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# Coupling of sequential injection with liquid chromatography for the automated derivatization and on-line determination of amino acids

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**Abstract**

The principle of sequential injection (SI) was exploited to develop a fully automated pre-column derivatization procedure combined on-line to liquid chromatography (LC). Using SI–LC derivatization 14 amino acids were determined fluorimetrically in pharmaceuticals with *o*-phthaldialdehyde (OPA) as the derivatization reagent. The SI system was used for the handling of samples and reagents, on-line mixing and introduction to the LC injection system. Chemical (pH and reagents concentrations) and instrumental variables (sample and reagent volumes, reaction time and flow rate) were optimized to attain the highest reaction yield and detector signal. Reversed phase chromatographic resolution of 14 amino acids was achieved within 35 min using gradient elution. The automated operation of the coupled SI–LC system resulted in very satisfactory performance. The method was applied for the simultaneous determination of amino acids in pharmaceutical formulations.
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*Keywords:* On-line derivatization; Sequential injection; LC; Amino acids; Pharmaceuticals

## 1. Introduction

Amino acids (AA) are biological compounds of fundamental significance playing an important role in food science and pharmaceutical industry. Separation techniques, such as liquid chromatography (LC) and capillary electrophoresis (CE) are the major analytical tools to determine amino acids in various samples in combination to UV, fluorescence spectrometry and electrochemistry [1,2]. Problems often encountered in the analysis are the big number of amino acid analytes, the varying molecular polarity and the detection sensitivity. Amino acids' molecules do not comprise chromophore groups, thus the molecular absorption coefficient is low and UV detection often provides insufficient sensitivity. Strategies to overcome this problem include either the utilization of alternative detection modes (evaporative light scattering, chemiluminescence, nuclear magnetic resonance (NMR), conductivity, refractive index and mass spectrometry) [3] or most commonly the development of derivatization protocols (both pre- and post-column) [4,5].

Pre-column derivatization is a useful way to improve both the sensitivity and the selectivity of the method. It is mostly performed off-line because of instrumentation simplicity (typically, on-line derivatization is performed in an autosampler). Off-line manipulation may be laborious, time-consuming and may result to low precision. In addition, it involves manual handling of toxic reagents and organic solvents. On the other hand, on-line derivatization offers automation, ease and high sampling rate, providing an attractive alternative.

SI can be characterized as "an effective on-line sample handling procedure" with high potential for laboratory automation. The principles of SI are similar to those of the well-established flow injection analysis: controlled sample dispersion and reproducible timing. Reagent and sample solutions are aspirated sequentially into a holding coil (HC) and the resulting mixture is propelled towards the flow cell of the detector. The "heart" of SI is the multi-port selection valve where each port of the valve carries out a different operation. SI offers significant advantages in terms of manifold simplicity, robustness and low reagent and sample consumption. Additionally, all major experimental

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parameters such as reagents and sample volumes, direction and rate of the flow, order of reagent mixing and reaction time can be computer-controlled. Typically sequential injection analysis is used as a single individual technique to perform solution chemistry; however, it has also been used as a sample pretreatment tool prior to separation techniques such as LC [6,7] and CE [8–10]. A comprehensive review covered the topic, demonstrating the impact of SI in on-line sample handling and pretreatment [11].

Recently, we have shown the potential of sequential injection analysis (SI) as a tool for automated derivatization coupled on-line to LC for the determination of γ-aminobutyric acid [6]. The present paper exploits the on-line coupling of SI to LC for the derivatization and determination of 14 amino acids. A fully automated sample derivatization protocol has been developed using OPA as the reagent. The SI is used for sampling, reagents mixing and introduction to LC loop. The derivatized amino acids are subsequently analyzed in gradient RP–LC within 35 min. Important method parameters (pH value, *c*(OPA), *c*(mercaptoethanol)) and instrumental variables (sample and reagent volumes, reaction time, flow rate) are studied and optimized. The method provides full automation and very satisfactory characteristics. The system is used for the determination of amino acids in pharmaceutical formulations.

## 2. Experimental section

### 2.1. Chemicals

Individual amino acids were purchased from Merck (Darmstadt, Germany). OPA was purchased from Fluka (Buchs, Switzerland) and 2-mercaptoethanol (MCE) ($d = 1.114\ g\ mL^{-1}$) was purchased from Sigma (St. Louis, MO, USA), 1,7-diaminoheptane (DAH) and HPLC-grade methanol used were provided by Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade and were also provided by Merck (Darmstadt, Germany). A Milli-Q water purification system (Millipore) was used to obtain HPLC-grade water.

Standard aqueous stock solutions of each amino acid: L-gloutamic acid (Glu), D,L-valine (Val), D,L-isoleucine (Ile), D,L-phenylalanine (Phe), D,L-tryptophan (Trp), D,L-alanine (Ala), L-serine (Ser), L-arginine (Arg), L-aspartic acid (Asp), D,L-threonine (Thr), L-tyrosine (Tyr), D,L-methionine (Met), glycine (Gly), L-gloutamine (Gln), D,L-histidine (His), L-asparagine (Asn) at $100\ mg\ L^{-1}$ and DAH ($50\ mg\ L^{-1}$) used as the internal standard—were prepared and kept stored at 4 °C for up to a month and were then discarded. Working standards were prepared by diluting the stock solutions. Borate buffer ($0.05\ mol\ L^{-1}$, pH 9.4) was prepared by dissolving 19.07 g of borax in water to 1000 mL. The pH was adjusted to the appropriate value by addition of small volumes of $1\ mol\ L^{-1}$ NaOH or $1\ mol\ L^{-1}$ hydrochloric acid. This buffer can be stored in ambient temperature for 2 weeks.

The OPA buffered reagent was prepared according to the Dorresteijn's method [12] with some modifications. 250 mg OPA and 400 μL of MCE (1.6 μL MCE/mg OPA) were added in a volumetric flask; the mixture was diluted to 50 mL with borate buffer ($0.05\ mol\ L^{-1}$ with pH adjusted to 9.6). Stability experiments showed that the above solution was stable for at least 1 month, if it was kept refrigerated and protected from the light. The working OPA buffered reagent was prepared by 5-fold dilution of the stock solution in borate buffer with the adjusted pH value.

All LC mobile phases were filtered through 0.2 μm membrane filters and sonicated for 15 min prior to use. Helium spargers were employed for continuous degassing of the mobile phases.

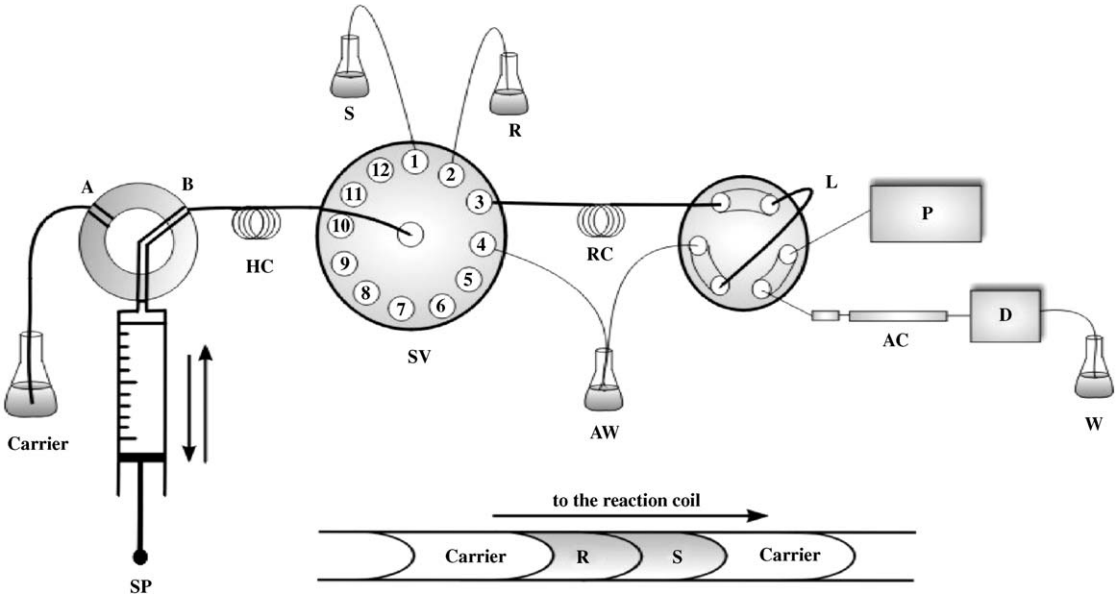


Fig. 1. SI–LC analysis instrumentation set-up, where Carrier = water, SP = syringe pump, SV = selection valve, HC = holding coil (300 cm/0.75 mm i.d.), RC = reaction coil (60 cm/0.75 mm i.d.), S = sample, R = OPA buffered reagent, AW = auxiliary waste, L = loop (250 μL), P = high pressure pump, AC = analytical column, D = fluorescence detector and W = waste.

### 2.2. Apparatus

A schematic diagram of the SI–LC setup is depicted in Fig. 1. The SI part consisted of the following parts: an Intellect 12-port valve (Kloehn Co. Ltd, Las Vegas, Nevada), and a Versa 6 syringe pump with 10 mL syringe's volume (Kloehn Co. Ltd, Las Vegas, Nevada). The flow system comprised 0.75 mm i.d. Teflon tubing throughout. The hardware was interfaced to the controlling PC through a serial port. The control of the system was performed through a Kloehn Control BETA 1.03 software program.

The derivative was injected to the LC analytical column through an automated injection valve system from ABI Spectroflow bearing a Rheodyne (Cotati, CA, USA) 7010 six port injection valve. LC separations were performed on a 250 mm × 4.6 mm i.d., $C_{18}$ Kromasil 4 μm analytical column obtained from MZ-Analysentechnik (Mainz, Germany) protected by a guard column ($C_{18}$ bonded silica). All separations were performed in ambient temperature. The mobile phase was driven through the column by an LCP 5020 gradient pump (INGOS, Czech Republic). The injection loop was 250 μL. The Shimadzu RF-551 spectrofluorimetric detector operated at $\lambda_{ext} = 340$ nm and $\lambda_{em} = 455$ nm. The response signal of the detector was acquired digitally and the data were saved in ASCII format for further manipulation (peak height/area measurement, digital filtering, etc.) using a home-made software programmed by Prof. P. Nikitas (Laboratory of Physical Chemistry, Department of Chemistry, Aristotle University Thessaloniki), running in Visual Basic® 6.0.

### 2.3. Determination of amino acids in aqueous solutions

The sequence of the determination of amino acids by the proposed method is shown in Table 1. Fifty microlitres of the OPA reagent and 50 μL of the standard/sample were aspirated in this order in the holding coil, through ports 2 and 1 of the selection valve, respectively. Blank analyses were performed by aspirating water instead of the aqueous amino acids solution. The two zones were propelled to the injection loop through port 3 at a flow rate of 1.2 mL $min^{-1}$. The amino acid–OPA derivatives were formed as the standard/sample and OPA zones were overlapped in tube towards to the LC injection loop. When the concentrated part of the plug of the derivative was tuned in the loop of the injection valve the flow of the syringe pump was stopped for 3 min to promote the reaction. Elution and effective separation of the amino acids and the internal standard (DAH) were achieved through a binary gradient elution program (Table 2), where solvent *A* was 0.02 mol $L^{-1}$ aqueous $CH_3COONH_4$ and solvent *B* was $CH_3OH$. Three replicate analyses were made in all instances. When changing between different samples or standard solutions, an additional washing step was performed in order to avoid carry-over effects; the new standard/sample was aspirated to the HC for 10 s at 1.2 mL $min^{-1}$ (200 μL), and then flushed through port 4 to the auxiliary waste (AW) for 60 s at 1.2 mL $min^{-1}$ (1200 μL).

Table 2
Gradient elution steps

| Time (min) | *A* (%) (v/v) | *B* (%) (v/v) | Flow rate (mL $min^{-1}$) |
|---|---|---|---|
| 0 | 70 | 30 | 1 |
| 13 | 55 | 45 | 1 |
| 13.1 | 55 | 45 | 1.1 |
| 35 | 20 | 80 | 1.1 |
| 35.1 | -End of cycle- | | |

Where the mobile phase *A* is the aqueous $CH_3COONH_4$ solution 0.02 mol $L^{-1}$ and *B* is methanol.

### 2.4. SI–LC determination of amino acids in pharmaceuticals

Each sample was filtered through a 0.45 μm membrane filter (Schleicher and Schuell, Dassel, Germany) and diluted 2000-fold with Milli-Q water. An aliquot of the diluted sample was spiked with the solution of DAH, used as internal standard, to provide a final concentration of 2 mg $L^{-1}$. Finally, the samples were analyzed using the above-described SI–LC procedure for aqueous solutions.

## 3. Results and discussion

### 3.1. Preliminary studies

Preliminary studies were done in order to check whether the derivatization reaction can be accomplished under SI flow conditions. For this reason, the reaction of each amino acid was performed individually using SI directly connected to the fluorimetric detector, omitting the LC separation step. The starting

Table 1
Sequence SI steps of a complete measurement cycle

| Time (s) | Pump action | Flow rate (mL $min^{-1}$) | Volume (μL) | Valve position | Action description |
|---|---|---|---|---|---|
| 1 | Off | – | – | 2 | Selection of OPA reagent port |
| 5 | Aspirate | 0.6 | 50 | 2 | Aspiration of reagent in holding coil |
| 1 | Off | – | – | 1 | Selection of sample port |
| 5 | Aspirate | 0.6 | 50 | 1 | Aspiration of sample in holding coil |
| 1 | Off | – | – | 3 | Selection of LC port |
| 20 | Deliver | 1.2 | 400 | 3 | Propulsion of reaction mixture to LC injection loop |
| 180 | Remaining in the injection loop to complete the reaction | | | | |
| | LC analysis | | | | |

values of the chemical and SI variables of these experiments were: $\gamma$(AA) = 1 mg $L^{-1}$ each, $c$(OPA) = 37.3 × $10^{-3}$ mol $L^{-1}$ $c$(MCE) = 114.4 × $10^{-3}$ mol $L^{-1}$ (pH 9.4, which is the pH of 0.05 mol $L^{-1}$ borate aqueous solution), the aspirated volume of each zone was $V$(S) = $V$(OPA) = 25 μL, respectively; the reaction coil (RC) was 60 cm/0.75 mm i.d., while the reaction mixture was propelled to the detector at a flow rate $q_v$ = 1.2 mL $min^{-1}$. These experiments confirmed that the reaction could proceed under flow conditions. The order of aspiration of sample and the reagent proved not to be critical, since negligible differences in the signals were observed when experimenting with the sequence of aspiration. Note that in order to avoid overpressure and/or bubble formation in the valve, the pump was stopped for 1 s between changing ports.

The second step of the preliminary studies was the on-line coupling of SI to LC. The SI system was connected to the LC system via a reaction coil (RC) (Fig. 1). The time between the start of the traveling of the derivative zone in the SI system and the actual injection into the LC system is evidently an important parameter. During this time the plug of the derivative goes through the reaction coil into the loop of the injection system of HPLC. The analyte concentration gradient is Gaussian-shaped with the highest analyte concentration being in the "head" of the traveling zone. Hence it is evident that a key issue of the coupling is the achievement of the optimum "tuning" of the reaction mixture in the injection loop of the LC. In other words, in order to attain maximum sensitivity it must be ensured that the most "concentrated" part of the sample will be the one injected into the LC column. It should be noted that a constant flow of the SI system was used during the time "tuning" experiments. The starting values of the chemical and SI variables of these experiments were the one mentioned in the previous paragraph. The feasibility of the methods' coupling was proven by applying the combined SI derivatization–LC determination scheme initially for two selected amino acids (Glu, Trp). These were selected in the basis of LC separation ease, as (for simplicity reasons) isocratic elution was used: aqueous solution $CH_3COONH_4$ 0.02 mol $L^{-1}$ 50/50 (v/v) $CH_3OH$ at a flow rate of 1 mL $min^{-1}$. This experimental study proved that the derivatives can be formed and subsequently analyzed in the LC using the on-line approach. For the assay of higher number of amino acids, systematic examinations of the SI and LC variables were performed.

### 3.2. Optimization of HPLC gradient elution

The simultaneous determination of a significant number of amino acid analytes requires the development of a gradient elution scheme. In the LC development stage, for simplicity reasons off-line derivatization and injection to LC was adopted. Hence, using the above mentioned reagent concentrations (AA, OPA, MCE) at a pH of 9.4, 200 μL of standard amino acid mixture solution were mixed with an equal volume of OPA. The mixture was left to react for 1 min and 50 μL of the derivatization mixture were injected manually in the LC column. Initially the amino acids were processed individually in order to identify the corresponding retention times. During the gradient optimization

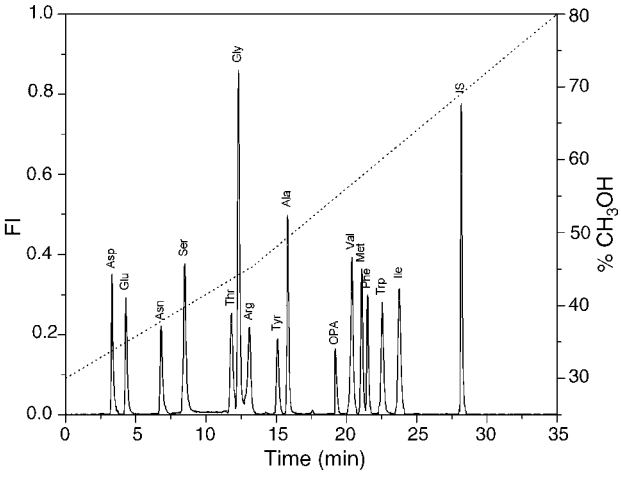


Fig. 2. Typical chromatogram of amino acid mixture containing 1 mg $L^{-1}$ of each amino acid. The abbreviation and the retention time of each amino acid were explained in the text. Where IS is the internal standard (DAH) and FI the fluorescence intensity. The right vertical axis represents to percentage of methanol in the gradient elution program. Chromatographic conditions are described in the text.

studies a mixture containing the 14 amino acids and the internal standard was used.

Several gradient elution protocols were tested to facilitate adequate elution and separation of the amino acids and the internal standard along with optimum peak shape and symmetry. To shorten the overall analysis time, further to the organic modifier alteration, an increase in the flow rate was applied: at 13 min the flow rate was increased to 1.1 mL $min^{-1}$. Finally, a binary elution system was chosen and applied (Table 2). Under the selected LC conditions each run (14 amino acids + internal standard) was completed in less than 30 min. The peaks of Glu–Gln and Ser–His, were partially separated, thus only one amino acid of each pair could be determined in real samples. The amino acids finally chosen were: Asp, Glu, Asn, Ser, Thr, Gly, Arg, Tyr, Ala, Val, Met, Phe, Trp, Ile. A typical chromatogram of a standard mixture of the selected amino acids and DAH in aqueous solutions is depicted in Fig. 2.

### 3.3. Optimization of reaction variables

Critical reaction parameters (pH, reaction time, $c$(OPA), $c$(MCE)) were studied and optimized in order to maximize the reaction yield and thus sensitivity. The univariate approach was used throughout the experiments. The starting values of the variables were pH 9.4, reaction time = 1 min, $c$(OPA) = 37.3 × $10^{-3}$ mol $L^{-1}$, $c$(MCE) = 114.4 × $10^{-3}$ mol $L^{-1}$.

The pH markedly affected the derivative reaction in the studied range (8.2–11.8) using direct SI-fluorimetry. It should be noted that a basic medium is required to keep the analytes unprotonated. The reaction rates for nearly all analytes were improved by increasing the pH of the buffer solution up to a value of 9.6 (Fig. 3). At a more basic medium, a decrease in the reaction yield was observed, probably due to partial hydrolysis of the derivatives. Therefore, the pH value of 9.6 was chosen and used for further experiments.

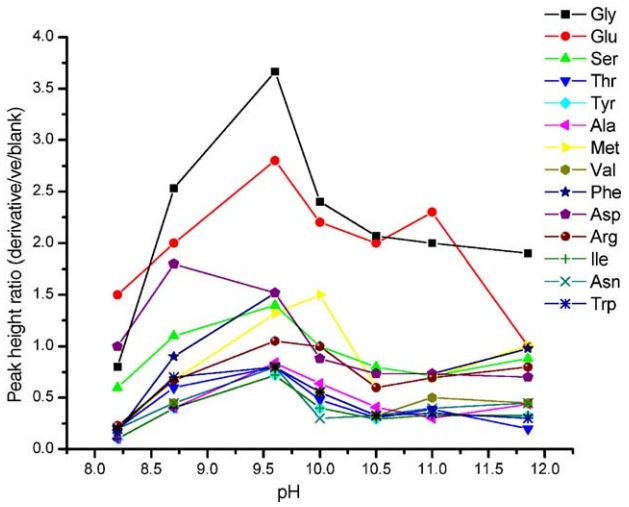


Fig. 3. Effect of pH value on the derivatization of amino acids. Reaction conditions described on text.

The OPA–MEC derivatizing reagent described in Section 2.1 was used as the starting reagent solution. However, a broad peak corresponding to OPA was found to interfere with the peak of valine. To minimize the excess concentration OPA–MEC, more diluted reagent solutions were tested. Five different dilution factors were studied to attain OPA concentrations of $1.87 \times 10^{-3}$–$37.3 \times 10^{-3}$ mol $L^{-1}$ and MCE concentrations of $5.7 \times 10^{-3}$–$114.4 \times 10^{-3}$ mol $L^{-1}$ (the ratio 1.6 μL MCE/mg OPA was kept constant). Borate buffer (pH 9.6) was used as the dilution medium. The optimum result was achieved at an OPA concentration of $7.46 \times 10^{-3}$ mol $L^{-1}$ and MCE concentration of $22.9 \times 10^{-3}$ mol $L^{-1}$ (1/5 dilution factor). The peak of valine–OPA was resolved from the OPA peak without sensitivity loss, as the peak areas for all amino acids remained constant.

Under the selected reagent concentration of $7.46 \times 10^{-3}$ mol $L^{-1}$, a study of the volume of the OPA reagent was conducted. Experiments were performed by aspirating 10, 25, 50 and 100 μL of the reagent mixture. The volume of the mixed sample solution was 25 μL and the concentrations studied were 1 and 5 mg $L^{-1}$ for each amino acid. In the series of experiments employing the lower sample concentration, the reagent volume did not affect the sensitivity. However, when derivatization was performed on the 5 mg $L^{-1}$ amino acid solution, an increase in the signal was observed up to the value of 50 μL. Surpassing this volume no gain in sensitivity was evident (equal peak areas), whereas the excess of the OPA–MEC reagent hindered the quantitation of neighboring peaks. Hence, the volume of 50 μL was finally selected. It should be pointed, that time tuning experiments preceded the alteration of the aspirated reagent volume.

Increase in the sample volume is theorized to result in increase in the peak signal. To verify this, several sample volumes were studied in the range 10–100 μL. The OPA reagent volume used was kept constant at 50 μL. Nonlinear increase was observed in the range 10–50 μL, leveling-off thereafter. Hence, the sample volume of 50 μL was selected as optimum.

In order to study the kinetics of the reaction, the reaction time was varied using a stopped-flow step. Reagent and sample zones were overlapped and mixed passing through the reaction coil (RC). The product zone was trapped in the LC loop, where it was stationed for 0, 1, 2, 3, 5 min. These experiments showed that the maximum reaction yield was accomplished at 3 min, reaching a steady state thereafter.

### 3.4. Analytical characteristics

Under the chosen SI and LC variables and using the SI–LC setup depicted in Fig. 1, calibration graphs for all amino acids in aqueous solutions were constructed. For this purpose the

Table 3
Analytical characteristics of the proposed method

| Amino acid | Calibration curves | $r^2$ | Linearity range (mg $L^{-1}$) | LOD[a] (μg $L^{-1}$) | Retention time (min) |
|---|---|---|---|---|---|
| Asp | $A^{b} = (0.139 \pm 0.006)\gamma[\text{Asp}] - (0.02 \pm 0.003)$ | 0.989 | 0.075–7 | 22 | 3.3 |
| Glu | $A = (0.131 \pm 0.005)\gamma[\text{Glu}] + (0.0025 \pm 0.003)$ | 0.998 | 0.08–8 | 24 | 4.3 |
| Asn | $A = (0.108 \pm 0.006)\gamma[\text{Asn}] - (0.011 \pm 0.002)$ | 0.996 | 0.06–6 | 17 | 6.8 |
| Ser | $A = (0.221 \pm 0.017)\gamma[\text{Ser}] - (0.030 \pm 0.005)$ | 0.995 | 0.08–9 | 18 | 8.5 |
| Thr | $A = (0.116 \pm 0.007)\gamma[\text{Thr}] + (0.012 \pm 0.002)$ | 0.995 | 0.05–8 | 26 | 11.8 |
| Gly | $A = (0.406 \pm 0.016)\gamma[\text{Gly}] + (0.067 \pm 0.009)$ | 0.999 | 0.07–7 | 10 | 12.3 |
| Arg | $A = (0.124 \pm 0.003)\gamma[\text{Arg}] + (0.036 \pm 0.001)$ | 0.997 | 0.1–10 | 30 | 13.1 |
| Tyr | $A = (0.098 \pm 0.004)\gamma[\text{Tyr}] + (0.017 \pm 0.002)$ | 0.996 | 0.08–5 | 25 | 15.1 |
| Ala | $A = (0.209 \pm 0.011)\gamma[\text{Ala}] - (0.016 \pm 0.006)$ | 0.998 | 0.09–9 | 18 | 15.8 |
| Val | $A = (0.227 \pm 0.013)\gamma[\text{Val}] + (0.025 \pm 0.005)$ | 0.998 | 0.08–10 | 20 | 20.4 |
| Met | $A = (0.165 \pm 0.006)\gamma[\text{Met}] - (0.041 \pm 0.006)$ | 0.989 | 0.1–9 | 23 | 21.1 |
| Phe | $A = (0.126 \pm 0.009)\gamma[\text{Phe}] + (0.014 \pm 0.002)$ | 0.997 | 0.05–5 | 25 | 21.5 |
| Trp | $A = (0.130 \pm 0.004)\gamma[\text{Trp}] + (0.021 \pm 0.003)$ | 0.999 | 0.07–7 | 21 | 22.5 |
| Ile | $A = (0.188 \pm 0.008)\gamma[\text{Ile}] - (0.032 \pm 0.005)$ | 0.998 | 0.08–9 | 20 | 23.7 |
| DAH[c] | – | – | – | – | 28.2 |

[a] Signal to noise (S/N) = 3.
[b] Amino acid to DAH peak area ratio.
[c] Mass concentration of DAH, γ(DAH) = 2 mg $L^{-1}$.

Table 4
Results from the analysis of pharmaceutical samples

| Amino acid | Aminoplasmal® L-5% | | | Vamine® glucose | | | Tonotil® | | |
|---|---|---|---|---|---|---|---|---|---|
| | Added[a] ($mg\,L^{-1}$) | Found[b] ($mg\,L^{-1}$) | $R$[c] | Added[a] ($mg\,L^{-1}$) | Found[b] ($mg\,L^{-1}$) | $R$[c] | Added[a] ($mg\,L^{-1}$) | Found[b] ($mg\,L^{-1}$) | $R$[c] |
| Asp | 650 | 630 | 96.9 | 4130 | 4060 | 98.3 | NC[d] | – | – |
| Glu | 2300 | 2280 | 99.1 | 9070 | 8880 | 97.9 | NC | – | – |
| Gln | NC | – | – | NC | – | – | 6000[e] | 6120 | 102.0 |
| Asn | 1640 | 1680 | 102.4 | NC | – | – | NC | – | – |
| Ser | 1200 | NS[f] | – | 7470 | NS | – | NC | – | – |
| Thr | 2050 | 2097 | 102.3 | 2930 | 2750 | 93.8 | NC | – | – |
| Gly | 3950 | 4120 | 104.3 | 2130 | 2040 | 95.7 | NC | – | – |
| Arg | 4600 | 4760 | 103.4 | 1400 | 1520 | 108.6 | 8270 | 8350 | 100.9 |
| Tyr | 300 | NS | – | 510 | 550 | 107.8 | NC | – | – |
| Ala | 6850 | NS | – | 2930 | 2810 | 95.9 | NC | – | – |
| Val | 2400 | 2320 | 96.6 | 4270 | 4350 | 101.8 | NC | – | – |
| Met | 1900 | 1820 | 95.8 | 1870 | 1800 | 96.2 | NC | – | – |
| Phe | 2550 | 2490 | 97.6 | 5470 | 5610 | 102.5 | NC | – | – |
| Trp | 900 | 890 | 98.9 | 1000 | 1060 | 106.0 | NC | – | – |
| Ile | 2550 | NS | – | 3870 | NS | – | NC | – | – |

[a] Amino acid mass concentration according to package label.
[b] Mean of three results.
[c] Percentage recovery.
[d] Not contained.
[e] Calculated by individual calibration curve $A = (0.124 \pm 0.009)\gamma[\text{Gln}] + (0.011 \pm 0.002)$, LOD: $45\,\mu g\,L^{-1}$.
[f] No baseline separation.

following analyte concentrations were used: 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 $mg\,L^{-1}$. The equations of the obtained calibration curves, the linear range, the concentration limits of detection ($c$LOD) and the retention time of each amino acid are shown in Table 3.

The within-day relative standard deviation of all amino acids varied between 2.7 and 7.0% at 1.0 and 5.0 $mg\,L^{-1}$ AA ($n = 8$). The within-day accuracy of the developed protocol also proved to be satisfactory, as the recoveries of the above-mentioned amino acid concentrations were in the range 94–107%.

The day-to-day reproducibility of the method was validated in a time period of 8 days ($n = 8$). The reproducibility of the method was very satisfactory and the relative standard deviation varied in the range 1.9–8.6%.

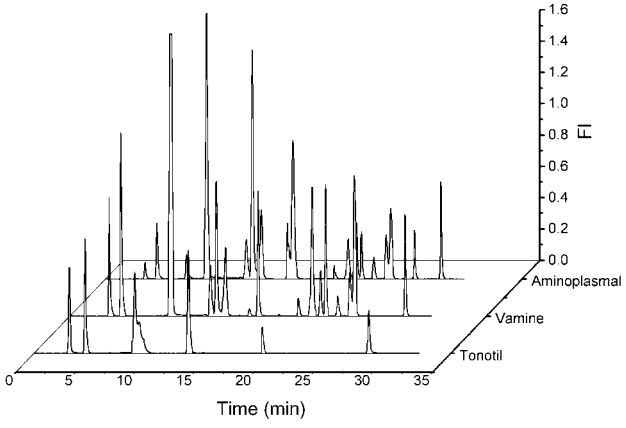


Fig. 4. Analysis of commercial pharmaceutical samples using SI–LC instrumentation. Where FI is the fluorescence intensity. Chromatographic conditions are described in the text.

### 3.5. Analysis of pharmaceutical samples

To demonstrate the potential of the developed coupling technology, the method was applied to the simultaneous determination of amino acids in pharmaceutical formulations (Aminoplasmal®, Vamine®, Tonotil®). All samples were used without any further pretreatment expect of filtration and a dilution step of 1/2000 in Millipore water. In these solutions appropriate volume of a stock solution of internal standard was added to reach a final concentration of 2 $mg\,L^{-1}$. The recoveries of the proposed assay were in the range 93.8–108.6% in all cases. The results of the analysis of pharmaceutical samples, Aminoplasmal® L-5%, Vamine® glucose and Tonotil® (contained four amino acids) are tabulated on Table 4. Characteristic chromatograms of each sample are depicted in Fig. 4.

## 4. Conclusions

SI can be used as an efficient and versatile tool for sample handling prior to LC. In the present work we provide further evidence on the potential of the method for on-line pre-column derivatization. The major advantages that this coupling offers, include instrumentation simplicity and at the same time significant flexibility in method development due to the possibility to perform multivariate studies in the same set-up. With this scheme, the SI and the LC may run in parallel mode: the LC may analyze a sample and at the same time the SI can process the next sample. As a result a sampling frequency of two samples per hour is achieved, including sample preparation (derivatization) and HPLC determination. The SI system is very easy to build and provides full handling automation. As a result the

developed SI–LC method is characterized by very satisfactory accuracy and precision.

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