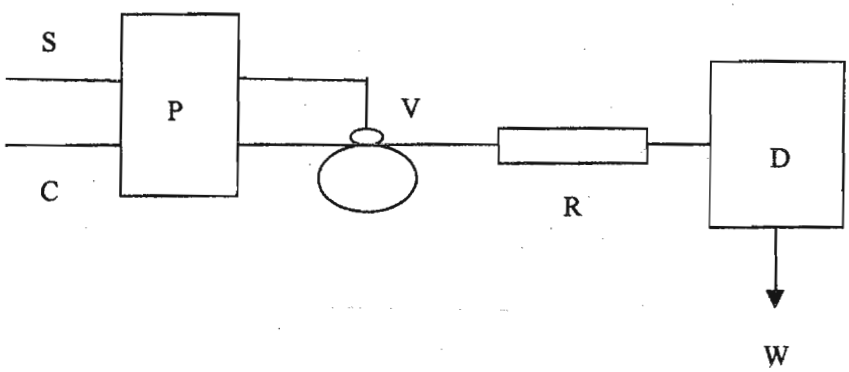

Figure 1.a Optimised flow assembly

C, carrier; P, pump; S, sample; R, reactor (immobilised copper(II) carbonate); D, detector and W, waste.

ter 38 seconds from injecting a sample with a resolution of 0.4 nm in the range between 190 and 320 nm at a scan rate 480 nm min^{-1}.

Results and discussion

Preliminary tests

The initial tests were aimed at shifting the spectrophotometric signal to the visible region and increasing the small differences in absorption between glutamic acid, alanine and glycine, which exhibit very similar spectra with strong bands in such a scarcely selective region as that around 200 nm. For this purpose, 0.5 mol dm^{-3} solutions of these non-aromatic aminoacids at pH 1-12 were prepared. The spectral profile changed very little with pH, the sole difference worth noting being a new absorption band appearing at 220 nm at pH 12. Figure 2 shows the spectra for glutamic acid, alanine and glycine at a variable pH.

In view of these results, the aminoacids were derivatized with ninhydrin or Cu(II). The reaction with ninhydrin always gave rise to spectra with two broad maxima at about 400 and 550 nm, both of which were associated to the chromophore.

Derivatizations with Cu(II) were initially performed in an FIA system where an aqueous solution of copper(II) ion was merged with one of the aminoacid concerned. The formation of Cu(II) complexes is especially favourable at an alkaline pH (about 9); however, precipitation of the metal as a hydroxide and spectral changes

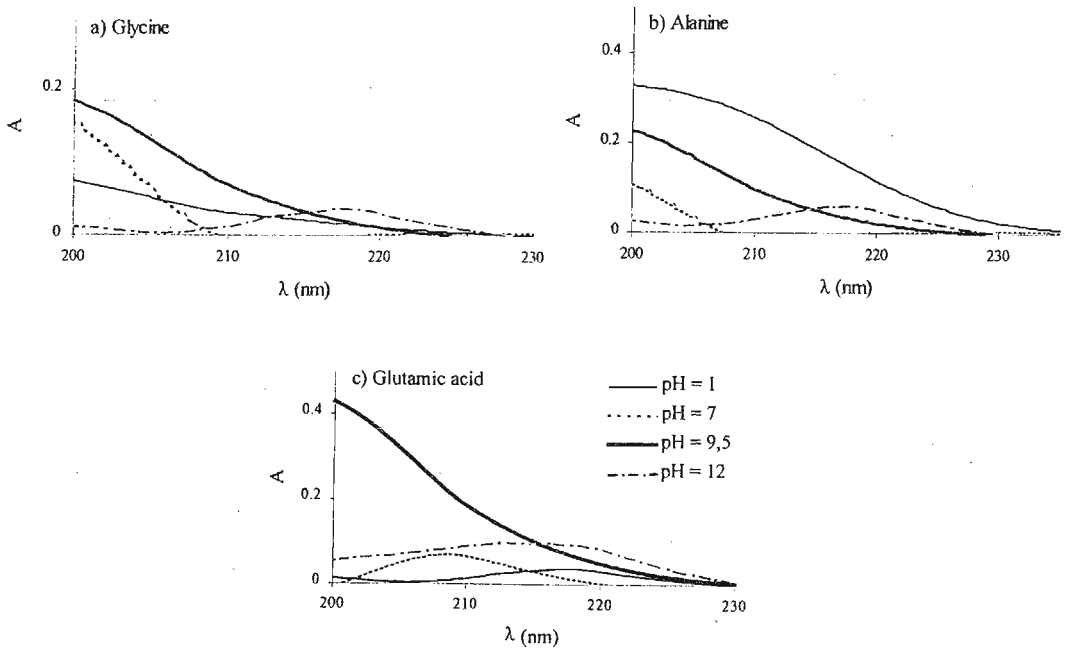

Figure 2. Influence of the pH on the absorbance spectra of glutamic acid, alanine and glycine.

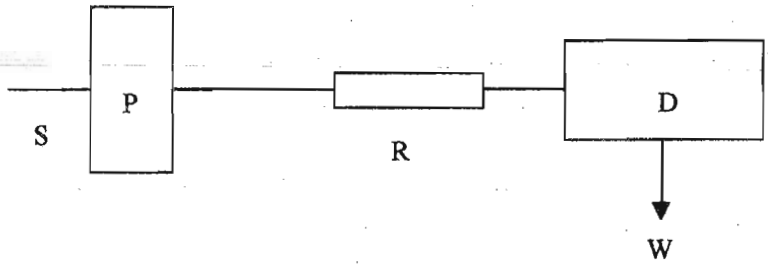

Figure 1 b FIA assembly employed in preliminary tests

S, sample; P, pump; R, reactor (immobilised copper(II) carbonate); D, detector and W, waste.

due to the formation of hydroxo complexes in the presence of excess cation make the use of copper(II) solutions to resolve aminoacid mixtures inadvisable. These shortcomings, however, can be circumvented by using a solid-phase reactor consisting of copper carbonate immobilised onto a polymer matrix. The metal ion can thus be released in a controlled manner from the reactor and only upon contact with a chelating agent (the aminoacid), with which it reacts in a stoichiometric ratio. In alkaline media, copper carbonate is insoluble and only the complex formed reached to the spectrophotometric cell (which avoids the presence of excess reagent). The polymer matrix of the reactor is inert under the working conditions and allows the obtainment of reagent-resin particles of appropriate size and hydrodynamic properties for continuous-flow operation.

A 20 cm long-1.5 mm i.d. reactor packed with particles 300-400 μm in size consisting of $CuCO_3Cu(OH)_2 \cdot 2\ H_2O$ and polyester resin in a 1.5:1 w/w ratio was prepared. A $5 \cdot 10^{-4}$ mol dm^{-3} solution of each aminoacid in a buffer of $Na_2B_4O_7 \cdot 10\ H_2O$ adjusted to pH 6-10 with NaOH or HCl as required was passed through the reactor to record spectra (see Figure 1.b).

Lower pH values resulted in dissolution of the immobilised reagent, whereas higher values led to gradual passivation of the reactor through formation of copper oxide, which is more insoluble than the starting reagent. Based on the results, formation of the aminoacid-Cu(II) complexes was quantitative (*i.e.* absorption was maximal) at pH 9.5; also, the maximum wavelength (*viz.* 230.8, 234.8 and 235.2 nm for glycine, alanine and glutamic acid, respectively) remained constant above pH 8.

Optimisation of the spectrophotometric operating conditions

The instrumental variables associated to the spectrophotometer (*e.g.* the scan rate and wavelength range used) can have a significant influence on the reproducibility and some features of the spectra obtained in each scan, particularly when the kinetic mode typical of FIA methodology is used and analyte spectra are virtually completely overlapped. Under these conditions, a minimal spectral difference may facilitate resolution of the mixture.

Subsequent experiments were aimed at establishing the most suitable detection conditions (*viz.* scan rate and wavelength interval for data acquisition). The influence of these variables was examined over the aminoacid concentration range $1 \cdot 10^{-4}$-$1 \cdot 10^{-3}$ mol dm^{-3}. The scan rates used were 240, 480, 960 and 1440 nm min^{-1}, and the wavelength intervals 0.2, 0.4, 0.8 and 1.2 nm. The best corrections for the blank (a borax/NaOH buffer at pH 9.5) were obtained at a scan rate of 240 nm and a wavelength interval of 0.2 nm. Higher scan rates (960 or 1140 nm min^{-1}) resulted in wide oscillations in the maximum absorption wavelength (greater than 1 nm in some cases). This was also the case at lower rates (240 nm min^{-1}), so 480 nm min^{-1} and an integration interval of 0.4 nm for the sample were chosen for subsequent experiments.

After the detector parameters were optimised, three calibrations were done using the same glycine solution on the same day. The slopes of the graphs were found to gradually decrease as the likely result of carry-over and an exceedingly long time of contact between the reactor and the sample solutions. The calibration curves obtained by using an assembly similar to that of Fig. 1*a* and a long enough coil to ensure a broad enough plateau to perform one scan had relative standard deviations less than 0.3%. In this new configuration, the carrier (borax/NaOH buffer at pH 9.5) acted as a flushing solution. This assembly, which included a 20 cm long-1.5 mm i.d. reactor packed with particles 300-400 μm in size consisting of $CuCO_3Cu(OH)_2\ 2H_2O$ and polyester resin in a 1.5:1 w/w ratio, was adopted for subsequent experiments.

Because of their strong mutual overlap, the absorption spectra for the three aminoacids could not be used to resolve their ternary mixtures. The zero-crossing approach was thus tested as an alternative. Figure 3 shows the absorption and first-derivative spectra for glutamic acid, glycine and alanine, all at a $0.5 \cdot 10^{-3}$ mol dm^{-3} concentration, obtained by using the above-described FIA manifold and detection conditions.

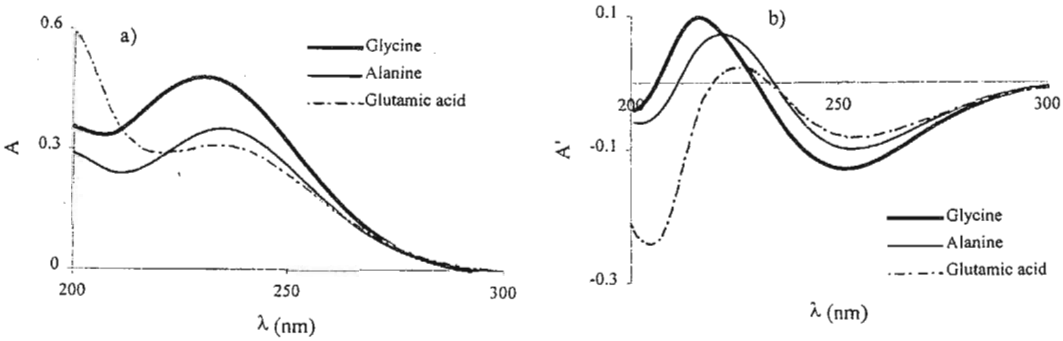

Figure 3 Absorption and first derivative spectra at pH 9.5 of glutamic acid, alanine and glycine.

Optimisation of the flow-rate and sample volume

The FIA variables to be optimised in the new manifold were the flow-rate and injected sample volume, both of which influence the residence time and the duration of the maximum. The optimum values for the operating variables associated to the solid-phase reactor (particle size, reagent-to-resin weight ratio, length and inner diameter) were taken from previous work [20,21]. FIA variables were optimised by the univariate method, using buffered solutions of each aminoacid at a $5 \cdot 10^{-4}$ mol dm^{-3} concentration, which gave absorbance values between 0.1 and 1.0. The flow-rate was varied from 0.62 to 2.8 cm^{-3} min^{-1}, using an injected sample volume of 929 µl and $t_{res} = t_{meas}$ - 12 s, where t_{meas} and 12 s were the times elapsed from insertion of the sample and beginning of the scan, respectively, to appearance of the absorption peak. A flow-rate of 1.77 cm^{-3} min^{-1} was found to be the best compromise between absorbance and throughput while providing reproducible spectra judging by the maximum absorbances and wavelengths obtained.

The injected volume was optimised at the above-mentioned optimum flow-rate, using solutions containing 0.5 10^{-3} mol dm^{-3} glycine, alanine or glutamic acid, and volumes over the range 19-929 µl. From Fig. 4 it follows that the most suitable injected volume was 929 µl (the largest tested), which yielded the highest absorbances. Above this level, however, did not increase in proportion but the residence time did. A sample volume of 803 µl was adopted as optimal because lower volumes resulted in significantly decreased analytical signals and durations of the maximum. The delay between successive injections was 1 min, which ensured

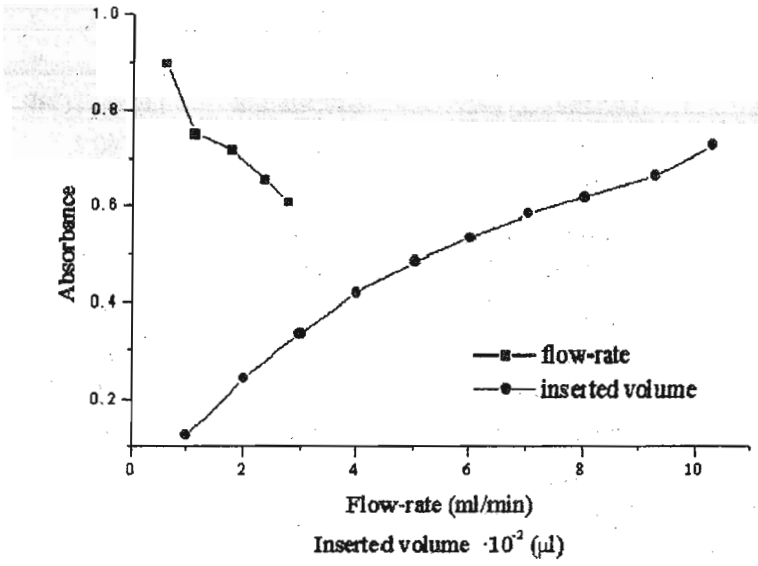

Figure 4 Influence of the carrier flow-rate and injected sample volume on the absorbance of glycine.

efficient flushing of the reactor with each fresh solution.

Selection of the measurement wavelength

The spectra obtained under the above-described optimum FIA conditions confirmed the maximum wavelengths observed in previous tests. Determining glycine from its first derivative spectrum at the zero-crossing wavelength for either alanine (234.8 nm) or glutamic acid (235.2 nm) was impossible owing to the small difference between both maxima (0.4 nm). This closeness between the zero-crossings of alanine and glutamic acid led us to explore the use of an intermediate wavelength (235.0 nm) in order to have the contributions of both aminoacids cancel each other. For this purpose, ternary mixtures of the aminoacids in different proportions were used to determine the error made in the determination of glycine, using the zero-crossing approach and the first-derivative of the spectrum at 235.0 nm. The composition of the mixtures and the errors made in their determination are shown in Table 1. As can be seen, the error in the determination of glycine depended on the alanine-to-glutamic acid concentration ratio and increased with increase in the aminoacid concentrations, probably as a result of the very high absorbance given by the mixture. The error in the determination of glycine was minimal when this aminoacid and alanine were in equimolar ratios in the presence of a small excess of glutamic acid, and also in mixtures containing excess glycine.

Table 1 Errors in the determination of the glycine content in synthetic ternary mixtures.

$[glycine]_{real}$ (M)	$[glycine]_{calc.}$ (M)	Gly:ala:glu	|Error| (%)
$2{,}0034 \cdot 10^{-4}$	$7{,}9862 \cdot 10^{-5}$	1:1:5	60.1
$2{,}0034 \cdot 10^{-4}$	$1{,}2037 \cdot 10^{-4}$	1:1:4	39.9
$2{,}0034 \cdot 10^{-4}$	$2{,}0717 \cdot 10^{-4}$	1:1:3	3.4
$2{,}0034 \cdot 10^{-4}$	$2{,}0486 \cdot 10^{-4}$	1:1:2	2.3
$2{,}0034 \cdot 10^{-4}$	$1{,}8056 \cdot 10^{-4}$	1:1:1	9.9
$2{,}0034 \cdot 10^{-4}$	$2{,}4306 \cdot 10^{-4}$	1:2:1	21.3
$2{,}0034 \cdot 10^{-4}$	$2{,}7662 \cdot 10^{-4}$	1:3:1	38.1
$2{,}0034 \cdot 10^{-4}$	$3{,}0556 \cdot 10^{-4}$	1:4:1	52.5
$2{,}0034 \cdot 10^{-4}$	$4{,}2014 \cdot 10^{-4}$	1:5:1	109.7
$2{,}0037 \cdot 10^{-4}$	$2{,}0071 \cdot 10^{-4}$	1:2:3	0.2
$4{,}0075 \cdot 10^{-4}$	$4{,}0809 \cdot 10^{-4}$	2:2:3	1.8
$3{,}6054 \cdot 10^{-4}$	$3{,}6381 \cdot 10^{-4}$	3:2:3	0.9
$4{,}8072 \cdot 10^{-4}$	$4{,}5932 \cdot 10^{-4}$	4:2:3	4.4

Analytical applications

Under the optimum operating conditions, the aminoacids exhibited a linear behaviour at concentrations over the range from $5 \cdot 10^{-5}$ and $1 \cdot 10^{-3}$ mol dm^{-3}; the results fitted the equation $y = -137.85x + 0.0014$ with $r = 0.998$, x being the glycine concentration, in mol dm^{-3}, and y the first derivative of the absorbance. Concentrations below $5 \cdot 10^{-5}$ mol dm^{-3} gave rise to exceedingly low values for analytical purposes. On the other hand, above 1 10^{-3} mol dm^{-3}, the absorbance exceeded unity.

The reproducibility of the method was assessed in two series of experiments. In one, four calibrations were done under the above-described optimum conditions on as many different days. The first two were carried out on two consecutive days and the last two after allowing the reactor to stand unused for 2 weeks. As can be seen from Table 2, the slope of the calibration curve changed little between consecutive days but varied significantly with lack of use. The decrease by 7.6% in sensitivity was the likely result of gradual passivation of the reactor.

In the second series of experiments, four different reactors prepared as described above were used. The relative standard deviation thus obtained for the calibrations was 2.6%.

The tolerance of the proposed method to substances usually accompanying glycine in its pharmaceutical preparations was studied by using 2 10^{-4} mol dm^{-3} solutions of this aminoacid containing different concentrations of potential interferents. As can be seen from Table 3, aminoacids such as valine, phenylalanine, histidine and tryptophan interfere with the determination at concentration ratios above 100:1.

The proposed method was used to determine glycine in the commercially available pharmaceutical Tebetane, from Elfar-Drag, S.A, the per capsule composition of which was 45 mg of glycine, 265 mg of glutamic acid, 100 mg of alanine and 30 mg of lipid complex from *Prunus arborea* or *Prunus africana*. The

Table 2 Study of the reproducibility between-days of the solid-phase reactor.

Experience number	Calibration line	r^2
1	A'= -137.85 C – 0.0005	0.9962
2	A'= -135.48 C + 0.0026	0.9941
3	A'= -121.38 C – 0.0024	0.9900
4	A'= -118.73 C + 0.0003	0.9943
1'	A'= -135.28 C + 0.0004	0.9938
2'	A'= -137.85 C - 0.0005	0.9962
3'	A'= -142.91 C + 0.0030	0.9984
4'	A'= -135.02 C + 0.0023	0.9995

Table 3.- Study of the influence of the potential interferents.

Compound	Concentration (M)	|Error| (%)
Ascorbic acid	$1.05 \cdot 10^{-5}$	5.0
Nicotinamide	$1.62 \cdot 10^{-6}$	1.1
Citric acid	$4.02 \cdot 10^{-4}$	2.2
Glucose	$5.00 \cdot 10^{-3}$	1.8
Saccharose	$1.00 \cdot 10^{-2}$	0.9
Caffeine	$7.99 \cdot 10^{-7}$	5.6
Acetylsalicylic acid	$8.06 \cdot 10^{-7}$	10.2
Sorbitol	$5.00 \cdot 10^{-3}$	2.5
Lysine	$2.40 \cdot 10^{-4}$	4.6
Valine	$4.01 \cdot 10^{-6}$	7.7
Phenylalanine	$2.01 \cdot 10^{-6}$	2.4
Arginine	$4.00 \cdot 10^{-4}$	3.9
Histidine	$6.00 \cdot 10^{-6}$	5.2
Cysteine	$2.99 \cdot 10^{-5}$	4.4
Tryptophan	$8.00 \cdot 10^{-7}$	18.5
Tyrosine	$2.06 \cdot 10^{-6}$	5.2

difference between the manufacturer's stated glycine content and the average of three replicate determinations was only 0.8%.

Conclusions

A simple and cheap procedure is proposed for the determination glycine in presence of alanine and glutamic acid is proposed. The method is based on the first derivative spectra of the complex glycine-Cu(II). The procedure shows a competitive selectivity.

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