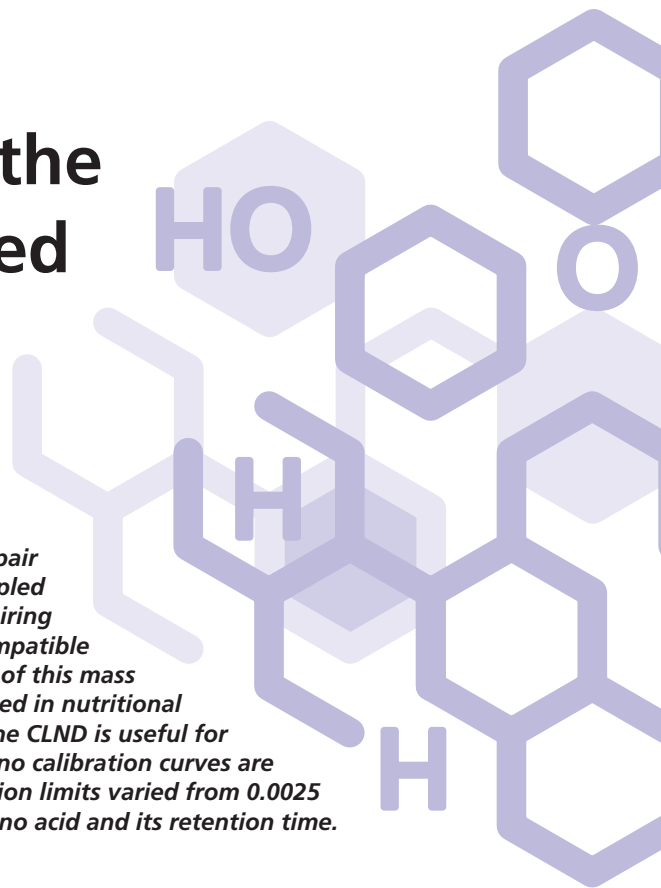


HPLC–CLND for the Analysis of Underivatized Amino Acids

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Underivatized amino acid analysis is demonstrated with ion-pair reversed-phase high performance liquid chromatography coupled with a chemiluminescent nitrogen detector (CLND). Volatile ion-pairing reagents (perfluorinated carboxylic acids) are shown to be compatible with this mode of detection. The linearity and equimolarity of this mass dependent detector is confirmed. Amino acids can be determined in nutritional serum, tobacco extract and wine with a single calibration curve. The CLND is useful for determining amino acid stoichiometry of peptide hydrolysates; no calibration curves are needed if the molecular weight of the peptide is known. Detection limits varied from 0.0025 mM to 0.0075 mM (0.33–0.86 mg/L) depending on the amino acid and its retention time.



Introduction

There is considerable incentive to develop new chromatographic methods for the direct analysis of amino acids without derivatization. The main advantages over derivatization-based methods are simplicity and ease of automatization, without any of the disadvantages such as derivative instability, reagent interference, inability of some reagents to derivatize the secondary amino group, long preparation times and increases in the void volume for postcolumn derivatization methods.

Traditionally, underivatized amino acids are separated by ion-exchange (1–4) or ion-pair reversed-phase high performance liquid chromatography (IP-RP-HPLC) (5–8). However, the above methods use non-volatile buffers (phosphate, lithium etc.) and/or non-volatile ion-pairing reagents (salts of alkylsulfonic acids, tetrabutylammonium, etc.). The appearance on the market of powerful new detectors such as the atmospheric pressure ionization-mass spectrometer (API-MS), the evaporative light-scattering detector (ELSD) and the chemiluminescent nitrogen detector (CLND), all requiring volatile mobile phases, changed the approach of analytical scientists for the selection of mobile phases.

For underivatized amino acids analysis Chaves-das-Neves and Braga-Morais (9–10) used mixtures of trifluoroacetic acid (TFA)–H₂O–acetonitrile (ACN) and column

switching or mixed-bed columns (reversed-phase cation exchange) coupled with ELSD. In another interesting approach, Peterson et al. (11) employed a cation exchange column and an aqueous TFA-ammonium acetate buffer with ELSD. We have demonstrated previously the usefulness of semi-long chain perfluorinated carboxylic acids as ion-pairing reagents (12–17) for the separation of underivatized amino acids. These reagents not only exhibited better selectivity compared with alkylsulfonic acids but were also volatile enough to be used with ELS and MS detection (12–16). This method allowed the simultaneous analysis of the 20 proteogenic amino acids (14–16) as well as less common amino acids (17–19). The simultaneous analysis of 15 chiral underivatized amino acids was also possible by LC–MS–MS using a teicoplanin chiral stationary phase (20).

The use of ozone-induced chemiluminescence detection in chromatography has been reviewed recently (21–24). Briefly, HPLC column effluent is nebulized with oxygen and argon or helium and introduced into a pyrolysis tube heated to 1050 °C. In an oxygen-rich environment the mobile phase and the solutes are oxidized into oxides (water, carbon dioxide and other oxides). All the nitrogen-containing compounds except from diatomic nitrogen are

converted to nitric oxide (NO). Nitric oxide is then mixed with ozone (O₃) to form nitrogen dioxide in the excited state (NO₂*), which rapidly decays to the ground state, releasing a photon (hν). A photomultiplier tube then detects the photon emission. The detected signal is directly proportional to the amount of nitrogen in the original sample.

While the nitrogen chemiluminescence detection technology as used in the CLND has existed for more than 25 years (25–27) the coupling of CLND with HPLC has been problematic (28). In 1992, a novel HPLC–CLND detector (29) was commercially introduced. Since then, the detector has been redesigned and upgraded for routine and uninterrupted operation (22–23). From 1992 (29) to date, some 20 papers have appeared, exploring the usefulness of this detector, such as in combinatorial chemistry (22–23,30) and peptide analysis (31–33).

While underivatized amino acid analysis with CLND may appear evident, almost all chromatographic methods previously described in the literature are incompatible with this mode of detection because the mobile phase contains either high concentrations of non-volatile buffers or nitrogen-containing solvents or additives (ACN, ammonium etc.). In a previous paper (12), we demonstrated that a great number of highly polar underivatized amino acids may be separated isocratically

using low concentrations (0.5–1 mM) of tridecafluoroheptanoic acid (TDFHA) or pentadecafluorooctanoic acid (PDFOA) in water.

In this study the analysis of underivatized amino acids with IP-RP-HPLC–CLND is evaluated. Equimolarity, quantification of multiple amino acids using a single nitrogen calibration curve and analysis of underivatized amino acids in complex matrices are also discussed.

Experimental

Reagents: HPLC-grade methanol (MeOH) was obtained from J.T. Baker (Noisy le Sec, France). Taurine (Tau), hypotaurine (Hpt), aspartic acid (Asp), hydroxyproline (Hyp), asparagine (Asn), serine (Ser), glycine (Gly), glutamine (Gln), cysteine (Cys), glutamic acid (Glu), threonine (Thr), alanine (Ala), proline (Pro), methionine (Met), tyrosine (Tyr), leucine (Leu), isoleucine (Ile), phenylalanine (Phe) and tryptophan (Trp)

were purchased from Sigma or Aldrich (St. Quentin Fallavier, Missouri, USA). TFA and TDFHA were obtained from Interchim (Montluçon, France). Heptafluorobutyric acid (HFBA), nonafluoropentanoic acid (NFPA) and PDFOA were purchased from Aldrich (St. Quentin Fallavier, France). Formic acid (HCOOH) was obtained from Sigma (St. Quentin Fallavier, France). The tetrapeptide Gly-Gly-Asp-Ala was purchased from Bachem (Torrance, California, USA). The nutritional serum and ground tobacco were kindly donated by their manufacturers. The Cabernet Sauvignon wine was obtained from a local market. 18 M Ω deionized water by an Elgastat UHQ II system (Elga, Antony, France) was used as HPLC-grade eluent and for the preparation of amino acid solutions.

Apparatus: The HPLC system was composed of a Beckman (Fullerton, California, USA) Model 128 System gold binary pump, a Gilson (Villiers le bel, France) Model Bio732 autosampler fitted with a 10 μ L loop, a Shimadzu (Kyoto, Japan) C-R6A integrator and an Antek (Houston, Texas, USA) Model 8060 chemiluminescent nitrogen detector (Alytech, Juvisy sur Orge, France). Gas settings: O₂ = 202 mL/min, He = 99 mL/min, Make-up = 50 mL/min, PMT voltage = –750 V.

Isocratic separations were performed on a Purospher RP-18e (125 \times 3 mm, 5 μ m) column. The flow was split postcolumn (0.15 mL/min to the nebulizer, 0.45 mL/min to waste) with a simple tee and appropriate length of PEEK tubing. A Purospher Star RP-18e (55 \times 2 mm, 3 μ m) column was used to study if the detector response was proportional to concentration or mass. Flow-rate was 0.2 mL/min and no split was used in this instance.

Sample preparation: Peptide hydrolysis: A slightly modified conventional acidic hydrolysis (34) was used to hydrolyse Gly-Gly-Asp-Ala into its free amino acids. A 1.3 mg sample of the tetrapeptide was digested for 24 h in 2 mL of 6 N HCl containing 0.5% phenol, heated at 110 °C. The sample was then lyophilized and reconstituted with 2 mL of mobile phase (0.5 mM PDFOA). The sample was further diluted 1:50 before injection.

Nutritional serum: The serum was diluted 1:100 and injected without further treatment. **Tobacco extract:** 500 mg of tobacco was mixed with 50 mL of water containing 10 mM of formic acid and stirred for 30 min. Subsequently, 30 mL of tobacco extract was ultrafiltered with a 1000 MW cut-off membrane to eliminate high

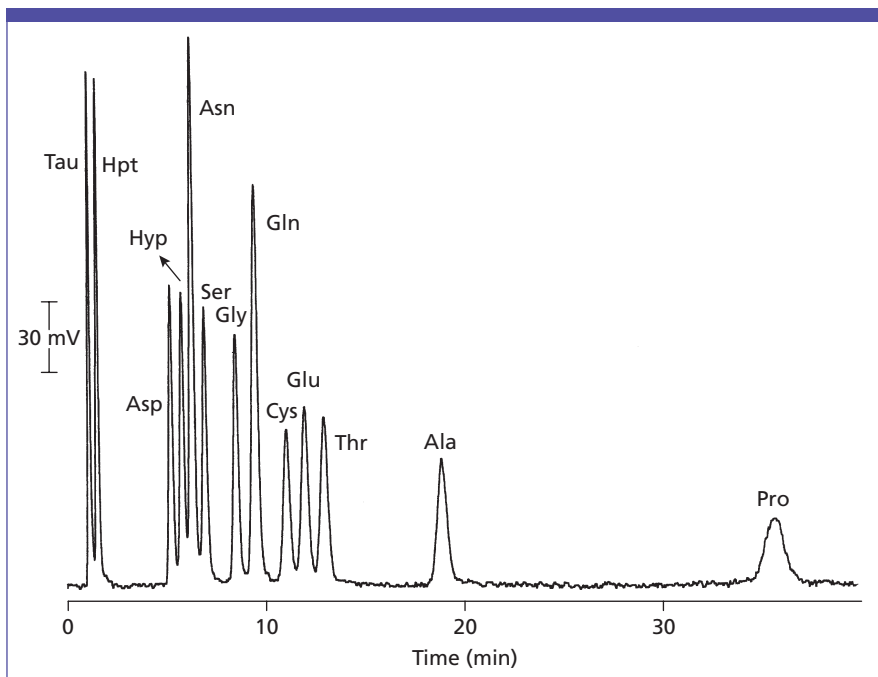


Figure 1: IP-RP-HPLC–CLND analysis of a standard solution of 13 underivatized amino acids under isocratic conditions. Column: Purospher RP-18e (125 \times 3 mm, 5 μ m); flow-rate: 0.6 mL/min, split 1/4 (0.15 mL/min to the detector); mobile phase: 0.5 mM pentadecafluorooctanoic acid; injected volume: 10 μ L; concentration of amino acids: 0.1 mM of each. For amino acid abbreviations and detection settings see experimental section.

Table 1: Equimolarity and Limits of Detection for Underivatized Amino Acids under Isocratic HPLC–CLND.

Amino acid	Area	Area per N atom	LOD (mM)	LOD (mg/L)
Tau	1729302	1729302	0.005	0.625
Hpt	1842565	1842565	0.005	0.545
Asp	1783914	1783914	0.005	0.665
Hyp	2020738	2020738	0.005	0.665
Asn	3515782	1757891	0.0025	0.33
Ser	2073198	2073198	0.005	0.525
Gly	1850649	1850649	0.005	0.375
Cys	1773565	1773565	0.005	0.605
Gln	3805475	1902737	0.0025	0.365
Glu	1992915	1992915	0.005	0.595
Thr	1894523	1894523	0.005	0.735
Ala	1844378	1844378	0.00625	0.556
Pro	2071109	2071109	0.0075	0.862
RSD%		6.3		

molecular weight compounds. A 10 mL aliquot of the ultrafiltrate was used for analysis without further treatment. *Wine*: A 30 mL sample of red wine was ultrafiltered in the same way as the tobacco extract.

Discussion

Is the CLND a concentration- or mass-dependent detector? Whether the CLND is a concentration- or a mass-sensitive detector is one of the most important aspects of performance for a chromatographic detector. We confirmed that CLND is a mass-sensitive detector by using a Purospher Star RP-18e (55 × 2 mm, 3 μm) column and 80:20 H₂O:MeOH containing 1 mM NPPA as mobile phase. 0.01 mM of Val was injected in triplicate in the following mobile-phase flow-rates: 0.1, 0.15, 0.2, 0.25 and 0.3 mL/min respectively. Val peak area was about the same (RSD = 6%) independent of the flow-rate, implying a mass-sensitive detector.

Choice of the ion-pairing reagents: Except for TFA, which is available in analytical grade, all the higher homologues contained 1–4% impurities. Furthermore, their purity varied between different lots (e.g., for PDFOA Aldrich reference 17146-8, lot number AS 07216 purity 98%, lot number HU 14404 purity 96%). Flow injection analysis with CLND (FIA-CLND) was used to verify if the impurities were nitrogen-containing compounds by injecting 10 mM (aliquoted in 80:20 H₂O:MeOH) of TFA, HFBA, NPPA, TDFHA and PDFOA (two lots). FIA-CLND confirmed that the 1–4% of impurities were not nitrogen-contaminants.

HPLC-CLND analysis of underivatized amino acids: It has been demonstrated (12) that baseline separation of 10 highly polar amino acids, Asp, Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala and Pro was possible with a Purospher RP-18e column and 0.5 mM PDFOA in water as the mobile phase. As shown in Figure 1 three other amino acids, Tau, Hpt and Hyp could be simultaneously separated under the same chromatographic conditions. The CLND detected these 13 underivatized amino acids without problem.

The equimolarity of this mode of detection was also tested by triplicate injection of the same concentration of each amino acid (0.1 mM). Table 1 shows that the detector response was about equal for all amino acids (RSD = 6.3%). Moreover, this RSD includes small errors that may come from amino acid weighing, solution dilution etc. The equimolarity obtained in this study is higher than that obtained previously for compounds

synthesized by combinatorial chemistry (RSD = 10%) (22). The detection limit (S/N = 3) depended on the amino acid (nitrogen number in the molecule) and its retention time (chromatographic dilution) varied from 0.0025 mM to 0.0075 mM (see Table 1). These detection limits will be improved by the use of smaller internal diameter columns; no split is then required. It must be pointed out here that any attempt at derivatization of the amino acids by non-nitrogen containing reagents will increase the limits of detection as the percentage of nitrogen will be decreased.

Finally, for the present chromatographic method, linearity studies showed a linear dynamic range (from the limit of detection (LOD) to saturation of the detector) of 2.5 orders of magnitude for the early eluting amino acids and ≥3 orders of magnitude for the late-eluting amino acids. The full four orders of magnitude linearity given by the manufacturer can be obtained by running one set from LOD to saturation to achieve about 2.5–3 linear dynamic range, and then decreasing the gain to extend the calibration curve (35).

HPLC-CLND for amino acid stoichiometry determination of peptide hydrolysates:

The importance of peptides in food chemistry, clinical chemistry and other fields is well known. The design of new drugs and other pharmaceutical compounds having a peptidic structure is the main research area of many pharmaceutical groups. Amino acid analysers and mass spectrometry are the main tools currently used for amino acid stoichiometry and peptidic sequence determination. Simpler, faster and automated methods are always welcome.

The basic advantage of this detector in comparison to all the other detection methods is its equimolarity. When applied to the determination of amino acid stoichiometry of peptide-protein hydrolysates, the need for calibration curves becomes unnecessary if the molecular weight of the peptide or protein is known (mass spectrometry in combination with deconvolution programmes provides this information with a simple infusion of the peptide-protein (36)). The amino acid stoichiometry of the hydrolysed peptide or protein analysed by HPLC-CLND will be given from Equation 1, where MW_p is the molecular weight of the peptide or protein (known or determined by ESI-MS), A is the multiplier factor to obtain the MW of the peptide or protein (A = 1, 2, 3 etc.), MW_{aa} – 18 is the molecular weight of amino acid – 18 dalton for the peptidic bond and n_{aa(n)} is

the detector response for an amino acid per nitrogen and normalized as if the hydrolysis was 100%.

In Equation 2, n_{aa(min)} is the amino acid(s) that gives the minimum n_{aa}, S_{aa} is the peak area of amino acid, a_{aa} is the percentage recovery of each amino acid (defined for a validated method of hydrolysis), N_{aa} is the number of nitrogens in the molecular structure of the amino acid and aa is the amino acid.

If the hydrolysate comes from a physiological protein with post-translational modification, the modified amino acids must be added in to the above equation (and separated chromatographically before their detection).

The tetrapeptide Gly-Gly-Asp-Ala was chosen as a simplified example. After hydrolysis (see experimental section) the hydrolysate was injected in triplicate (see Figure 2). The peak area was 2445005 for Gly, 1288279 for Ala and 1175836 for Asp. Recovery of these particular amino acids with this hydrolysis method is 100% (37). The molecular weight of the peptide is 318. Equation 1 then gives $318 = A \times \{[(57 \times 1.98) + [71 \times 1.04] + [115 \times 0.95)] + 18\} \rightarrow 318 = A \times (313.8)$ (the mean area of Asp and Ala was taken as least peak area). The whole number that 313.8 must be multiplied by to give approximately 318 is 1, so the correct amino acid stoichiometry will be Gly: 2, Ala: 1, Asp: 1. The Bachem certificate of analysis gives: Gly: 2, Ala: 1, Asp: 0.98. If the peptide was the octapeptide analogue Gly-Gly-Asp-Ala-Gly-Gly-Asp-Ala Equation 1 will potentially give $636 = A \times (313.8)$, A = 2 and stoichiometry is doubled.

The conventional acid hydrolysis used in this study could be replaced by microwave radiation-induced hydrolysis in order to

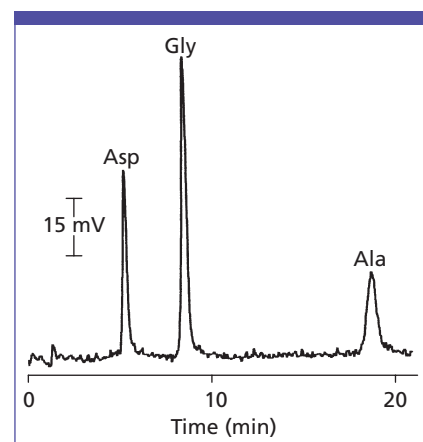


Figure 2: Isocratic analysis of the tetrapeptide Gly-Gly-Asp-Ala hydrolysate by IP-RP-HPLC-CLND. Same experimental conditions as in Figure 1.

reduce hydrolysis time (1–30 min) and allow the use of single autosampler vials for sample hydrolysis. This represents an important step towards process automation (38, 39). Another interesting approach comes from Fountoulakis and co-workers (39, 40), who developed apparatus for large-scale hydrolysis using multiple hydrolysis vessels, each accommodating 56 vials.

HPLC–CLND analysis of underivatized amino acids in complex matrices: The limits of detection obtained in this study are low enough to make this method applicable to most physiological samples. Furthermore, the equimolarity of the detector allows the quantification of amino acids with a single nitrogen calibration curve. Figure 3 shows calibration curves obtained for two orders of magnitude. It can be clearly seen that the slope obtained for Asn (which contains two nitrogen atoms per molecule) is the double of the slope of Tau or Asp, which contains one nitrogen atom per molecule. On a per-nitrogen-atom basis, the three calibration curves are practically superimposable. This demonstrates the ability of CLND to quantitate with a single nitrogen calibration curve. Figure 4(a) shows the

analysis of a nutritional serum. The serum also contains additional hydrophobic and basic amino acids that are not eluted under these experimental conditions. Table 2 shows the quantification of amino acids in the nutritional serum by using Tau or Asp or Asn as calibration curves (Asn equation was divided by two) in comparison to the values and the norms given by the serum certificate of analysis. All the amino acids except Cys show good correlation. The lower than expected results from Cys maybe the result of its partial oxidation to cystine, which is not eluted under these chromatographic conditions. The RSD among the values calculated by a single calibration curve were less than 1.66% for all amino acids except Tau (RSD = 2.15%).

Nutritional sera generally contain glucose, which under these chromatographic conditions is eluted at the void volume (as is Tau). Glucose does not contain nitrogen and, therefore, is not detected by CLND; however, the possibility of perturbation of Tau quantification was investigated. Four standard solutions were prepared all containing 0.01 mM Tau with and without glucose (0, 1000, 10 000 and 100 000 mg/L of glucose). Table 3 shows that peak area and retention time of Tau was

not perturbed until the concentration of glucose reached 100 000 mg/L. There was then a small perturbation in both retention time and peak area but not peak shape. It should be noted that 100 000 mg/L of glucose is well above the concentration generally used in serum (0.5%). Moreover, (see experimental section) in order to avoid saturation of the detector, serum samples were diluted 1:100 to further decrease glucose concentration.

Finally, amino acids were quantified in physiological samples, such as tobacco extracts and red wine (Figures 4(b) and 4(c), respectively). The chromatographic method and the specificity of this mode of detection precluded interference of matrix components with detection of the amino acids present, except for several nitrogen compounds eluting close to the void volume. Calculated amino acid values were in good agreement with amino acid values given by the manufacturer. In the wine sample Asp may have co-eluted with an unknown compound. From Figure 4(c) it can be seen that proline is the major amino acid contained in the wine, which is in accordance with published data (41).

Conclusion

In this study, underivatized amino acids are analysed by HPLC–CLND. Volatile perfluorinated carboxylic acids are used as ion-pairing reagents and found to be compatible with the detector. In addition, the low detection limit (<1 mg/L), the specificity of the detection and the selectivity of the chromatographic system allowed the analysis of underivatized amino acids even in complex matrices, such as wine, tobacco extract and peptide hydrolysate.

The principal advantage of this detection system stems from its equimolar response, which allows:

- quantification of many underivatized amino acids using a single nitrogen calibration curve
 - determination of amino acid stoichiometry in a peptide-protein (after hydrolysis) without the need for calibration curves, if the molecular weight of the peptide-protein is known.
- This second advantage will be particularly helpful for the control of solid-phase synthetic peptides for which amino acid composition is known and a rapid verification of amino acid stoichiometry can be obtained using HPLC–CLND. Furthermore, elimination of the time-consuming derivatization and calibration curves steps makes the automation of the

Equations:

$$MW_p = A \left[\left[(MW_{aa1} - 18) \frac{n_{aa1}}{n_{aamin}} + (MW_{aa2} - 18) \frac{n_{aa2}}{n_{aamin}} + \dots + (MW_{aan} - 18) \frac{n_{aan}}{n_{aamin}} \right] + 18 \right] \quad [1]$$

$$n_{aa(n)} = \frac{S_{aa(n)}}{a_{aa(n)} N_{aa(n)}} \quad [2]$$

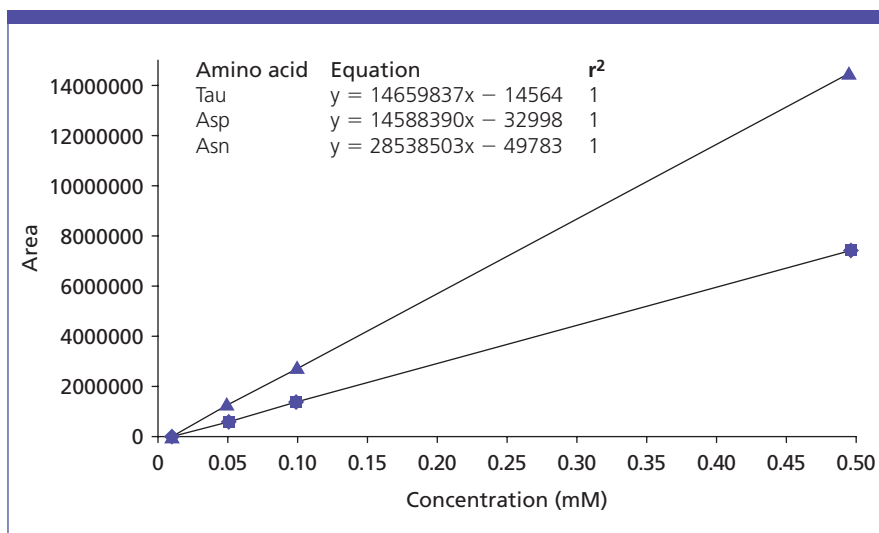


Figure 3: ◆ = Tau, ■ = Asp and ▲ = Asn calibration curves (injections in triplicate). Excellent linearity, double response factor value for Asn when compared with Tau or Asp.

system particularly easy and minimize analysis time.

Future studies with CLND will include development of a rapid liquid chromatographic method compatible with CLND for the separation of the 18 proteogenic amino acids (and if possible

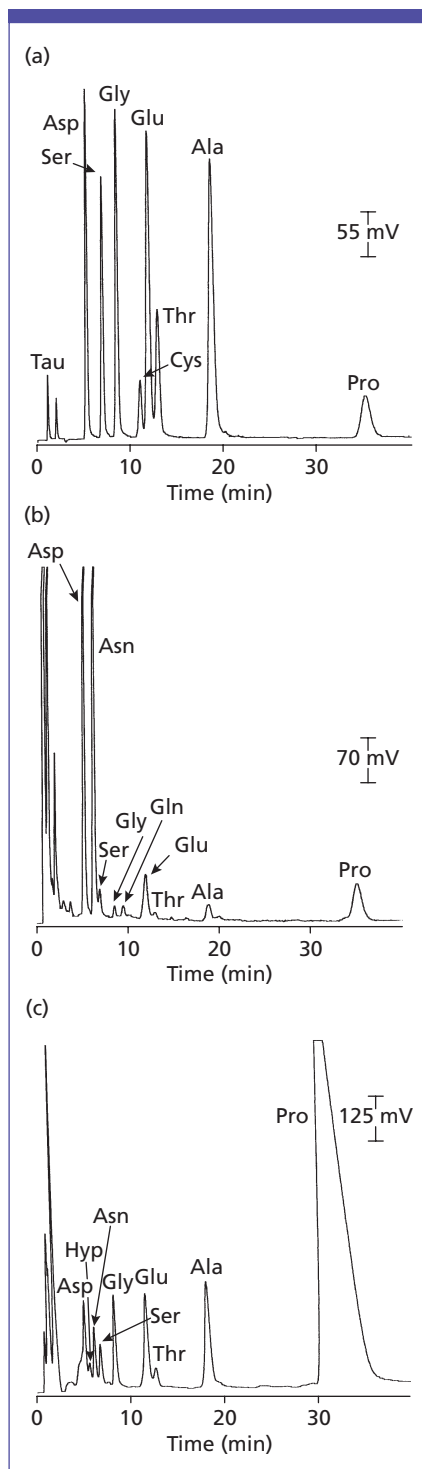


Figure 4: IP-RP-HPLC-CLND isocratic analysis of (a) a nutritional serum sample, (b) a tobacco extract and (c) wine. For extraction methodology see experimental section. Same experimental conditions as in Figure 1.

amino acids after post-translational modifications) obtained after acidic hydrolysis and validation of new methods for amino acid stoichiometry of protein-peptide hydrolysates without the need for calibration curves. The possibility of constructing a totally automated hydrolysis instrumentation coupled with an automated HPLC-CLND system will also be investigated. Lastly, the potential use of the above system, in tandem with mass spectrometry for proteomic research, will be investigated by decreasing the detection limits through the use of microbore-chromatographic columns to minimize the amount of sample required.

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Table 2: HPLC-CLND Quantification of Underivatized Amino Acids in a Nutritional Serum using a Single Calibration Curve and by Comparison with Certificate of Analysis Values.

Amino acid	Single calibration curve using:			Certificate of analysis	
	Tau	Asp	Asn	Values	Norms
Tau	0.60	0.62	0.62	0.59	0.54–0.66
Asp	5.72	5.76	5.88	6.06	5.4–6.6
Ser	3.81	3.84	3.92	3.95	3.6–4.4
Gly	3.77	3.80	3.88	3.92	3.6–4.4
Cys	1.35	1.37	1.39	1.82	1.7–2.08
Glu	9.94	10.01	10.23	10.34	9.0–11.0
Thr	3.71	3.74	3.82	3.64	3.3–4.07
Ala	8.07	8.12	8.30	7.9	6.84–8.36
Pro	2.91	2.94	3.00	2.97	2.7–3.3

All values are expressed at mg/L

Table 3: Potential Perturbation Resulting from the Coelution of a Non-nitrogen Containing Compound (Glucose) on the Quantification by HPLC-CLND of a Nitrogen-Containing Compound (Taurine).

Tr	0 ppm glucose		1000 pm glucose		10000 ppm glucose		100000 ppm glucose	
	Tr	Area	Tr	Area	Tr	Area	Tr	Area
Tau	1.055	3910280	1.057	3864862	1.057	3879758	1.066	3467009

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