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REVIEW

Post-column labeling techniques in amino acid analysis by liquid chromatography

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Abstract Amino acid analysis (AAA) has always presented an analytical challenge in terms of sample preparation, separation, and detection. Because of the vast number of amino acids, various separation methods have been applied taking into consideration the large differences in their chemical structures, which span from nonpolar to highly polar side chains. Numerous separation methods have been developed in the past 60 years, and impressive achievements have been made in the fields of separation, derivatization, and detection of amino acids (AAs). Among the separation methods, liquid chromatography (LC) prevailed in the AAA field using either pre-column or post-column labeling techniques in order to improve either separation of AAs or selectivity and sensitivity of AAA. Of the two approaches, the post-column technique is a more rugged and reproducible method and provides excellent AAs separation relatively free from interferences. This review considers current separations combined with post-column labeling techniques for AAA, comparison with the pre-column methods, and the strategies used to develop effective post-column methodology. The focus of the article is on LC methods coupled with post-column labeling techniques and studying the reactions to achieve optimum post-column derivatization (PCD) conditions in order to increase sensitivity and selectivity using various types of detectors (UV–Vis, fluorescence, electrochemical etc.) and illustrating the versatility of the PCD methods for practical analysis.

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Introduction

There is considerable need to develop new separation methods for the direct analysis of underivatized amino acids (AAs). This is derived from the main advantages over derivatization-based methods such as the simplicity and ease of automation. It has been almost 55 years since the first automated procedure for the analysis of underivatized AAs by IEC was published [1]. Ninhydrin was used as a post-column reagent at approximately 130 °C to detect underivatized AAs in the visible region of light after their separation in an ion-exchange column. The reaction with primary and secondary amino group AA forms a blue ninhydrin–AA derivative detectable at 570 nm and at 440 nm for the ninhydrin–secondary amine AA derivatives (e.g., proline, hydroxyl proline). Separation was achieved with cation-exchange chromatography on a sodium or lithium ion-exchange column using a gradient with sodium or lithium citrate buffers with increasing pH and temperature. Regeneration with sodium or lithium hydroxide is needed at the end of the run.

The transformation of AAs by a chemical reaction with ninhydrin, as was applied the first time for the detection of AAs after ion-exchange chromatography (IEC), resulted in the formation of stable derivatives with improved optical properties and a linear relationship between the color intensity and AA concentration [2]. The goal of derivatization was to gain selectivity and sensitivity in the AA analysis by liquid chromatography (LC). Ninhydrin-based derivatization was among the first derivatization techniques that were studied extensively over the years for the detection of AAs after LC separation [2–18]. Different terms such as chemical derivatization or labeling were coined [19–22]. Chemical derivatization is applied using post-column (after separation) [20–27] or

pre-column (before separation) labeling techniques [28, 29]. Both post-column and pre-column derivatization techniques focus on improving analyte detection. They also focus on the selectivity improvement, while pre-column is involved in the separation mechanism. Pre-column derivatization may be considered as less demanding because AA derivatization is complete before separation is started and, therefore, will not impact on band spreading as the post-column technique does [30, 31]. In contrast, this potential benefit is often gained at the expense of complex and time-consuming sample clean-up [32, 33]. Furthermore, because the derivatization process is administered to all AAs simultaneously, conversion may not be uniform and the relative concentration of AA derivatives will be different from their original AAs in the untreated sample. If this is the case the pre-column technique may be problematic for quantitation. The disadvantages such as derivative instability, reagent interference, inability of some reagents to derivatize the secondary amino group, and long preparation times might decrease the reproducibility and precision of the technique. The post-column technique applies derivatization to each AA separately, so that given an excess amount and activity of the reagent, conversion is uniform across all AAs. The conversion takes place on-line as each AA exits the column and travels to the detector flow cell. It imparts distinct UV–Vis, fluorescent chemiluminescence, or electrochemical properties to AAs that can easily be detected by conventional LC detectors. Sensitivity of the detection is increased by several orders of magnitude up to a certain extent when the increase in the void volume does not impact on the decrease in resolution. The derivatization reactions are selected in order that the AAs can be easily seen against a complex background. Therefore, the post-column derivatization technique is used to increase sensitivity and selectivity in high-performance liquid chromatography (HPLC) AA analysis. Because the derivatives are formed post-column, the chromatographic separation is preserved, i.e., the post-column derivatization (PCD) technique must be carefully configured and controlled so as to maintain chromatographic resolution. Sample preparation is relatively simple compared with pre-column techniques, and the overall process can be adapted to automation.

Cation-exchange chromatography, when combined with PCD and UV–Vis detection, is considered the most accurate and reproducible method for the determination of the AA composition of pure protein, feeds, or biological fluids [20]. Usually the kinetics of ion-exchange processes are slower than the reversed phase resulting more often in long chromatographic runs [30]. However, an accelerated single column system using lithium citrate buffer was developed for determination of 99 AAs and other ninhydrin-positive compounds in less than 2 h [22]. IEC removes most of the contaminants before separation of AAs begins [19] and minimal sample pretreatment is needed compared to pre-column techniques using reversed-phase columns. Underivatized AAs can also be determined using ion-interaction agents [34–37] and in limited cases reversed-phase chromatography has been applied for certain AAs [38–41]. Post-column labeling techniques enhance the UV–Vis detection properties of AAs, convert AAs to fluorescent AA derivatives, or through the chemical reaction chemiluminescent light is produced and transforms photochemically inactive AAs to electrochemically active species in order to improve the sensitivity, specificity, reproducibility, or simplicity and ruggedness of the AA analysis [38, 42–50]. Fundamental developments in the derivatization of AAs were reviewed by Toyo'oka et al. [51] and Coppex et al. [52]. These articles provide excellent reviews of current literature on the analysis of AAs by HPLC combined either with post-column or pre-column derivatization techniques.

The focus of this review is the post-column labeling techniques applied to AA analysis with the purpose of increasing sensitivity and selectivity. AA analysis separations are discussed together with the post-column labeling technique used. Extensive reviews of most of the post-column labeling techniques are presented. Detailed review of the fundamental reactions occurring during PCD analysis of AAs is presented in order to better understand the variables that affect the post-column technique and the application of optimum conditions. PCD optimization conditions are reviewed. In conclusion, future trends are discussed.

Separation of underivatized amino acids

Underivatized AAs are mainly separated either by ion-exchange [1, 3, 7, 19–27, 34, 53–55] or ion-interaction chromatography (IIC) [34–37, 56–62] or to some extent by reversed-phase chromatography [49, 50, 63–70] for some AAs having hydrophobic moieties and hydrophilic interaction chromatography (HILIC) [71–73]. Application of lithium or sodium citrate buffers and gradient elution with an increasing pH gradient and ionic strength allows the separation of AAs achieved on polystyrene–divinylbenzene sulfonated resins based on a cation-exchange mechanism [20–25]. AAs are commonly detected either by UV–Vis, fluorescence, or electrochemical detectors. In anion-exchange chromatography AAs are separated on a polymeric resin with an alkylammonium moiety, using gradient elution with sodium hydroxide–sodium acetate eluents and electrochemical detection [26–28]. In IIC, ion pairs are formed between the acidic or basic moiety of AAs and a cationic or anionic surfactant. They are usually separated on a reversed-phase column. Alkylammonium [34] or alkylsulfonate salts [35] were initially used as IIC reagents for the separation of AAs coupled with post-column labeling techniques with fluorescence detection. Volatile perfluorocarboxylic acids were also later used as IIC ions allowing the separation of AAs by LC coupled to electrospray ionization

mass spectrometry (ESI-MS) [36, 37, 60–62]. In addition the application of HILIC for the separation of AAs using ESI-MS was reported, too [71, 72, 74]. Because the focus of this review is post-column labeling techniques further literature on the application of volatile IIC ions or HILIC coupled to MS are included in reports [36, 37, 73].

Owing to the ionic properties of AAs (cationic, anionic, or even zwitterionic), most of the work concerning separation of multiple AAs involves the use of ion-exchange or ion-interaction liquid chromatography (IIC). Separation of underivatized AAs combined with PCD has attracted the interest of investigators because the elimination of pre-column derivatization brings separation power, flexibility, reproducibility, and automation with less sample preparation. Cation-exchange chromatography of underivatized AAs is the method that is most commonly coupled with PCD techniques. Why cation-exchange chromatography? Pickering et al. [19] describes that IEC of free AAs suffers a lot less from matrix interferences than the reversed-phase chromatography. Reversed-phase chromatography with pre-column derivatization usually requires extensive sample pretreatment to remove interferences for complex samples such as foods and biological fluids. In various matrices, compounds such as carbohydrates, organic acids, flavonoids, fats, and proteins will change the retention time of AAs under reversed-phase conditions. In a partitioning environment of a reversed-phase column all species in solution compete with each other for adsorption in the stationary phase. In addition, the retention of any species in solution will be affected from all other compounds in solution. Instead, in IEC the retention of ionic species is matrix insensitive. Owing to the high ion-exchange capacity and the initial acidic pH of the eluent all positively charged AAs are strongly retained by the anion-exchange sites. Although various retention mechanisms might be present the ion-exchange mechanism is the most dominant. Therefore, the injected AAs except acidic ones are retained in a fixed narrow band in the column while all other matrix components move rapidly through the ion-exchange column before the separation of AAs and are discarded through the post-column system resulting in better performance.

In cation-exchange chromatography three to six eluent buffers are used [27, 32]. They are either sodium or lithium citrate buffers. Sodium eluents are mainly used for analysis of up to 20 AAs in hydrolyzed samples, whereas lithium eluents are used for more than 40 AAs in physiologic fluids or non-hydrolyzed samples [20, 21]. Owing to the high complexity of native samples the higher resolution power of Li cation is needed. Both types of eluents are used in the same manner. Step or continuous linear gradients of changing pH, ionic strength, and temperature are applied. For continuous gradients up to three eluents are required, whereas more than three eluent buffers are needed for step gradients. Ion-exchange separation of AAs is performed on a sulfonated copolymer of polystyrene and divinylbenzene which is more stable in a wider pH range than silica backbone columns. Because of the multiple mechanisms (ion-exchange, partitioning, adsorption, ion exclusion etc.) that take place with the ion-exchange columns, gradient elution of changing pH, temperature, and cation concentration provides easier and effective separation of more than 40 AAs. A low-pH citrate buffer with low cation concentration is the first eluent, whereas the second to fifth eluents have either an increased pH and/or ionic strength with high cation concentration and less buffer capacity. The final eluent is a high-pH eluent based on the cation hydroxide and is of similar cation concentration as the first eluent used mainly to regenerate the column. Linear gradients are preferred over step gradient elution because step gradients can cause a front that can interfere with the separation and integration of AAs peaks or create refractive index change causing osmotic shrinking of the resin. The use of continuous linear gradients achieves complete AAs separation based on pH, ionic strength increase, or temperature. It has been recognized that, owing to the strong influence of partitioning mechanism, the addition of an organic modifier affects the separation of certain AAs [20, 21] (addition of 2–5 % organic solvent improves resolution between serine and threonine). The detection of AAs is accomplished using a photometric (UV–Vis), fluorescence, chemiluminescence, or electrochemical detector based on post-column labeling reaction. Despite the dramatic changes in pH and ionic strength gradient during separation, flat and stable baselines are obtained, which is a crucial issue for high sensitivity analysis.

Three main parameters can influence the separation of AAs in cation-exchange chromatography. Increase in pH causes AAs to go from a positive to a neutral charge state (isoelectric point) and even with further increase of pH to anionic charge state and AAs are released from the stationary phase. A low pH is used (pH 2.2) in AA samples before they are loaded on the column in order to ensure that all are positively charged and retained on the column. The second parameter is the competition with the eluting counter cations (H^+, Li^+, or Na^+) for exchange sites. The positive AAs are strongly bound to the anionic sites of the stationary phase because they show a higher affinity for the ion-exchange sites than the eluting cations do. They are gradient eluted by the continuous flow of cation or by increasing cation concentration (ionic strength increase). The third important parameter is the column temperature which affects the kinetics of the ion-exchange reaction. A temperature gradient speeds up the AAs separation dramatically [75].

Separation of underivatized AAs can also be achieved by IIC. IIC using alkylammonium or alkylsulfonic salts [34, 35] has the capability of separating multiple AAs using post-column labeling techniques with fluorescent detection, whereas the use of perfluorocarboxylic acids as ion-interaction agents has been used either with MS or evaporative light

scattering detector (ELSD) and chemiluminescent nitrogen detector (CLND) detectors [36, 37]. Although IIC has some limitations such as long equilibration times, retention time variations, permanent modification of the column, and it does not have the same separation power as IEC, the use of perfluorocarboxylic acids as IIC agents with MS detection enables the determination of 25 underivatized AAs [62].

The pre-column derivatization techniques offer the advantage of improved chromatographic separation of derivatized AAs usually on reversed-phase separation columns. The derivatized AAs are compounds with different chromatographic properties than the AAs themselves. Pre-column derivatization, intrinsically as a more sensitive technique, has been applied successfully to the analysis of protein hydrolysates. The advantage of the pre-column labeling technique is a shorter analysis time, but it does not separate all the AAs achieved by IEC [28, 34]. However, analysis of AAs in more complex samples such as physiologic fluids has been proven to have poor peak resolution and matrix interferences and requires extensive sample pretreatment procedures [32, 33]. An early extensive review of AA analysis using pre-column and post-column methods in combination with LC was reported by Deyl et al. [30]. A detailed review of several post-column techniques is described along with their sample preparation. Chow et al. [31] compared the two derivatization techniques where improved sensitivity and selectivity is attained by both derivatization techniques. In the pre-column derivatization technique the mixture of AAs is reacted with a reagent, and the resulting AA derivatives are chromatographed on a reversed-phase column and detected by UV–Vis, fluorescence, or electrochemical detectors. In the post-column technique, which is the subject of this article, the mixture of free AAs is first chromatographed by ion-exchange or ion-interaction chromatography. After their elution from the column, the AAs can be derivatized on-line. They can be detected by UV–Vis, fluorescence, chemiluminescence, and electrochemical detectors.

Post-column labeling techniques and detection

The workflow of the technique involves the separation of AAs followed by on-line PCD (labeling) and subsequent detection of the formed derivatives. Various post-column labeling techniques for AAs analysis are differentiated on the basis of the detector or the reactor type used. The most common type of reactor is the tubular design that has been used in the post-column addition of various chemical reagents to the flowing stream in order to enhance the detectability of the analyte by forming highly detectable chromophores and/or fluorophores.

The most common type of PCD reaction of AAs involves their reaction with a reagent yielding derivatives which either absorb UV–Vis light or emit fluorescence. The reactor may be open tubular design and either heated (e.g., ninhydrin) or at ambient temperature (e.g., *o*-phthalaldehyde). When a three-dimensional knitted design is applied then peak broadening becomes independent of the flow rate [76]. Open tubular reactors are used for reactions with reaction times of less than 1 min, whereas knitted reactors are used for reactions with reaction times of a few minutes. If several minutes (10–15 min) residence time is required for the reaction then air segmentation is recommended. Figure 1a shows a schematic of the essential components of a PCD system connected to a quaternary gradient HPLC system. A dual pump is displayed in PCD for AA analysis either with *o*-phthalaldehyde (OPA), fluorescamine or 4-fluoro/4-chloro-7-nitrobenzofurazan (NBD-F/NBD-Cl) using fluorescence detection.

The second type of labeling reaction of AAs is the photochemical reaction which usually does not involve the addition of a reagent [38, 39, 42, 77–80]. These reactions may be photolytic or photocatalytic in the presence of TiO_2. Although photochemical reactors may be used with virtually any detection mode, the most common applications have used fluorescence detection (FL) or electrochemical (amperometric) detection (ECD). In most AA applications using photochemical reactions the effluent of the post-column photochemical reactor is directed to a conventional electrochemical detector which may be in either a single or dual electrode configuration. If photolytically generated species become electroactive on irradiation, then the selective and specific determination of analytes is achieved at oxidative potentials that are either non-electroactive or reductively electroactive. Photo-ECD may also be used to selectively detect these photogenerated species at lower oxidation potentials where fewer possible matrix interferences can be oxidized. Often photochemical derivatization may be conducted without additional reagents. An additional reagent may be added if a second reaction is required. Reagent may either be added prior to the reactor, e.g., photosensitizers, or after the photochemical reactor, e.g., derivatization reagent that reacts with the photolysis product. Figure 1b shows a schematic of the essential components of a PCD system connected to a quaternary gradient HPLC system. A knitted coil is housed inside the UV lamp compartment. A single pump and a heated coil are needed for reagent introduction after the photolysis.

The third type is PCD systems with labeling reactions emitting chemiluminescence. Figure 1c shows a schematic of the essential components of a PCD system connected to a quaternary gradient HPLC system. A dual pump is displayed for PCD of AA analysis using chemiluminescence detection. For a given flow rate of the eluent and reagent stream(s), the dead volume of the mixing tee and the detector cell becomes very important because of the chemiluminescence half-time.

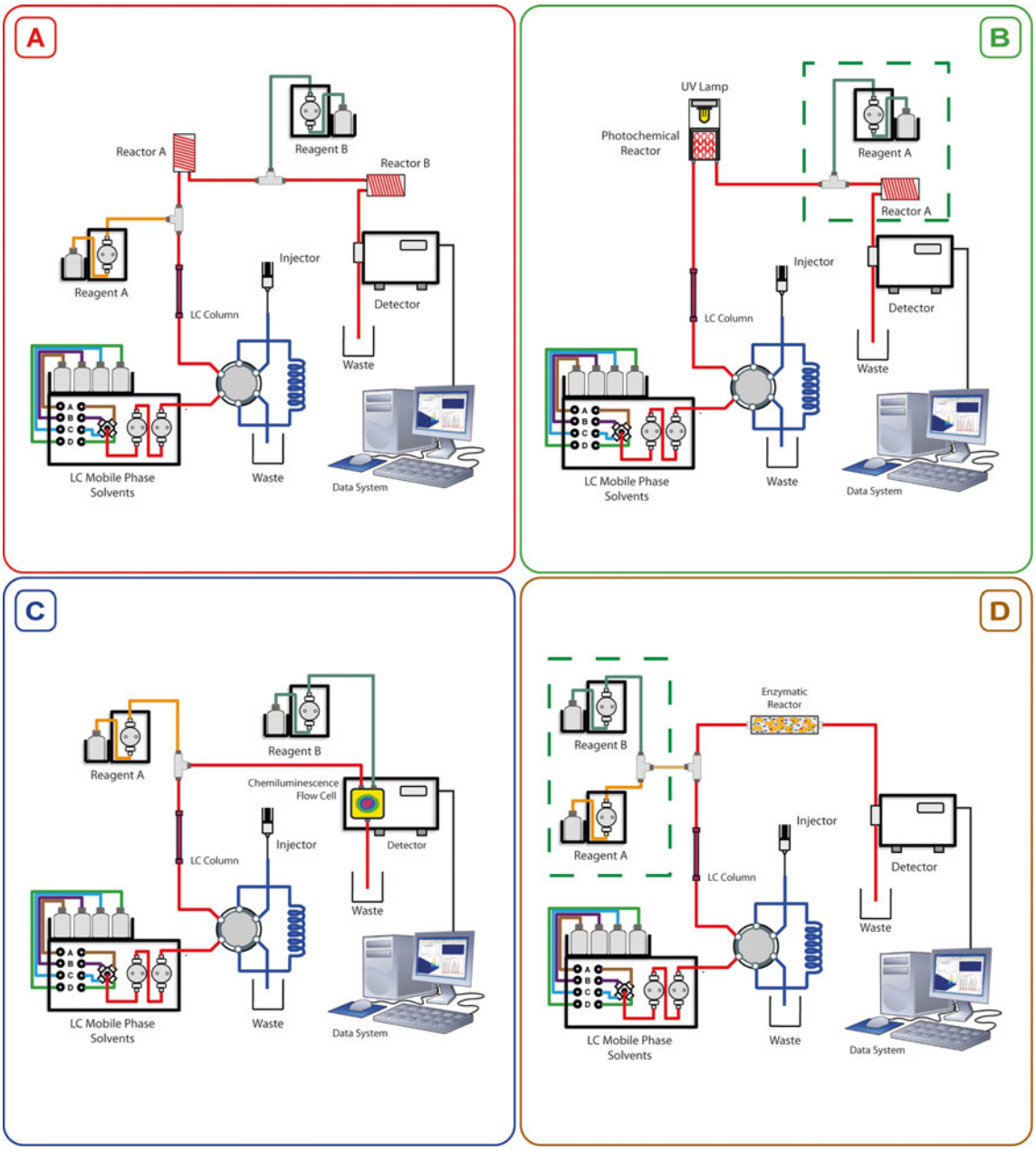


Fig. 1 HPLC-PCD system with **A** two reagents/two heated coils and UV–Vis or fluorescence detector, **B** photochemical reactor and optional one reagent/one heated coil and EC detector, **C** two reagents mixed in the chemiluminescence detector cell, **D** IMER and optional two reagents mixing and fluorescence detector

Usually the two reagents are mixed inside the flow cell coil because of the short lifetime of chemiluminescence.

Liquid chromatography can be coupled to various selective post-column reaction detection devices using immobilized enzymes as components of the selective detection device in LC. The enzymes are immobilized on a solid support and they are contained in a packed-bed immobilized enzyme reactor (IMER). Packed-bed reactors are preferable to open tubular reactors with regard to dispersion and pressure drop. After separation of the analytes, the column effluent is mixed with a make-up flow containing the substrates necessary for the enzymatic conversion in the IMER. With most of the enzymes used in flow systems, the products of enzymatic post-column reactions are NADH or H_2O_2 [81]. The most frequently used detection principles for these products are fluorescence and chemiluminescence. Figure 1d shows a schematic of the essential components of a PCD system connected to a quaternary gradient HPLC system. A dual-pump PCD system is needed in addition of the substrate and buffer with fluorescence or chemiluminescence detection.

Post-column derivatization with UV–Vis detection

AA analysis with IEC became a reality for the first time using PCD chemistry with ninhydrin [2–4] and UV–Vis detection. It is now one of the most common widespread detection techniques employed for quantitative AA analysis [1, 20–23, 27]. Adriaens et al. [23] studied the elution behavior of 145 ninhydrin-positive substances, most of which were naturally occurring AAs. Friedman et al. [82, 83] described in detail the chemistry and mechanisms of ninhydrin reactions with AAs. A Li-based cation-exchange resin is employed for the separation of AAs in the more complex physiological samples, and the faster Na-based cation-exchange resin is used for the simple AA mixtures obtained with protein hydrolysates (typically containing 18–20 AA components). Separation of the AAs on an ion-exchange column is accomplished through a combination of gradient in pH and cation strength. A temperature gradient is often employed to enhance separation and increase speed.

Ninhydrin as a selective oxidizing agent causes oxidative decarboxylation of AAs, releasing CO_2, NH_3, and an aldehyde with one less carbon atom than the parent AA. Then the reduced ninhydrin form reacts with the liberated ammonia to form Ruhemann's purple, a complex which has characteristic purple color with a maximum absorption at 570 nm. The imino acids (secondary amine AAs) such as proline and hydroxyproline yield a yellow color with a maximum absorption at 440 nm. There is a linear relationship between the color intensity and AA concentration [2]. Hydrindantin, as a reduced form of ninhydrin, is required in order for the reaction mixture to obtain optimal color formation [18]. The presence of hydrindantin in the nihydrin reagent may result either from a direct addition of this substance or from the addition of a reducing agent that converts ninhydrin to hydrindantin. The two major drawbacks of the direct addition of the hydrindantin is its easy oxidation by air during the preparation of the reagent [6] and its high price. In the early stages of the development of ninhydrin reagent, stannous chloride [2] was added to the reagent mixture. This reagent was applied in the photometric detection of AAs after their separation from ion-exchange resins [1, 3, 7]. High reaction temperatures (100–130 °C) are required to accelerate the reaction between the AA and ninhydrin, and the derivative is produced by the destruction of each AA [2]. A modification involved the elimination of the stannous chloride which was previously added to form reduced ninhydrin (hydrindantin) in the reagent solution. Hydrindantin [6] itself was added directly in order to avoid the precipitation of tin salts which occurred during chromatography. It was applied in the photometric detection of AAs after their separation from ion-exchange resins [4]. The replacement of methyl cellosolve (ethylene glycol monomethyl ether), which is used as a solvent, by dimethyl sulfoxide (DMSO), and of lithium acetate buffer by sodium acetate buffer enhanced the stability of the reagent [9]. DMSO proved to be a better solvent for the reduced form of ninhydrin (hydrindantin) than methyl cellosolve is. The resulting reagent is a ninhydrin–hydrindantin solution in 75 % DMSO/25 % 4 M lithium acetate buffer at pH 5.2. However, the reagent with hydrindantin must be kept under inert atmosphere [14] and in the refrigerated compartment of the analyzer [6]. The use of reagents other than hydrindantin has, as a result the formation of hydrindantin by the reduction of ninhydrin, been applied in the detection of AAs. One of these reagents is KCN [10, 11] as the reducing agent and the replacement of sodium acetate by sodium propionate to prevent clogging [10]. Other reducing reagents involve the use of ascorbic acid [15–17] and titanous chloride [12–14]. However, the limited stability of the reagent and clogging problems prevented further use of these reducing reagents in ninhydrin reagents for AA analysis.

A stable ninhydrin reagent which, unlike the other reducing agents, does not form precipitates was obtained when sodium borohydride was used as the reducing agent for ninhydrin in a system with DMSO as the solvent [84]. A drawback of this reagent is the considerable drift of the baseline when basic AAs were eluted from the column. This drift is associated with some not yet known mechanisms causing a change of color of the ninhydrin reagent in the alkaline region, e.g., in the region of citrate buffers used for basic AAs elution. The problem was overcome when the ninhydrin reagent with sodium borohydride used methyl cellosolve as a solvent with sodium acetate buffer at pH 5.2 and the instability of the reagent in the segment of basic AAs was resolved [85]. A very stable reagent was produced, without the formation of precipitate in the flow-through system of the analyzer and usable within the whole range of the AA separation. Maturation of the reagent is not needed. The shelf life is up to 3 months when kept at laboratory temperature. Storage in a refrigerator is not necessary for practical applications.

Ninhydrin reagents are buffered with lithium or sodium acetate at pH 5.2 and 5.5 respectively to increase the stability of the derivatives and to provide better baseline results. A buffer is required because the derivatization reaction is pH dependent. Besides ninhydrin, hydrindantin and a water-miscible organic solvent such as methyl cellosolve or sulfolane [86] are also included in the final reagent. The organic solvent is necessary to maintain both the hydrindantin and the new purple or yellow chromophores in solution. The presence of lithium ion in the formula prohibits the use of eluents containing phosphate, because lithium phosphate would precipitate at the point of mixing and may clog the reaction coil. The ninhydrin derivatization detection mode was the first developed approach and is still considered suitable for pure proteins, feeds, and complex mixture analysis. However, one remaining limitation is the relative instability due to oxidation of the ninhydrin reagent, limiting the use of

the ninhydrin/hydrindantin/acetate buffer mixture to approximately 2 weeks while the hydrindantin/$NaBH_4$/acetate buffer mixture can be extended up to 3 months. Application of the latest reagent to AA analysis is shown in Fig. 2 [27]. To reduce the potential for oxidation it is of critical importance to maintain the reservoir under an inert gas (He or N_2). Recent commercial developments extend the shelf life of the ninhydrin reagent by using special formulations that require mixing before its use. These formulations extend the shelf life up to 12 months [87].

The fact that the ninhydrin reaction requires a certain amount of the reduced ninhydrin (hydrindantin) be present in the reagent along with its instability due to air oxidation led Hori and Kihara [88] to propose a flow-through electrolysis system for the in situ preparation of a reduced ninhydrin reagent. Hydrindantin is continuously generated in situ by a controlled current electrolysis of 3.0 mA and the resulting solution is reacted with the AAs eluted from the cation-exchange column. The formation of 1 mole of hydrindantin is derived from 2 moles of ninhydrin and 2 electrons (Table 1). The electrolytically prepared ninhydrin reagent using DMSO as organic solvent and lithium acetate buffer at pH 5.2 was identical to that prepared by chemical reduction.

For sulfur-containing AAs separated with IEC and detected by ninhydrin, the formation of Ruhemann's purple derivative is not always the preferred derivative [89–91]. The organic solvent of the ninhydrin reagent was DMSO. For AAs containing both amine and thiol groups, the thiol group is more reactive than the amino group. Owing to the proximity of the amine and thiol nucleophiles in cysteine, a chemical reaction takes place between these species and ninhydrin, resulting in the formation of either a thiazolidine or cyclic thiazine compound, thereby preventing or minimizing formation of Ruhemann's purple [83, 89]. In an acidic medium, the reaction products are pink in color with a maximum wavelength at approximately 560 nm. Therefore, for the determination of cysteine along with other AAs, the suggested approach is prior oxidation to cysteic acid and monitoring at 590 nm. For L-2-thiolhistidine the SH group is attached to a rigid aromatic imidazole ring which is sterically hindered and thiazolidine or thiazane formation takes place to a lesser extent, thereby favoring the normal ninhydrin reaction. If the color yield of the reaction products of various SH AAs is compared to that of leucine, then a progressive increase is obtained for the ninhydrin derivatization of L-Cys (0.12), the dipeptide α -L-glutamyl-L-cysteine (0.348), L-2-thiolhistidine (0.64), and L-GSH (0.72). One explanation is that the increase in these values is probably due to either the increase in distance between the NH_2 and the SH groups in the compounds or steric hindrance which minimizes the extent of the thiazane ring

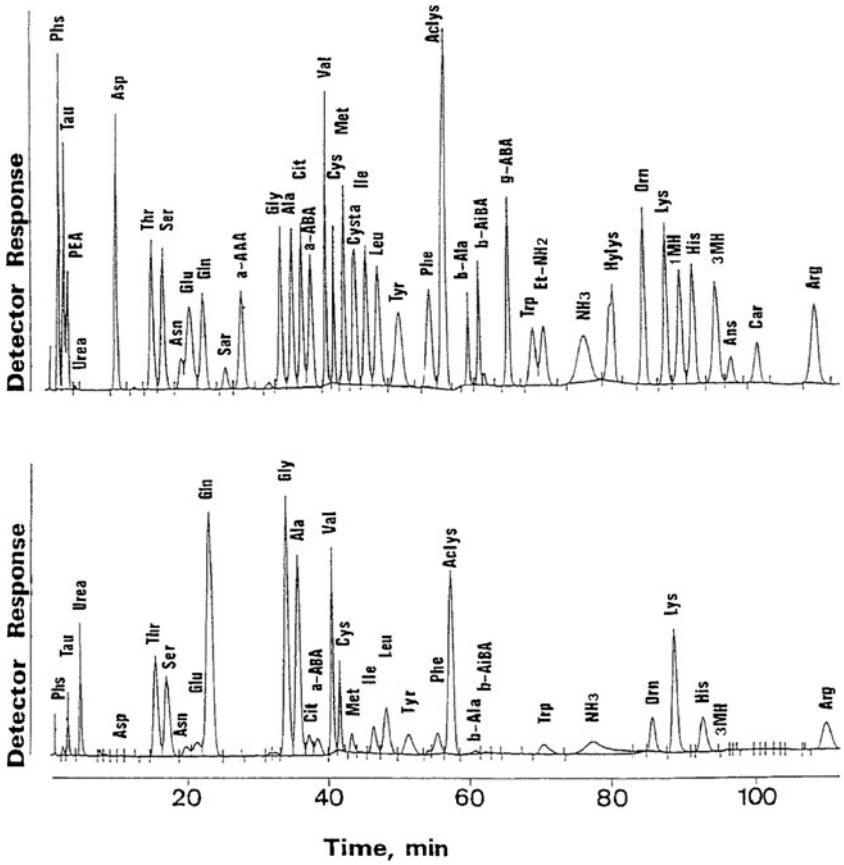


Fig. 2 Profile of elution of a 100 pmol/mL synthetic physiological mixture of AAs supplemented with Gln (*top*) and of a human deproteinized plasma sample from one healthy subject (*bottom*) assayed with an Hitachi L-8500A using IEC and detection with hydrindantin/$NaBH_4$/acetate buffer. Reprinted with permission from reference [27]

Table 1 Post- column derivatization chemistries and conditions of amino acids analysis using photometric (UV–Vis) detection

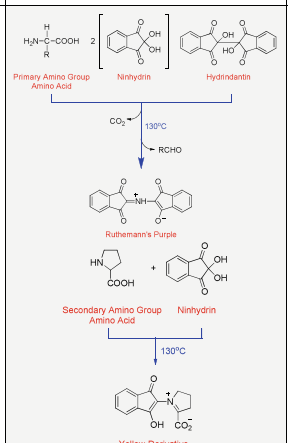
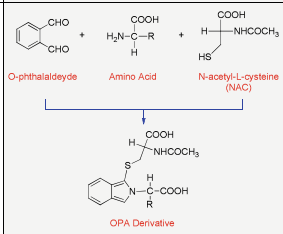
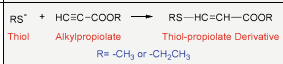
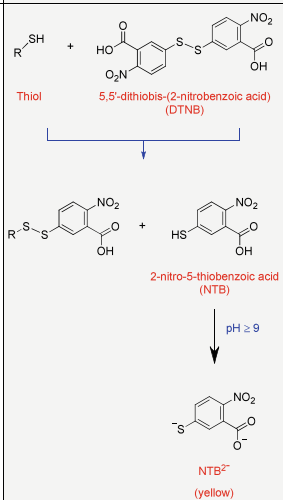
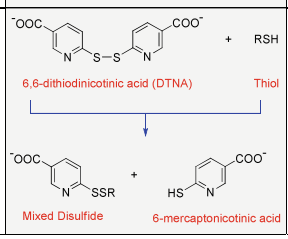
No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
1.	Primary and Secondary amino group AAs	Ninhydrin/Hydrintatin/ Sulfolane R1: 56 g LiOH.H_2O, 145 mL Glacial Acetic acid in 200mL water (3.9M Lithium Acetate buffer) mixed with 10 g ninhydrin, 0.18 g of hydrindantin in 200 mL Sulfolane. Flow rate: 0.3 mL/min		One coil 0.5mL & 130°C	Simultaneous monitoring in two wavelengths is required for both primary and secondary AA. Ammonia causes interferences in separation. Cysteine requires oxidation to cysteic acid. PCD reagent must be purged with N_2 or He.	Cation Exchange Chromatography	UV-Vis 570 nm for primary amine AA derivatives and 440 nm for secondary amine AA derivatives	sub-nanomole	[86, 127]
2.	Asp, Gln, Thr, Cys, Ala, Tyr, Val, Met, Phe, Leu, Lys, His, Trp and Arg	o-phthalaldehyde (OPA)/ N-acetyl- L-cysteine (NAC) R1: 0.05 mM OPA / 0.05 mM NAC, 10% EtOH, 0.1 M Borate Buffer (pH=9.6) Flow rate:0.3 mL/min		One coil 0.15 mL & 40°C	14 AA are separated using sodium dodedecyl sulfate at a concentration higher than the critical micellar concentration and detected with OPA/NAC reagent.	Micellar Liquid Chromatography	UV-Vis 335 nm	Not reported	[109]
3.	Glutathione (GSH) N-acetylcysteine (NAC)	Ethyl or Methyl propiolate R1: Britton–Robinson (B-R) buffer consisted of a mixture of H_3PO_4, CH_3COOH, and H_3BO_3 (0.1mol/L each) pH=11.5[131] or pH=11.0[132] Flow rate: 0.25 mL/min R2: 15 mM Ethyl[101] or 20 mM Methyl propiolate [132] Flow rate: 0.25 mL/min		Two mixing tees and one coil 0.12 mL [131] or 0.18 mL [132] & Ambient Temperature	The thiol in order to act as nucleophile, it should be firstly be deprotonized which occurs when the pH of the reaction medium is greater than the pKa of the thiol group. Ethyl or methyl propoiolate reagent was not stable when dissolved in the alkaline medium therefore a dual PCD system is required	Reverse Phase Chromatograph	UV-Vis 285 nm	GSH [131] 0.1 μM NAC [132] 40 μg/L	[97,98]
4.	Sulfur-containing AA (Thiols) Cysteine (Cys), Glutathione (GSH), N-acetylcysteine (NAC)	5,5,-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellmann's reagent) R1: 40 mg/L 5,5,-dithiobis(2-nitrobenzoic acid) (DTNB), 80mg/L hexadecyl-trimethylammonium bromide(HTAB) in 0.5 M phosphate buffer, pH=9 Flow rate: 0.25 mL/min		One coil 3.5 mL & Ambient Temperature	Thiols react with DTNB, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB^-), which ionizes to the NTB^{2-} dianion (yellow color) in water at neutral and alkaline pH. NTB dianion has a molar extinction coefficient of ~14,150 $M^{-1}cm^{-1}$ and can be detected at 412 nm. The presence of cationic micelles of HTAB in the reaction mixture enhanced the sensitivity of the detection by nearly two-fold.	Reverse Phase Chromatograph	UV-Vis 412 nm	19 μg/L GSH, 26 μg/L Cys 18 μg/L NAC	[102]
5.	Sulfur-containing AA (Thiols) Cysteic Acid (CySH) Cysteine (Cys), Glutathione (GSH), Homocysteine (Hcy), Thiolhistidine (This), Ergothioneine (Erg)	6,6′-dithiodinicotinic acid (DTNA) R1: 1.5 mM 6,6′-dithiodinicotinic acid (DTNA), 0.2M sodium phosphate buffer pH=7.0 Flow rate: 1.0 mL/min		One coil 0.12 mL & Ambient Temperature	The post-column reaction with DTNA is favorable of generating stable product over a wide range of physiological pH even though the molecular extinction coefficient of 6-mercaptonicotinic acid at 344nm (~10,000$M^{-1}cm^{-1}$) is lower than that of NTB at 412nm.	Reverse Phase Chromatograph	UV-Vis 344nm	0.1nmol Cys, GSH, Hcy	[103]

Table 1 (continued)

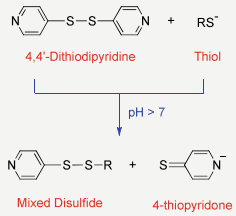
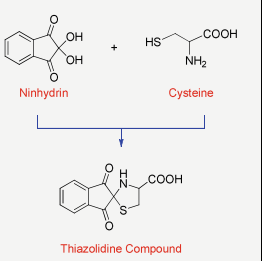
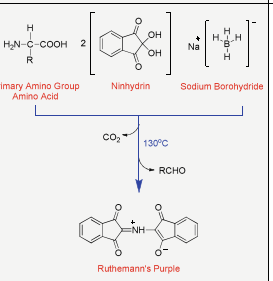
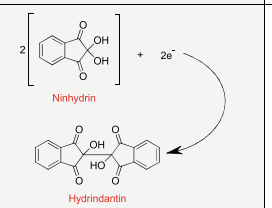
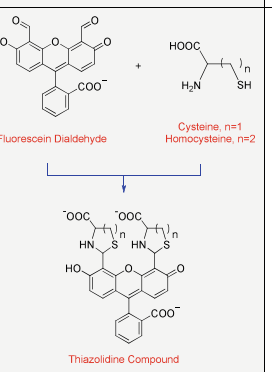
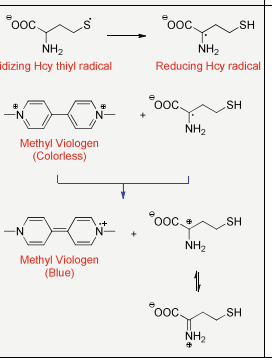
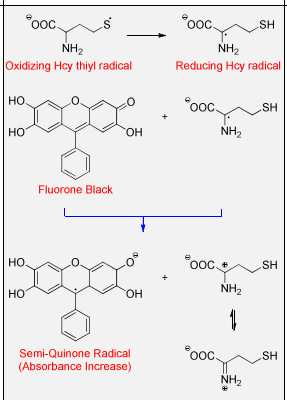
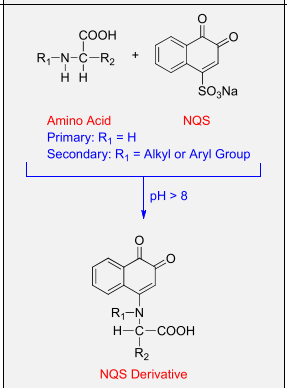
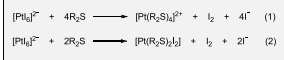
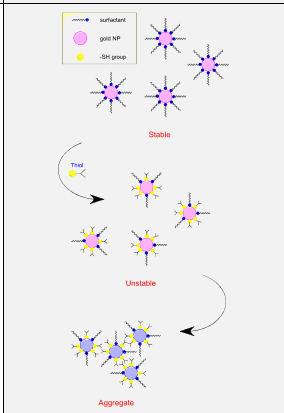
No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
6.	Sulfur-containing AA (Thiols) Cysteine (Cys), Glutathione (GSH), N-acetylcysteine (NAC)	4,4′-dithiodipyridine (DTDP) R1: 3 mL of 10mM 4,4′-dithiodipyridine(DTDP) in 0.01M HCl/MeOH (97/3), 300mL of 0.3M Tris base containing 1mM EDTA, (adjusted to pH=8.5 with H_3PO_4) Flow rate: 0.4 mL/min		One coil 0.46mL & Ambient Temperature	A thiol reacts with 4,4′-dithiodipyridine to form a mixed disulfide and 4-thiopyridone. The reaction product has the largest extinction coefficient (~21,400 $M^{-1}cm^{-1}$) than that of DTNB & DTNA	Ion Interaction Chromatography (IIC)	UV-Vis 324nm	50nM (3pmol)	[105]
7.	Sulfur-containing AA	Ninhydrin/Hydrintatin/Dimethyl-sulfoxide (DMSO) R1: 20 g/L ninhydrin, 0.625 g/L of hydrindantin in Dimethylsulfoxide (DMSO): 4M aqueous lithium acetate buffer, pH= 5.2 3:1 Flow rate: 0.3 mL/min		One coil 0.5 mL & 130°C	AAs that contain another nucleophilic group (e.g sulfhydryl) close to the reacting amino group may form cyclic intermediates that are not converted to Ruhemann's purple. In Cys the proximity of the amine and thiol nucleophiles allows a reaction between these species with ninhydrin, resulting in the formation of a thiazolidine derivative which absorbs at 560 nm. In case of 590 nm detection Cys is oxidized to Cysteic acid.	Cation Exchange Chromatography	UV-Vis 590 nm	sub-nanomole	[89-91]
8.	Primary and Secondary amino group AAs	Ninhydrin/$NaBH_4$/Methyl cellosolve R1: 20 g/L ninhydrin, 0.08 g/L of sodium borohydride in methyl cellosolve (ethylene glycol monomethyl ether):4 M aqueous sodium acetate buffer, pH= 5.5 3:1 Flow rate: 0.3 mL/min		One coil 0.5 mL & 90°C	The reagent does not form any precipitate in the flow lines of the analyser. No maturation of the reagent is necessary. The shelf life is at least 1 month and up to 3 months when kept at ambient temperature. Basic pH does not induce a base line shift when basic amino acids are eluted off.	Cation Exchange Chromatography	UV-Vis 570 nm for primary amine AA derivatives and 440 nm for secondary amine AA derivatives	sub-nanomole	[85]
9.	Primary and Secondary amino group AAs	Ninhydrin/Electrogenerated Hydrindatin (1.75 mA) R1: 20 g/L ninhydrin, in Dimethylsulfoxide (DMSO): 4 M aqueous lithium acetate buffer, pH= 5.2 3:1 Flow rate: 0.25 mL/min		One coil 9.8 mL & 100°C	Hydrindatine is continuously generated in situ by a controlled current electrolysis and the resulting solution is reacted with the amino acids eluted from the LC column.	Cation Exchange Chromatography	UV-Vis 570 nm for primary amine AA derivatives and 440 nm for secondary amine AA derivatives	sub-nanomole	[88]
10.	Cysteine (Cys), Homocysteine (Hcy)	Fluorescein dialdehyde R1: 6.4 X 10^{-6} M Fluorescein Dialdehyde in 0.125 M carbonate buffer, pH=9.5 Reagent delivery pressure: 60psi		One coil 0.6 mL & 80°C	The reaction of Fluorescein dialdehyde with Cys or Hcy produces bright yellow to brownish-orange thiazolidine derivative. As the concentration of Hcy or Cys increases, quenching of fluorescence emission is observed too. Other sulfur-containing amino acids (such as Met and GSH) and other amino acids do not respond.	Reverse Phase Chromatography	UV-Vis 510nm	Not reported	[94]
11.	Cysteine (Cys), Homocysteine (Hcy), Glutathione (GSH)	Methyl viologen R1: 10 mM Methyl viologen in 0.25M carbonate buffer (pH=9.5)		One coil 0.6 mL & 80°C	Thiols are oxidized to form sulfenyl radicals. An intramolecular hydrogen abstraction takes place to form a reducing α-amino carbon-centered radical through electron-transfer reaction with Methyl viologen dication upon heating in alkaline conditions turns blue. At neutral pH, the reaction is highly selective for Hcy.	Reverse Phase Chromatography	UV-Vis 610nm	sub-nanomole	[94]

Table 1 (continued)

No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
12.	Cysteine (Cys), Homocysteine (Hcy), Glutathione (GSH)	Fluorone black R1: 12.5 X 10^{-6} M Fluorone black in 50/50 (v/v) mixture of MeOH and 0.25 M carbonate buffer (pH=9.5) Reagent delivery pressure: 40 psi		One coil 0.6 mL & 80°C	In a similar way with Methyl viologen the electron-accepting Fluorone black reacts with Cys, Hcy and GSH selectively. Other amino acids and the disulfide Hcy do not respond.	Reverse Phase Chromatography	UV-Vis 505 nm	sub-nanomole	[94]
13.	Primary and Secondary amino group AAs	NQS (1,2-naphthoquinone-4-sulfonate) R1: 0.015 M $NaHCO_3$/0.185 M Na_2CO_3 R2: 1.2 mM NQS (1,2-naphthoquinone-4-sulfonate) in 0.1 M HCl		One coil 3.8mL & 65°C	Due to the instability of this reagent in alkaline medium (pH=10) a dual pump PCD system is required in order to be mixed on line with the basic buffer solution	Ion-Interaction Chromatography	UV-Vis 305 and 480 nm	sub-nanomole nmol AA 0.16 Ser 0.10 Gly 0.08 Pro 0.11 Tyr 0.20 Met 0.14 Trp 0.26 His 0.09 Lys 0.33 Arg	[106]
14.	Sulfur-containing AA Cysteine (Cys), N-acetylcysteine (NAC), Cystine ($(Cys)_2$), Methionine (Met)	Hexaiodoplatinate reagent R1: 100μM $H_2PtCl_6{\cdot}6H_2O$, 10mM KI in 0.1M sodium phosphate buffer at pH=2.2 Flow rate: 0.6 mL/min		One coil 0.5 mL & 40°C	Hexaiodoplatinate reacts with Sulfur-containing AA via ligand exchange reactions, resulting in absorbance decrease at 500nm.	Ion-Interaction Chromatography	UV-Vis 500 nm	~20 pmol	[110, 111]
15.	Cysteine (Cys), Homocysteine (Hcy)	Zonyl FSN-capped Gold nanoparticles (GNP) R1: 4.9nM colloidal solution of FSN-capped GNP (12nm), 0.1M Sodium Phosphate Buffer pH=6.0 Flow rate: 0.5mL/min		One coil 1.0mL & 70°C	Colloid solutions of gold NPs are stabilized with nonionic fluorosurfactant molecules (Zonyl FSN). Aggregation of the GNPs is induced by either Hcy or Cys selectively, resulting in an absorption decrease of the colloidal solution at 525 nm and an absorption increase at a longer wavelength (680 nm)	Reverse Phase Chromatography	UV-Vis 680 nm	80 pmol Hcy	[112]
16.	Sulfur-containing AA (Thiols) Cysteine (Cys), Homocysteine (Hcy), Glutathione (GSH), Methionine (Met)	Brij 35-capped Gold nanoparticles (GNP) R1: 4.9 nM colloidal solution of Brij 35-cappedAuNP (12nm), 60 mM NaCl, 0.07% Trifluoroacetic acid (TFA) Flow rate: 0.35 mL/min	Same as in application 15	One coil 1.0 mL & 70°C	Colloid solutions of gold NPs are stabilized with nonionic surfactant molecules Brij 35. The sulphydryl-containing molecules were attached to the GNPs via strong Au–S bonds and displaced the surfactant ligands, resulting in the decrease of colloidal solution stability, and rapid aggregation of the GNPs show an absorption increase at 680 nm.	Ion-Interaction Chromatography	UV-Vis 680 nm	40 pmol Cys 40 pmol Hcy 80 pmol GSH	[113]

formation in the higher homologues and steric hindered L-2-thiolhistidine. Bowie et al. [90] measured sulfur-containing AAs in quantities as low as 10–100 pmol; 10 pmol of cystine could be measured with a comparable precision to 1 nmol. Total plasma homocysteine (Hcy) was determined using IEC with ninhydrin post-column derivatization. Increased levels of

Hcy were determined after oral methionine loadings revealing inherited defects of Hcy metabolism and increased risk of cardiovascular disease [92].

The PCD system involves a single pump and the derivatives produced from the reaction between ninhydrin and AA eluted off the column are monitored with a dual wavelength visible detector simultaneously at 440 and 570 nm. The obtained chromatogram is used for the determination of AA composition. Other ninhydrin-positive components and contaminants retained on the IEC column by the buffer solutions are in the chromatogram region in which the basic AAs elute. Therefore, there are baseline disturbances due to the ammonia peak. In addition prior oxidation of cysteine to cysteic acid is required.

Overall the ninhydrin post-column labeling technique is the most widespread derivatization technique with detection limits for most AA derivatives in the range of 50–100 pmol AAs on-column [93]. The response linearity range is 100 to 1,000 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, protein/peptide samples larger than 1 μg before hydrolysis are required for good AA composition.

One of the major drawbacks of the ninhydrin reaction system is the low color yield of cysteine. The reaction of an aldehyde with cysteine (Cys) or Hcy also produces thiazolidine compounds. Strongin and coworkers [94] studied the interactions between Cys and Hcy and a new fluorescein dialdehyde compound (Table 1). They found that the UV–Vis absorbance is increased and its fluorescence is quenched. Other sulfur-containing compounds (such as methionine and GSH) or other AAs do not induce any absorbance or fluorescence changes. They have used fluorescein dialdehyde as a PCD reagent for HPLC determination of Cys and Hcy [94]. The reagent conditions are listed in Table 1 and detection was accomplished at 510 nm.

Oxidation of thiol-containing AAs often leads to the formation of sulfenyl-type radicals. One of the reactions involves the formation of a reducing disulfide radical anion [94] and the other reaction involves an intramolecular hydrogen abstraction where a reducing α-amino carbon-centered radical can be generated (Table 1). A particularly favorable reaction for Hcy as compared to Cys and GSH molecules takes place with methyl viologen and fluorone black. The intramolecular formation of captodatively stabilized α-amino alkyl radical in Hcy was favored via a five-membered ring transition state [94]. The electron-transfer reactions of the Hcy carbon-centered radicals with methyl viologen and fluorone black are shown in Table 1, applications 11 and 12. For the dication methyl viologen under the basic conditions used (pH 10.5), no colorimetric selectivity among GSH, Cys, and Hcy was noted. The formation of the blue methyl viologen radical cation can be detected via changes in the UV–Vis absorbance spectra. However, at neutral pH, the reaction is highly selective for Hcy [94]. In the case of electron-accepting fluorone black, the formation of semiquinone radical between the reagent molecule and Hcy leads to an increase in absorbance and fluorescence emission. A similar, but relatively smaller, absorbance change is observed for Cys, GSH. Strongin's group applied these electron-transfer reactions in HPLC post-column determination of Cys and Hcy [94]. In these initial experiments, the two reagents were used in the post-column detection of Cys and Hcy after their HPLC separation. Both reactions proceeded fast enough for efficient post-column conditions at temperature of 80 °C. The wavelength of the UV–Vis detection was 610 nm or 505 nm for methyl viologen or fluorone black, respectively. The post-column detection mode with these two reagents showed selective responses towards Cys and Hcy, although the sensitivity is relatively low (limit of detection, LOD, around several hundred picomoles) and further optimization is required.

Propiolate esters react with AAs having thiol groups under mild alkaline conditions forming thioacrylates according to the reaction scheme in Table 1 [95]. The reaction mechanism under basic conditions involves the nucleophilic attack of the thiolate ion to the α-carbon atom of the triple bond resulting in a stable alkylthioacrylate compound that absorbs in the UV region [96]. The resulting thioacrylate from the reaction of Cys with ethyl propiolate shows a very substantial UV spectrum with maximum absorbance at 280 nm (molar absorbance 12,500 M^{-1} cm^{-1}) as opposed to the virtual transparency of the precursor propiolate. Both methyl and ethyl propiolate have been applied as post-column reagents for the determination of *N*-acetylcysteine (NAC) and glutathione (GSH) [97, 98]. Owing to their instability in basic buffer solutions, a dual reagent PCD system was employed for the pumping of a basic buffer and the propiolate ester. The propiolate PCD scheme reacts almost instantaneously, with mild reaction conditions (aqueous solutions, room temperature), the reagent is cost-effective, selective for thiols, and offers adequate sensitivity with an LOD of 0.1 μmol/L and 40 μg/L for GSH and NAC, respectively. Owing to the nature of the post-column labeling reaction, (2-carboxyethyl)phosphine (TCEP) was used as reducing agent of the oxidized form of GSH without affecting the derivatization reaction.

Thiol-type AAs react with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) via a sulfhydryl/disulfide-exchange reaction to 2-nitro-5-thiobenzoic acid (NTB) which under basic conditions is converted to the yellow dianion (NTB^{2-}). DTNB, well known as the Ellman's reagent, is highly specific for the detection of thiols (see Table 1, application 4). Half of the NTB dianion is attached to the thiol and the remaining free NTB can be detected at approximately 412 nm. DTNB is widely used for the post-column determination of thiol-containing AAs such as CySH, Cys, Hcy, GSH, and NAC [99–101]. Typical post-column application of DTNB reagent in the determination of glutathione, cysteine, and NAC in rabbit eye tissues [102] is shown in Table 1, application 4. The presence of cationic micelles of hexadecyl-trimethylammonium bromide (HTAB)

in the reaction mixture enhanced the sensitivity of the detection by nearly twofold.

Nishiyama et al. [103] reported a similar post-column reaction with 6,6'-dithiodinicotinic acid (DTNA), as shown in Table 1, application 6. However, the post-column reactions of thiols with DTNA are conducted under wide pH range compared those of DTNB which are carried out only under mild alkaline conditions (pH≥9). The reaction between DTNA and thiols produces a mixed disulfide and 6-mercaptonicotinic acid with an absorption maximum at 344 nm. Although 6-mercaptonicotinic acid at 344 nm has a molecular extinction coefficient of approximately 10,000 M^{-1} cm^{-1}, i.e., a bit lower than that of NTB (approximately 14,150 M^{-1} cm^{-1}) at 412 nm [104], the post-column reaction with DTNA has the advantage of generating stable products over a wider range of physiological pH. This property provides flexibility in choosing mobile phase eluents with low pH in order to handle difficult separation of thiols and to protect them against oxidation during separation [103]. The reaction of DTNA with ring thiols such as thiolhistidine (This) and ergothioneine (Erg) was not quantitative, and had a smaller detectable response than with GSH. Another similar reagent 4,4'-dithiodipyridine (DTDP) has also been applied as a post-column reagent for the detection of Cys, GSH, and NAC (see Table 1, application 6) [105]. A mixed disulfide and 4-thiopyridone are the products of reactions of thiol-type AAs with DTDP. Owing to the stabilizing effect exerted by the thione anion, the reaction equilibrium favors the formation of 4-thiopyridone. The reaction rates for Hcy, Cys, and GSH were found to be similar. The product (4-thiopyridone) of this reaction has a larger extinction coefficient (approximately 19,500 M^{-1} cm^{-1}) [101] than that with DTNB and DTNA. During pre-column derivatization of Cys and Hcy with DTNB and DTDP the peaks for the thione anion (324 nm) were larger than expected. Total, free, and reduced forms of Hcy, Cys, and GSH in human plasma have been quantified by using this post-column reagent [105]. The photometric detection was accomplished at 324 nm. The detection limit for Hcy was determined to be approximately 50 nM.

1,2-Naphthoquinone-4-sulfonate (NQS), a water-soluble reagent, reacts with primary and secondary amines in basic media, by a Michael addition, in relatively mild conditions [52]. The derivatives can be detected by UV–Vis at 305 nm and 480 nm [52]. A chromatographic method was applied using IIC with PCD with NQS and photometric detection of AAs [106]. The derivatization reaction takes place at 65 °C in a reaction coil. The derivatives are not stable and immediate injection after reaction is needed when this method is applied using the pre-column derivatization technique [107]. The detection limits for AAs are in the sub-nanomole range. Owing to the instability of this reagent in alkaline media (pH 10), a dual-pump PCD system is required in order mix a basic buffer solution on-line with NQS before the derivatization reaction takes place (Table 1). NQS has advantages over other reagents, because it is soluble in water, reacts with primary and secondary amino group AAs under milder conditions than ninhydrin derivatization reagent, and is relatively cheap [108]. Both primary and secondary amino group AAs can be detected photometrically at the same wavelength with linearities up to 40 nmol and LODs ranging from 80 to 330 pmol [106], without the need of multiple wavelength detection as is required with ninhydrin.

Micellar liquid chromatographic separation of AAs using sodium dodecyl sulfate (SDS) and post-column OPA/NAC derivatization [109] of 14 free AAs was achieved while the OPA/NAC isoindole derivatives were poorly resolved; only eight AAs could be separated satisfactory. Detection was accomplished by UV–Vis at 335 nm. The main advantage of the PCD is the direct injection of free AAs into the LC column, which avoids the decomposition of the isoindole derivatives before their injection or inside the chromatographic system. NAC is a better alternative to the use of 2-mercaptoethanol (2-ME) and producing more stable AA isoindoles.

Sulfur-containing AAs such as NAC and Cys can be determined in rat plasma using HPLC with PCD using a hexaiodoplatinate reagent [110]. This sulfur-specific detection reagent undergoes a ligand-exchange reaction that replaces iodine atom(s) with sulfur atom(s), resulting in absorbance decrease at 500 nm (Table 1, application 14, reactions 1 and 2) [108]. The substitution reaction is rapid and takes place at relatively low temperature (40 °C). Dithiothreitol (DTT) was used to reduce low molecular weight disulfides and mixed disulfide conjugates of thiols with proteins. A side reaction between hexaiodoplatinate and DTT may lead to the formation of a contaminating substance that may be adsorbed to the inner walls of the HPLC tubing as a yellow-brown substance and induce peak-broadening and tailing due to its affinity for NAC, Cys, and thioproline (internal standard) [110]. Although hexaiodoplatinate has a broad reactivity, interferences such as other AAs (cystine, methionine), diacetylcystine, reduced GSH, thioethers, thiazolidines, and other reductants such as ascorbic acid are separated under the chromatographic conditions used (IIC). A detection limit of approximately 20 pmol was achieved for NAC and Cys.

Gold nanoparticles (GNPs) have found increased use as novel post-column reagents in LC [70, 112, 113]. Owing to the distance-dependent optical properties and large surface of GNPs, their colloid solutions exhibit extinction coefficients of approximately three orders of magnitude larger than those of organic dyes. The colloids are generally stabilized by capping with surfactant molecules. However, the interactions of GNPs with some compounds may destabilize their colloidal solution and may lead to aggregation of the GNPs. The compound-induced aggregation shifts the surface plasmon resonance absorption maximum of GNPs towards longer wavelengths. This change of the optical property can be used as an advantage in the photometric measurement of the analyte. GNPs capped with

nonionic fluorosurfactant molecules (Zonyl FSN) were used in a colloidal solution as a probe reagent for the post-column photometric detection of Hcy [112]. FSN-capped GNPs with an average diameter of 12 nm exhibit excellent stability in aqueous solutions, even in the presence of high salt concentrations. The colloidal solution shows a strong surface plasmon resonance absorption peak at 525 nm. The aggregation of the GNPs could be induced by either Hcy or Cys, resulting in an absorption decrease of the colloidal solution at 525 nm and an absorption increase at longer wavelengths (600–700 nm). Therefore, by measuring the changes of absorbance at 525 nm or at a longer wavelength (680 nm), the analyte can be quantified. The height of the new absorption peak (680 nm) is proportional to the concentration of the thiol-type AA. In the post-column application the aggregation reaction could be accelerated by increased salt concentration and elevated temperature (70 °C). The aggregation reaction is complete within 1 min. The reaction process involves the initial attachment of the thiol species forming Au–S bonds on the NPs by displacement of the adsorbed surfactant molecules and a subsequent aggregation of the NPs (Table 1, application 15). The colloidal solution is destabilized, and the aggregation is driven by the London–van der Waals attraction force. An efficient capping process of GNPs with the nonionic fluorosurfactant molecules (Zonyl FSN) is always crucial for the rapid and selective interaction of the colloids towards the thiols. The analyte-induced aggregation of the NPs can be accelerated by salt [112]. The kinetics of the thiol-induced aggregation is affected by the GNP size [114]. Detection limits of 80 pmol were obtained for Hcy. The type of capping surfactant plays an important role in the selective responses of GNPs. Probably because of steric hindrance FSN-GNPs do not respond rapidly to other sulfur-containing molecules, such as Cys-Gly and GSH. Therefore, the colloid reagent can be used as a specific post-column reagent for HPLC assay of Cys and Hcy only [112]. Another application of GNPs in post-column reagents involves the use of Brij-35, as a nonionic surfactant capping agent of NPs [113]. Sulfur-containing AAs such as Cys and Hcy induce aggregation of the post-column reagent and shift absorption at 680 nm where detection without interferences can be achieved. Other analytes that have been tested along with Hcy and Cys to induce aggregation are Cys-Gly, γ-Glu-Cys, GSH, and Met. They are all resolved chromatographically. Low picomole detection limits (40–80 pmol) were obtained [113]. In general the application of GNPs in post-column photometric detection is characterized by low background noise, high selectivity, and greatly reduced interferences from other species.

Selected applications along with PCD reagents and conditions (flow rates, coil volume(s), temperature etc.), various post-column labeling chemistries, type of separation, and detection are summarized using photometric detection of AAs in Table 1.

Post-column derivatization with fluorescence detection

In general, a 'fluorogenic labeling' reagent is composed of a non-fluorescent compound which reacts with the functional group of AAs to form fluorescent derivatives. OPA reacts with primary AAs in the presence of a thiol compound to form highly fluorescent thio-2-alkyl-substituted isoindole products [115]. The derivatization reaction is performed at ambient temperature in a mixture of borate buffer (pH 9.7–10) and methanol or ethanol. This reaction is utilized either with pre-column or PCD in analysis of AAs after their separation by ion-exchange [44, 116–119], ion-interaction [34, 35, 57, 120, 121], reversed-phase [29, 66, 122, 123], and HILIC methods [124]. The rules governing the separation are the same as for the method with ninhydrin reagent. Many modifications of this methodology exist and details of the chemistry, PCD reagents, conditions, and detection are described in Table 2.

Although OPA does not react with secondary amine group AAs (imino acids such as proline, hydroxyproline etc.) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amino group AAs to react with OPA [118]. The mechanism of the conversion of secondary amines by sodium hypochlorite (NaOCl) to compounds that form fluorescent adducts with OPA/ME was thoroughly investigated [125, 126] and it is illustrated for proline in Table 2, application 3. In this case the oxidation product is 4-amino-*n*-butyraldehyde which reacts with OPA/2-ME. *N*-Methyl AAs such as sarcosine produce methylamine and/or glycine as products [126]. Although *N*-chlorosuccinimide follows a similar oxidation mechanism for proline it cannot be used because it oxidizes OPA, making it useless for fluorophore formation [118]. The complete procedure usually employs a strongly acidic cation-exchange column or reversed-phase column with an ion-interaction agent for separation of free AAs followed by post-column oxidation with sodium hypochlorite at increased temperature (60 °C) and PCD using OPA and a thiol compound as a co-reagent at ambient temperature. Co-reagents used are ME, ethanethiol, 2-dimethylamino ethanethiol, 3-mercaptopropionic acid, and NAC [43, 116–119, 127–130]. OPA, in combination with ME, is currently the most widespread OPA labeling technique for the fluorescent detection of picomole quantities of primary amino group AAs [57, 117–119, 124]. Ethanethiol has produced derivatives with slightly higher fluorescence intensities than those with ME [122, 131]. It is responsible for the increased stability of the adducts and resistance to spectral responses due to changes in solvent polarity without appreciably changing the observed fluorescence intensity [43]. Co-reagent 2-dimethylamino ethanethiol is a low-odor nucleophile and superior substitute for ME in the preparation of OPA reagents. It forms a more stable reagent and longer-lasting fluorophores with OPA than ME does, maintaining the same fluorescence

Table 2 Post- column derivatization chemistries and conditions of amino acids analysis using fluorescence (FL) detection

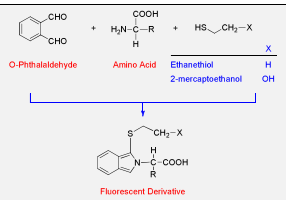
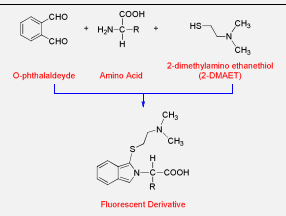
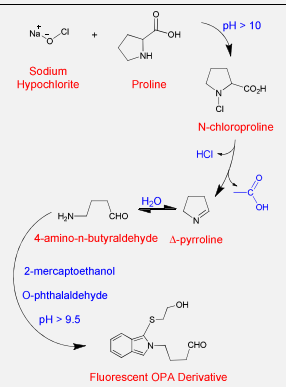
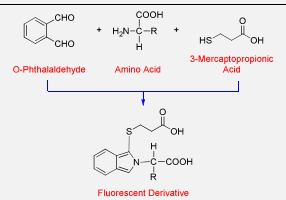
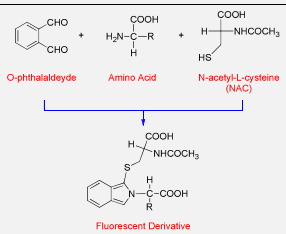
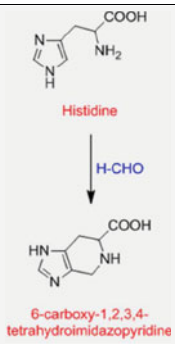
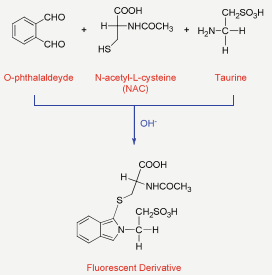
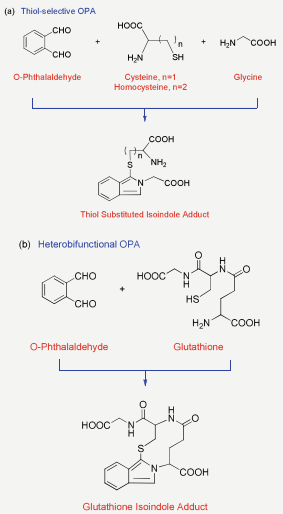
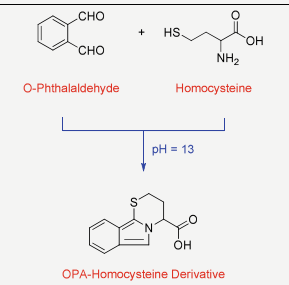
No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
1.	Primary amino group AAs	o-phthalaldehyde (OPA)/ 2-mercaptoethanol (2-ME)- or 2- ethanethiol (ET) R1: 0.8 g o-phthalaldehyde (OPA) / 2.0 ml of 2-mercaptoethanol (2-ME), 10 mL EtOH, 1.0 g Brij-35 in 990 mL of 0.4 M Potassium borate buffer (pH=9.7) Flow rate:0.14 mL/min		One coil 0.15 mL & Ambient Temperature	Ammonia causes interferences in separation. Brij-35 is necessary to prevent fluorescence quenching of the Lys and His derivatives. Cys gives a poor fluorescence and requires oxidation to cysteic acid. ET produces derivatives with slightly higher fluorescent yield than 2-ME.	Cation Exchange Chromatography	FL Ex. 330 nm Em. 465 nm	picomole	[43, 117, 122]
2.	Primary amino group AAs	o-phthalaldehyde (OPA)/ 2-dimethylamino ethanethiol (2-DMAET) R1: 0.3 g o-phthalaldehyde (OPA) /2.0 g of 2-dimethylamino ethanethiol (2-DMAET), 10 mL MeOH, 3.0 ml Brij-35 (30% w/v) in 945 mL of 0.4 M Potassium borate buffer (pH=10.4) Flow rate:0.3 mL/min		One coil 0.15 mL & 45°C	Use of 2-DMAET, a solid and odorless nucleophile, provides superior substitute for 2-ME in the preparation of OPA reagent. It forms a more stable reagent and longer-lasting fluorophore with OPA than 2-ME.	Cation Exchange Chromatography	FL Ex. 330 nm Em. 465 nm	picomole	[127]
3.	Primary and secondary amino group AAs (Pro, OH-Pro)	NaClO /o-phthalaldehyde (OPA)/ 2-mercaptoethanol (2-ME) R1: 0.1% NaClO solution (available chlorine 5%), 0.2M Potassium borate buffer (pH=10.5) Flow rate:0.07 mL/min R2: 0.8 g o-phthalaldehyde (OPA) / 2.0 ml of 2-mercaptoethanol (2-ME), 10 mL EtOH, 1.0 g Brij-35 in 990 mL of 0.4 M Potassium borate buffer (pH=9.7) Flow rate:0.14 mL/min		Two coils 1) 0.07 mL & 60°C 2) 0.14 mL & Ambient Temperature	Use of NaClO as the oxidizing reagent enables the detection with secondary amine AA such as Pro and OH-Pro. NaClO solution was introduced after the elution of Glu by means of a three-way valve and discontinued after elution of Pro [64]. Baseline separation between Glu and Pro is required. Heating of R1 increase fluorescence response of Pro and OH-Pro. N-chlorosuccinimide cannot be used since it oxidizes OPA, making it useless for fluorophore formation. If NaClO concentration is equal to 2-ME concentration a non-switching method can be applied without loss of AA sensitivity [65]	Cation Exchange Chromatography	FL Ex. 340 nm Em. 455 nm	picomole of AAs 3 pmol Pro 5 pmol OH-Pro	[117-119]
4.	Primary and secondary amino group AAs	NaClO/o-phthalaldehyde (OPA)/ 3-mercaptopropionic acid (3-MPA) R1: 0.2% (v/v) NaClO solution (available chlorine 5%), 0.35M Potassium borate buffer, 1.5% (w/v) K_2SO_4, (pH=10.4) Flow rate: 0.35 mL/min R2: 3.2 g o-phthalaldehyde (OPA) / 2.4 ml of 3-mercaptopropionic acid (3-MPA), 40 mL EtOH, 2.0 ml Brij-35 (30% w/v) in 960 mL of 0.35 M Potassium borate buffer (pH=10.4) Flow rate:0.35 mL/min		Two coils 1) 0.06 mL 2) 0.14 mL & 68°C	Good sensitivity in the determination of secondary amino acids. The highest Pro, Met and Tyr responses were found with 0.2% NaClO and 0.32% OPA. Adducts of Met and Tyr produce higher fluorescence with the use of 3-MPA. The molar ratio of 3-MPA to OPA in the derivatizing solution was kept at 1:1 as with NaClO/OPA/NAC method.	Cation Exchange Chromatography	FL Ex. 340 nm Em. 455 nm	10 pmol each AA	[44, 130]
5.	Primary and Secondary amino group AAs	NaClO/o-phthalaldehyde (OPA)/N-acetyl- L-cysteine (NAC) R1: 0.2% NaClO in 0.34 M Na_2CO_3/0.216 M H_3BO_3/0.18 M K_2SO_4 buffer Flow rate:0.2 mL/min R2: 1.6 g o-phthalaldehyde (OPA) 2.0 g N-acetyl- L-cysteine (NAC) 75 mL EtOH, 4 mL of 10% Brij-35 in 980 mL of 0.34 M Na_2CO_3/0.216 M H_3BO_3/0.18 M K2SO4 buffer Flow rate:0.2 mL/min		Two coils 1) 0.04 mL 2) 0.40 mL & both coils at 55°C	Sensitive determination is achieved for secondary amine AA such as proline hydroxyproline, and sarcosine. The fluorophore is more stable to hypochlorite which has been used for the oxidative cleavage of the imino linkage. The sensitivity for proline was about 20 times that of the OPA/2-ME method, and it was approximately the same order as those for other AAs.	Cation Exchange Chromatography	FL Ex. 348 nm Em. 450 nm	picomole	[128, 129]
6.	Primary and Secondary amino group AAs	NaClO/o-phthalaldehyde (OPA) /2-mercaptoethanol (2-ME) Hollow-Fiber Membrane Reactor (HFMR) R1: 0.8 mL NaClO solution (available chlorine 10%), 0.5M NaOH, 0.2M Sodium tetraborate R2: 0.8 g o-phthalaldehyde (OPA) / 1.0ml of 2-mercaptoethanol (2-ME), 5mL EtOH, in 95mL of 0.4M Potassium borate buffer (pH=9.2)	Same as in application 1	Three HFMRs (M1, M2 & M3) Two HFMR (Dionex AFS-2 aminated and CFS1-2 sulfonated) 20 & 30 cm Immersed in R1. One HFMR (Dionex CFS1-2 sulfonated) 200cm Immersed in R2 & 60°C	The method does not require an additional pump for delivering the post-column reagent; just a mixing tee and reactor for post-column reaction. The first HFMR M1 is used for introduction of NaClO; M2, for adjustment of the eluant pH; and M3, for introduction of OPA/2-ME.	Ion Interaction Chromatography	FL Ex. 340 nm Em. 450 nm	0.4-20 pmol	[120, 121]

Table 2 (continued)

No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
7.	N-Methylhistidine	Formaldehyde /o-phthalaldehyde (OPA) / 2-mercaptoethanol (2-ME) R1: 2.5 M formaldehyde in borate buffer (0.4 M, pH= 10.6) Flow rate:0.6 mL/min R2: 12 mM OPA, 26 mM mercaptoethanol, and 0.12 M methanol in borate buffer (0.4 M, pH= 10.6) Flow rate:0.6 mL/min		Two coils 1) 0.04 mL 2) 0.40 mL & both coils at 45°C	The fluorescence of the product of the reaction of OPA with histidine which elutes very closely to N-methylhistidine was drastically reduced by the addition of formaldehyde. This is due to its condensation reaction with HCHO and the formation of spinacine (6-carboxy-1,2,3,4-tetra hydroimidazopyridine) which does not react with OPA. N-methylhistidine does not react with HCHO.	Ion Interaction Chromatography	FL Ex. 348 nm Em. 460 nm	0.3 nmol	[45, 141-144]
8.	N-acetylcysteine [87] Cysteine (Cys), Glutathione (GSH), [75]	o-phthalaldehyde (OPA)/ Taurine(TAU) R1: 1 mM OPA in 0.05M borate buffer (pH= 11) Flow rate:0.5 mL/min R2: 1 mM Taurine(TAU) in 0.05 M borate buffer (pH=11) Flow rate:0.5 mL/min [87] or R1: 25 mM OPA in 50% MeOH Flow rate:0.5 mL/min R2: 10 mM Taurine(TAU) in 0.4 M borate buffer (pH=10) Flow rate:1.2 mL/min [75]		Two mixing tees and one coil 0.4 mL [87] 0.15 mL [75] & Ambient Temperature	The eluent from the column first is mixed via a tee with OPA in borate buffer and then via a second tee is mixed with Tau in borate buffer and then through a reaction coil before the OPA derivative is detected fluorometrically. Cys gave weak or no fluorescence upon reacting with OPA in the absence of other primary amine.	Reverse Phase Chromatography [87] or Anion Exchange Chromatography [75]	FL Ex. 365 nm Em. 442 nm	10 nM NAC [87] 30 pmol Cys, GSH [75]	[46, 66]
9.	Sulfur-containing AA (Thiols) Cysteine (Cys), Glutathione (GSH), N-acetylcysteine (NAC), Homocysteine (Hcy)	o-phthalaldehyde (OPA)/Glycine R1: 0.8 g/L OPA, 0.4% (v/v) Brij-35 in 0.35 M borate buffer (pH=10.5) Flow rate: 0.3mL/min		One knitted coil 0.60 mL & Ambient Temperature	A co-reagent such as an amine is needed for the selective derivatization of a Cys, NAC and Hcy. Glycine is added to the mobile as a co-reagent phase at a concentration of 0.5mM. No addition of a nucleophile reagent (e.g. mercaptoethanol) for GSH. It contains both thiol and primary amine moiety and reacts directly with OPA (heterobifunctional OPA reaction).	Ion Interaction Chromatography (IIC)	FL Ex. 340 nm Em. 440 nm	0.4ng (1.30pmol) GSH	[145-147]
10.	Homocysteine (Hcy)	o-phthalaldehyde (OPA) R1: 6 mM OPA in 0.5 M NaOH Flow rate: 0.5 mL/min		One knitted coil 2.0 mL & Ambient Temperature	Hcy is reacted with OPA in the absence of an added thiol reagent to form a stable fluorescent derivative. A heterobifunctional OPA reaction of Hcy can proceed rapidly at pH 13 and produces a high fluorescent adduct.	Ion Interaction Chromatography (IIC)	FL Ex. 370 nm Em. 480 nm	100fmol	[149]

properties [127]. The use of 3-mercaptopropionic acid and NAC as co-reagent of OPA after NaClO oxidation enables the detection of imino acids proline and hydroxyl proline [119, 128] using increased temperature (55–68 °C) in both reaction coils. Böhlen et al. [118] added NaOCl to the column eluent only when proline was eluted (switching flow method) because the continuous addition of NaOCl resulted in a marked (10 %) reduction in the sensitivity of α-AAs. This is probably due to

Table 2 (continued)

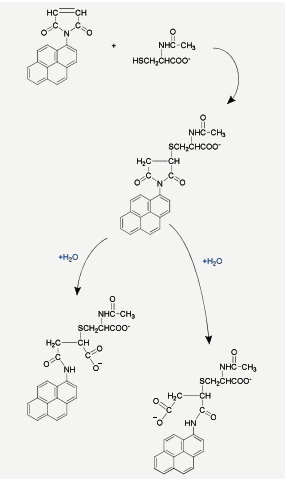
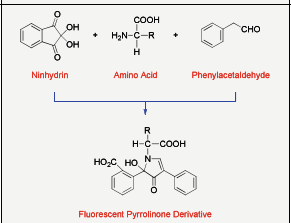
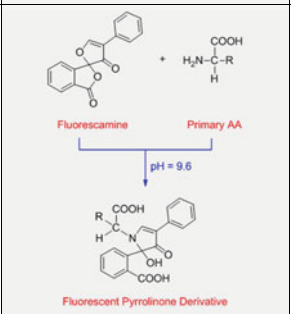
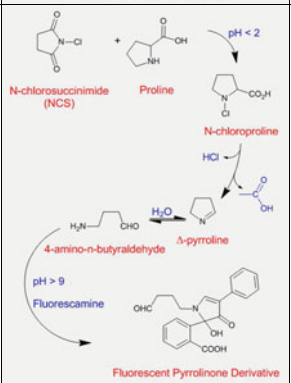
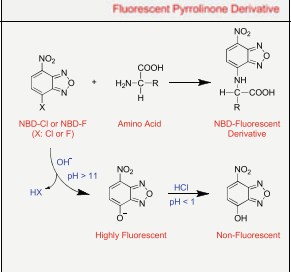
Table 2: POST COLUMN DERIVATIZATION CHEMISTRIES AND CONDITIONS OF AMINO ACIDS ANALYSIS USING FLUORESCENCE (FL) DETECTION									
No.	**Amino Acids**	**Derivatization reagent(s)**	**Chemistry & Derivative(s)**	**Reaction Coil(s) & Temperature**	**Comments**	**Chromatography**	**Detector**	**Detection Limits**	**References**
11.	Sulfur-containing AA (Thiols) Cysteine (Cys), Glutathione (GSH), N-acetylcysteine (NAC)	N-(1-Pyrenyl)maleimide R1: 0.05 M Borate buffer (pH= 11) Flow rate: 1.0 mL/min (RP-[88]) 0.5 mL/min (IIC-[94]) R2: 5×10^{-6} M N-(1-Pyrenyl)maleimide in acetonitrile Flow rate: 0.2 mL/min or R1: 2% (v/v) triethylamine – 1% (v/v) Brij–35 in CH_3CN:H2O 30:70 Flow rate: 0.3mL/min R2: 0.015g/L N-(1-Pyrenyl)maleimide in acetonitrile Flow rate: 0.3 mL/min [98]		Two mixing tees and one knitted coil 0.3 mL & Ambient Temperature	The reaction between NAC and pyrenemaleimide consists in a nucleophilic addition of the NAC, Cys, GSH to the activated C=C double bond. The derivative formed is sensitive to hydrolysis at the NC=O positions in the maleimide ring. The rate of hydrolysis is faster with an electron-attracting substituent at the N position, while an electron-donating substituent stabilizes the derivative. Small disulphides were determined after reductive cleavage in plasma with dithiothreitol.	Reverse Phase Chromatography (RP) & Ion Interaction Chromatography (IIC)	FL Ex. 342 nm Em. 389 nm	10 pmol of NAC (RP) 0.15 μM of NAC (IIC)	[67, 146, 160]
12.	Primary amino group AAs	Ninhydrin/Phenylacetaldehyde R1: 33.3 mM Ninhydrin, 3.33 mM Phenylacetaldehyde in 2:1 EtOH:H2O Flow rate: 0.3 mL/min R2: 0.2M Phosphate buffer (pH=7.5) Flow rate: 2.0 mL/min		R1 and R2 are mixed before they react with the AA One coil 65 mL & 65°C A cooling coil at 17°C was placed before the FL detector	Primary amino acids react with ninhydrin and phenylacetaldehyde to give a ternary product which is highly fluorescent. Ammonia does not react at all. The fluorescent ninhydrin procedure is 10 to 100 times more sensitive than the calorimetric ninhydrin procedure.	Cation Exchange Chromatography	FL Ex. 390 nm Em. 480 nm	sub-nanomole to picomole	[153]
13.	Primary amino group AAs	Fluorescamine (4-Phenylspiro-[furan-2(3H),1-phthalan]-3,3′-dione) R1: 0.1 6M Sodium borate buffer (pH=9.6) Flow rate: 0.2 mL/min R2: 150mg/L Fluorescamine in acetone Flow rate: 0.17 mL/min		Two coils 1) 0.02 mL 2) 0.18 mL & Ambient Temperature	In the presence of secondary AA non fluorescent derivatives are produced. Ammonia causes small baseline disturbances less than the one with ninhydrin derivatization.	Cation Exchange Chromatography	FL Ex. 390 nm Em. 475 nm	sub-nanomole to picomole	[125, 156, 157]
14.	Primary and Secondary amino group AAs	N-Chlorosuccinimide/Fluorescamine R1: 0.1 mM N-Chlorosuccinimide in 0.05M HCl Flow rate: 0.1 mL/min R2: 0.1 M Sodium borate buffer (pH=9.7) Flow rate: 0.4 mL/min R3: 300 mg/L Fluorescamine in acetone Flow rate: 0.3 mL/min		Three coils 0.015 mL each & Ambient Temperature	Use of N-chlorosuccinimide as oxidizing reagent enables detection of secondary AA. N-chlorosuccinimide solution was introduced after the elution of glutamic acid by means of a three-way valve and discontinued after elution of proline.	Cation Exchange Chromatography	FL Ex. 390 nm Em. 475 nm	sub-nanomole to picomole for all AA 100 pmol Pro	[158]
15.	Primary and Secondary amino group AAs	NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole)/HCl R1: 0.5 M Borate (pH=12.0) Flow rate: 0.2 mL/min R2: 0.2% NBD-F in EtOH Flow rate: 0.26 mL/min R3: 3 M HCl in MeOH:H2O 1:1 Flow rate: 0.8 mL/min		Two coils (The eluent is mixed first with reagent R1 and then with R2 before the reaction coil) 1) 0.25 mL (Reaction Coil) & 50°C 2) 0.05 mL (Cooling Coil) & 5-10°C	The hydrolyzed product (NBD-OH; 4-hydroxy-7-nitrobenzo-2,1,3-oxadiazole) of the NBD-F has a high fluorescence at basic pH. It loses its intensity at an acidic pH<1. There are no interferences due to ammonia. Trp is not detected since it forms a non-fluorescent derivative.	Cation Exchange Chromatography	FL Ex. 470 nm Em. 530 nm	Picomole (20 pmol for most of AA, 50 pmol Tyr, 5 pmol Pro)	[164, 169]

Table 2 (continued)

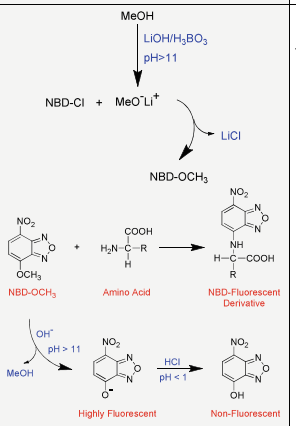
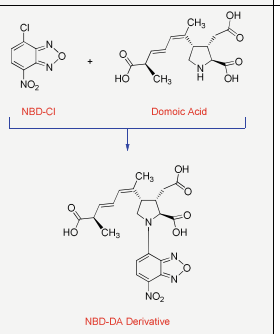
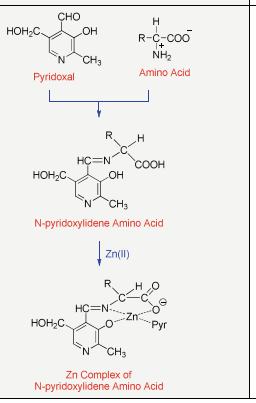
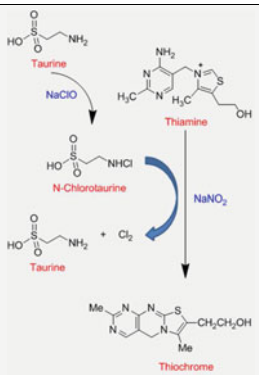
No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
16.	S-sulfocysteine ($CysSO_3H$), OH-Pro, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Ala, Tyr, Val	NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole)/HCl R1: 0.4 M Borate (pH=10.5) R2: 1.25 M NBD-Cl in EtOH Combined Flow rate of R1 & R2: 0.2 mL/min R3: 1 M HCl in Methylcellosolve Flow rate: 0.7 mL/min	Same as in application 15	One coil (The eluent is mixed first with reagent R1 and then with R2 before the reaction coil) 1.0 mL & 70°C	This method is one of the most sensitive fluorogenic post-column detection technique for AA with secondary amino group such as Pro and OH-Pro with moderate sensitivity for AAs with primary amino groups.	Cation Exchange Chromatography	FL Ex. 465 nm Em. 520 nm	30 pmol Gly 1 pmol Pro	[164]
17.	Primary and Secondary amino group AAs	NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole)/ HCl/H_5IO_6 ↓ NBD-OCH3 (4-methoxy-7-nitro-2,1,3-benzoxadiazole)/HCl/H_5IO_6 R1: 10 mM NBD-Cl, 0.09 M Borate buffer, 0.25% LiCl in MeOH (pH=11.5) Flow rate: 0.3 mL/min R2: 3 M HCl, 4 mM H_5IO_6 in Methylcellosolve Flow rate: 0.3 mL/min		Two coils (The eluent is mixed first with reagent R1 and then with R2 before the reaction coil) 1) 1.5 mL (Reaction Coil) & 120°C 2) 0.01 mL (Cooling Coil) & 5-10°C	NBD-Cl is commercially available in larger quantities than NBD-F. Under the mobile phase conditions is converted to NBD-OCH_3. High temperature and HIO_3 enables detection of Trp which forms a fluorescent derivative.	Cation Exchange Chromatography	FL Ex. 470 nm Em. 530 nm	Picomole to sub-nanomole	[165]
18.	Domoic acid	NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole) R1: 9.0 mM NBD-Cl 0.065M Borate buffer in MeOH (pH=10.0) Flow rate: 0.3 mL/min R2: 1 M HCl in Ethylacetate Flow rate: 0.6 mL/min		Two coils 1) 1.5 mL & 90°C 2) 0.05 mL & Ambient Temperature	NBD-Cl reacts in basic medium with domoic acid, giving a fluorescent derivative. Under reverse phase conditions most AA elute in the void volume while hydrophobic AA such as tryptophan are not detected since its NBD-derivative is not fluorescent	Reverse Phase Chromatography	FL Ex. 469 nm Em. 529 nm	25 ppb	[171]
19.	Sulfur-containing AA (Thiols) Glutathione (GSH), Cysteine (Cys), Homocysteine (Hcy)	NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) R1: 0.05% NBD-F in EtOH Flow rate: 0.13 mL/min R3: 3 M HCl in MeOH:H_2O 1:1 Flow rate: 0.8 mL/min	Same as in application 15	Two coils (The eluent is mixed first with reagent R1 and then with R2 before the reaction coil) 1) 0.10 mL (Reaction Coil) & 60°C 2) 0.05 mL (Cooling Coil) & 5-10°C	NBD-F reacts in acidic medium only with thiols, giving stronger fluorescence than AA which react in basic medium.	Cation Exchange Chromatography	FL Ex. 450 nm Em. 520 nm	150pmol GSH 10pmol Cys 100pmol Hcy	[164]
20.	Primary amino group AAs (except Trp)	Pyridoxal hydrochloride/Pyridine R1: 0.01% (w/v) Pyridoxal hydrochloride [3-Hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinecarboxaldehyde hydrochloride], 0.1% (w/v) $Zn(CH_3COO)_2$, 2% Pyridine(Pyr) in CH_3OH Flow rate: 2.0 mL/min		One coil 23.5 mL & 65-75°C	The fluorescent reaction is based on the formation of chelates between, N-pyridoxylidene amino acids and Zn(II) containing (1:1:1) each one molar of amino acid, pyridoxal, and Zn(II) ion. Citrates as strong chelating agents for Zn(II) interfere. Therefore, citrate buffers cannot be used as mobile phase eluents. Instead Acetate/Zn acetate buffers are used as eluents. Pro, OH-Pro, Trp and ammonia do not produce any fluorescence.	Ligand Exchange Chromatography	FL Ex. 365 nm Em. 485 nm	pmoles	[176-179]

Table 2 (continued)

No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
21.	Taurine	NaClO/Thiamine R1: 0.06% NaClO, 0.01% BriJ, in 0.1M Phosphate buffer (pH=12.0) Flow rate: 0.3 mL/min R2: 0.59 mM Thiamine hydrochloride, 0.33 M $NaNO_2$ in 0.1M Phosphate buffer (pH=7.0) Flow rate: 0.3 mL/min		Three coils 1) 0.60 mL 2) 1.00 mL (reaction coils) & both coils at 65°C 3) 0.20 mL (cooling coil) & 25°C	The derivatization is based on the chlorination of taurine using NaClO to produce N-chlorotaurine, which reacts with thiamine to yield fluorescent thiochrome.	Ion Exclusion-Reverse Phase Chromatography	FL Ex. 370 nm Em. 440 nm	6ng	[103]
22.	Primary and Secondary amino group AAs	$Cu(L\text{-}Trp)_2$ R1: 0.05 mM $Cu(L\text{-}Trp)_2$, 3 mM Sodium borate buffer (pH=8.70) Flow rate: 1.5 mL/min	$Cu(L\text{-}Trp)_2^{2+} + nAA \rightarrow Cu(AA)^{2+} + 2(L\text{-}Trp^*)$ Where AA is the amino acid molecule; L-Trp* is the fluorescent form of L-Trp and n is the number of the analyte molecules coordinated to Cu(II).	One mixing tee of 3 µL & Ambient Temperature	Fluorescence of L-tryptophan (L-Trp), is quenched in the presence of Cu(II) due to the formation of a $Cu(L\text{-}Trp)_2$ complex. Eluted AAs replace L-Trp from the complex and its fluorescence is analogous to AA concentration.	Anion Exchange Chromatography	FL Ex. 280 nm Em. 350 nm	3.8 pmol Cys Other amino acids are <10 pmol	[180]
23.	Sulfur-containing AA Cysteine (Cys), Glutathione (GSH), Cystine ($(Cys)_2$), Methionine (Met)	Palladium(II)–calcein R1: 10 µM Palladium(II)–calcein, 10 µM $ZnSO_4$ in 0.4 M NaOH-0.4 M NaH_2PO_4 (29:50) (air segmented) Flow rate: 0.6 mL/min	$Pd(Calcein)_2 + Cys \rightarrow Pd(Cys)_2 + Calcein$ Fluorescent	Two glass coils 2.0 mm i.d Reaction time 9 min & 55°C	A post-column ligand exchange reaction between Palladium(II)–calcein and sulfur-containing AA releasing free calcein providing an indirect measure of the compounds via fluorescence detection. An increase in fluorescence intensity was observed when adding Zn(II) to either the mobile phase or the reagent solution The reaction is not specific for sulfur-containing AA.	Cation Exchange Chromatography	FL Ex. 492 nm Em. 512 nm	0.5ng Cys 5ng GSH 3ng $(Cys)_2$ 3ng Met	[182]

the production of ammonia during to their oxidation [126]. In contrast, Ishida et al. [119] developed the "non-switching flow method" in which NaOCl is always added to the column eluent. These authors claimed that the addition of NaOCl had little or no influence on the sensitivities of α-AAs and that a few picomoles of α-AAs could be determined. However, the results from Nakamura and his coworkers [126] suggest that as far as the sensitivity is concerned the switching flow method is more recommendable for the determination of α-AAs than the non-switching flow method. Addition of either sodium SDS [132] or Brij-35 [117] to the derivatizing agent improves the stability and response of lysine and hydroxylysine derivatives and prevents their fluorescence quenching. There are baseline disturbances due to ammonia. Prior oxidation of cysteine to cysteic acid is required as is needed with ninhydrin, too.

The majority of AAs separations are accomplished via IEC or IIC and after PCD of eluted AAs with OPA the produced derivatives pass through a fluorometric detector. The fluorescence intensity of OPA-derivatized AAs is monitored with an excitation wavelength of 330 nm and an emission wavelength of 465 nm. For detection of both amino and imino acids a dual-pump configuration is required. A comparative study of two PCD techniques for AA determination has been reported [133]. Conventional HPLC was used to separate AAs on either sodium or lithium form cation-exchange columns, then derivatized with either OPA or ninhydrin. AA detection limits with OPA were near 5 pmol for primary amine group AAs and 100 pmol for secondary amine group AAs such as proline. Detection limits with ninhydrin are near 100 pmol. Response linearities obtained with OPA are in the range of a few picomoles to a few tens of nanomoles. To obtain good compositional data, samples larger than 500 ng before hydrolysis are required for the AA analysis of protein/peptide. The OPA system should be the system chosen whenever the detection of secondary AAs is unimportant. The single-pump OPA system is more sensitive and presents fewer technical problems than the ninhydrin system but does not allow for detection of secondary amines.

Baseline noise due to the mixing of different effluents (mobile phase and PCD reagents) has always been a problem. The contribution to noise due to the pulsation and flow irregularities of the piston pumps is usually very significant in PCD techniques. Haginaka and Wakai [120, 121] developed an HPLC method using a passive hollow-fiber membrane reactor (HFMR) to introduce the NaClO and OPA/2-ME reagent. One aminated and two sulfonated HFMRs immersed in a

thermostated bath were used for NaClO introduction, for pH adjustment of the effluent, and for the introduction of OPA and 2-ME, respectively. The reaction of AAs with OPA and 2-ME proceeds to the same extent in both types of post-column reactors. The HFMR has less band broadening than the conventional one. The proposed method using HFMRs required ten times higher OPA, five times higher 2-ME, and four times higher hypochlorite concentrations than those used in the conventional method. The detection limits achieved with this method were 0.4 to 20 pmol at a signal-to-noise ratio of 3. The advantage of the proposed method is that it does not require an additional pump for delivering the post-column reagent, mixing tees, and reactors for post-column reaction.

The technique of pre-column labeling of AAs using OPA with ME at pH 9.5–10.0 was successfully described by Jones et al. [123]. The techniques of pre-column labeling with OPA/thiol have been applied extensively in HPLC analysis of AAs [134, 135] and provide faster separations. However, the reproducibility is poor in comparison with the post-column techniques combined with IEC.

Other analogues of OPA such as 2,3-naphtalenedialdehyde (NDA) [136, 137], 3-(2-furoyl)quinoline-2-carbaldehyde (FQCA) [138], 3-benzoyl-2-quinoline-2-carbaldehyde (BQCA) [139], and 3-(4-carboxybenzoyl)-2-quinolinecarbaldehyde (CBQCA) [140] provide more stable derivatives than OPA and lower detection limits but they have been used only for pre-column labeling techniques owing to reasons of availability in large quantities for post-column use, instability of the reagent, and longer reaction times.

N-Methylhistidine (3-methylhistidine) is used as an index of skeletal muscle protein breakdown [141]. Measuring small amounts of *N*-methylhistidine in the presence of large amounts of histidine using LC has restricted the usefulness of the method because of their close elution [142]. A modified method using IIC and PCD with OPA has been proposed [45, 141]. In this method a formaldehyde reagent was used before the OPA reagent. The fluorescence of the product of the reaction of OPA with histidine was drastically reduced by the addition of formaldehyde. Histidine undergoes condensation reaction with HCHO (Table 2, application 7) to form spinacine (6-carboxy-1,2,3,4-tetrahydroimidazopyridine), a stable imino acid [143, 144] which does not react with OPA, whereas *N*-methylhistidine does not react with HCHO [142]. In this manner, 0.3 nmol of *N*-methylhistidine can be quantitated [141].

A reversed-phase HPLC method for the determination of NAC [66] and an anion-exchange chromatography for the determination of Cys and GSH [46] have been developed using a modified OPA method. The compounds are detected fluorometrically after derivatization with OPA in the presence of a primary amine AA (taurine). The analyte itself plays the role of thiol in the OPA reaction. OPA and taurine could not be used in a mixture as the PCD reagent owing to their reaction which generated a red color. Therefore, the column effluent was first mixed with OPA solution and then with the taurine solution (Table 2, application 8). Addition of sodium sulfite in the second reagent with taurine enabled the detection of disulfides [145]. Low pmole detection limits were achieved [46, 66]. In the OPA PCD of thiols a primary amine is needed as a co-reagent. Glycine is commonly used as the OPA co-reagent [146]. The co-reagent needs to be mixed first, prior to the OPA derivatization because a chemical reaction between OPA and the AA compound may occur. The isoindole adduct of Cys exhibits relatively weak fluorescence (Table 2, application 9). When OPA is used without co-reagent, only GSH [147, 148] and Hcy [149] can be detected (heterobifunctional reaction). Owing to steric hindrance, this type of reaction occurs very rarely. The requirements of the distance between the amino and thiol groups need to be fulfilled in order to obtain a highly fluorescent adduct (Table 2, applications 9 and 10). A 10-membered ring fluorescent adduct is produced between GSH and OPA [147, 148]. However, the heterobifunctional OPA reaction of Hcy is not efficient under conditions of about pH 10, and in order to proceed rapidly pH 13 is required to produce a six-membered ring adduct which is highly fluorescent [149]. Therefore, an OPA reagent in alkaline conditions (0.5 M NaOH) is used to obtain femtomole detection limits for Hcy.

Investigation of the fluorometric assay of phenylalanine with ninhydrin in the diagnosis of phenylketonuria [150] has led Samejima and coworkers [151] to a new reaction involving the condensation of ninhydrin, certain aldehydes, and primary amines to yield highly fluorescent ternary products. The most intense fluorescence was obtained with phenylacetaldehyde. Weigele et al. [152] studied the synthesis and the structure of the fluorescent pyrolinone product of this condensation reaction among equimolar quantities of ninhydrin, phenylacetaldehyde, and ethylamine in aqueous methanol solution. The condensation reaction was applied to the detection and quantitative assay of peptides, AAs, and amines in paper, thin-layer chromatography and commercial AA analyzers [153]. Ninhydrin and phenylacetaldehyde were mixed with phosphate buffer (pH 7.5) and reacted with the eluted AAs in a reaction coil at 65 °C for 30 min. A cooling coil was used before the products were detected by the fluorescence detector. One great advantage of the ninhydrin fluorescence technique is that ammonia was not detected, whereas a drawback is that this ternary condensation reaction does not go to completion and heating is required at 65 °C for a long time. Later, Weigele and Leimgruber [152] synthesized a spirolactone type compound, 4-phenylspiro [furan-2(3*H*),1'-phthalan]-3,3'-dione. The spirolactone compound reacts with amines at room temperature [154, 155] to form an identical fluorophore to that obtained with ninhydrin and phenylacetaldehyde. The reaction is binary rather the ternary one of the fluorescent ninhydrin method. Ammonia reacts to a slight extent but the conditions of this binary reaction produced considerably more intense fluorescence for the same amount of fluorophore. The development of this spirolactone compound

has led to a new reagent for AA detection with the commercial name of fluorescamine [156].

Fluorescamine is intrinsically non-fluorescent and reacts rapidly with primary amine group AAs to yield a blue-green-fluorescent derivative. This fluorogenic compound (with no inherent fluorescence property) reacts with primary amine group AAs in a borate buffer (pH 9.5–10) at ambient temperature. The binary condensation reaction is complete in a few minutes [156] and forms a fluorescent pyrrolinone derivative, whereas the reagent and its degradation products are non-fluorescent (Table 2). Fluorescamine offers enhanced fluorescence detection of primary AAs after separation using a cation-exchange column [157]. The fluorescamine PCD reaction requires a dual-pump configuration for the aqueous buffer and reagent which is quickly hydrolyzed in water; degradation products are non-fluorescent. Use of *N*-chlorosuccinimide as oxidizing reagent enables the detection of secondary AAs [125, 158]. Fluorescent derivatives are not very stable in aqueous eluents and sensitivity is two to four times lower than in the OPA reaction. This reagent is specific for primary AAs, whereas secondary AAs produce non-fluorescent derivatives. Peptides show higher fluorescence yields with their reaction with fluorescamine than single AAs at pH near 7 [156]. The application of fluorescamine in post-column labeling of small peptides was reported, too [159].

OPA has several advantages over fluorescamine as reagent [117]. (1) OPA yields greater fluorescent quantum yields (by nearly one order of magnitude). (2) OPA is soluble and stable in aqueous buffers without loss of sensitivity or increased fluorescence background, whereas fluorescamine is hydrolyzed. (3) The OPA PCD system is simpler because it requires only one reagent for primary amine group AAs. (4) Less noisy baselines are observed, because mixing of two aqueous solutions is not impaired by the simultaneous addition of organic solvents. This may cause gas formation and precipitation problems in the flow cell and can create baseline artifacts and increased noise. (5) Fluorescamine is considerably more expensive than OPA [108] resulting in AAs analysis at a higher cost. Owing to the high cost of fluorescamine, it is used only rarely today, limited mainly to microbore systems and in peptide detection because of its higher reactivity and sensitivity than OPA towards peptides.

N-(1-Pyrenyl)maleimide (NPM) or OPA can be used as a post-column labeling reagent for thiol-type AAs (Table 2) [67, 145, 160]. NPM is unstable in aqueous solution and, therefore, the post-column reagent has been prepared in a water miscible organic solvent (i.e., acetonitrile). A dual-reagent PCD system is required with the fluorescence detector operating at excitation and emission wavelengths of 342 and 389 nm, respectively. The NPM post-column system was applied to the determination of Cys, NAC, and GSH and similar detection limits were obtained [160]. The derivatization for the N-substituted maleimide is based on a nucleophilic addition of the thiol group to the carbon–carbon double bond of the maleimide reagent. The reaction rate depends on the pK_a value of the thiol because the thiolate ion is a stronger nucleophile and increases with pH [67, 160]. The reaction products of NPM with thiols are detected fluorometrically even though the reagent itself shows weak fluorescence. The maleimide/thiol conjugates are unstable and may undergo further hydrolysis, leading to the formation of two ring-cleaved products, as shown for NAC in Table 2, application 11. This is why several chromatographic peaks may appear under reversed-phase conditions of the derivatives [161]. NPM labeling reagent exhibits low selectivity towards thiol-type AAs because cross-linking to amines may cause interferences. These labeling reaction systems are used extensively in pre-column derivatization and the advantage of NPM labeling is the low response for DTT (dithiothreitol) that is often used in excess as a reducing reagent for disulfides [146]. Another maleimide reagent that has been applied as a post-column reagent is *N*-(9-acridinyl)maleimide (NAM). Linear relationships were obtained over the concentration range 0.05–25 nmol for cysteine and 0.1–100 nmol for glutathione, respectively. However, owing to high background fluorescence observed with this method it did not find extensive use [162].

Halogenobenzofurazan reagents, such as 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [163] and 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) [164, 165], react with both primary and secondary amino group acids at 60–70 °C under alkaline conditions (pH 8–12). The reaction with NBD-F is 10 times faster than with NBD-Cl [52, 166] and was complete within 1 min. Ahnoff et al. [167] have shown that the reactivity of NBD-Cl with primary amino group AAs was much lower than that with imino acids such as proline and hydroxyproline. Imai et al. [168] compared the reactions of proline with three halogenated 4-nitrobenzo-2-oxa-1,3-diazoles (NBD-R where *R*=F, Cl, Br) and the reactivities of NBD derivatives decreased in the order of NBD-F, NBD-Cl, and NBD-Br. However, the hydrolysis product, 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH), may cause problems because it fluoresces strongly (λ_{ex}=470 nm, λ_{em}=555 nm) under basic conditions. When an acid reagent at pH≤1 is added to the effluent the NBD-O^- anion is converted to NBD-OH (Table 2, application 15) and the background fluorescence is decreased substantially [163]. Therefore, PCD of AAs proceeds in a dual-coil system [163, 169], where in the first coil the reaction between AAs and NBD-F or NBD-Cl takes place, and in the second the fluorescence of NBD-OH is suppressed.

Amino and imino acids [163] after separation on a cation-exchange column are reacted with the NBD-F in an alkaline medium (pH 12) reagent to produce highly fluorescent NBD derivatives, and these are detected using fluorescence (λ_{ex}=470 nm, λ_{em}=530 nm). The first advantage of this system is that it detects directly both primary and secondary AAs, whereas fluorescamine [156] and OPA [117] react only with primary AAs. The second advantage of the system is that

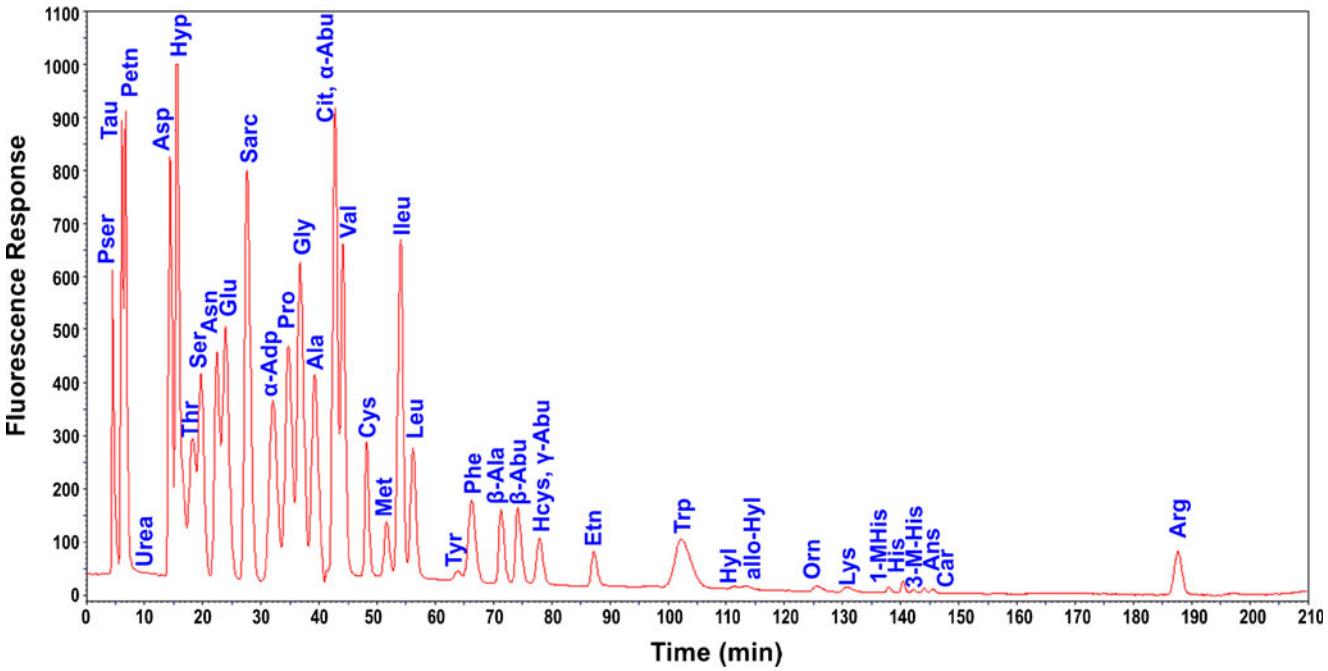


Fig. 3 Separation of 39 physiologic amino acids using ion- exchange chromatography with gradient elution and lithium citrate buffers and NBD-Cl as a post-column reagent. Mobile phase: A,: Li citrate buffer 0.7 %, LiCl 0.6 %, sulfolane 0.5 %, MeOH 2 %, pH=2.75; B,: Li citrate buffer 0.9 %, LiCl 2.0 %, sulfolane 0.5 %, MeOH 2 %, pH =7.50; C,: regenerant LiOH 0.4 %, LiCl 0.6 %, sulfolane 0.5 %, MeOH 2 %;, column temperature,: 37 °C;, flow rate,: 0.33 mL/min; column,: Lithium AA Analysis Column Li^+ 5 μm, 3 mmX×250 mm (Transgenomic);, detailed PCD conditions are listed in Table 2, application 17

ammonia is not present on the chromatogram [163], mainly because of the slow reaction of ammonia with NBD-F. With ninhydrin, OPA, or fluorescamine, ammonia contamination produces a peak that interferes with other AAs such as lysine and histidine, requiring extra purification of buffers and good degassing procedures, which are tedious and time-consuming. Some thiols, e.g., cysteine, homocysteine, and glutathione, are also quantitated with NBD-F in an acidic medium [163]. The obtained detection limits for glutathione, cysteine, and homocysteine were 150, 10.0, and 100 pmol, respectively. One of the disadvantages of using NBD-F as a post-column reagent is that it is unable for detecting tryptophan owing to the non-fluorescent properties of the NBD-tryptophan derivative. Picomole sensitivities (10 pmol) of most AAs and 1 pmol for proline were achieved.

The application of the NBD-Cl reagent to the post-column provides detection of picomole quantities of primary and secondary amino groups [164]. The reagent has been applied in the determination of proline and hydroxyproline in blood [170]. It is the most sensitive fluorogenic post-column detection technique known for proline and hydroxyproline with equivalent sensitivity to an OPA method for AAs with primary amino groups. In another application, NBD-Cl was used in the determination of domoic acid [171] at increased temperature of 90 °C (the NBD-Cl reagent requires higher post-column reaction temperatures than the NBD-F reagent). A tryptophan interference peak was not observed. Although tryptophan is not detected at lower temperatures (60 °C) [160], it can be detected fluorometrically at higher temperatures (120–130 °C) [165]. Under the reagent preparation conditions NBD-Cl was converted to NBD-OCH_3 which reacts 3.5 times faster with AAs than NBD-Cl does [167, 172]. Tryptophan detection was achieved using high temperature and the oxidative action of H_5IO_6 which was added in the reagent. Separation and detection of close to 40 AAs were achieved (Fig. 3). The PCD conditions with reactions and detection details are described in Table 2, application 17.

Other benzofurazan-type reagents such as DBD-F (4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole) [173], DBD-NCS (7-[(*N*,*N*-dimethylamino)sulfonyl]-2,1,3-benzoxadiazol-4-yl isothiocyanate) [174], and DBD-COCl (4-(*N*-chloroformylmethyl-*N*-methyl)-amino-7-*N*,*N*-dimethylaminosulfonyl benzofurazan) [175] provide better sensitivity with amines and AAs. In spite their large potential to be candidates for post-column reagents they have been used in pre-column labeling techniques for the same reasons described above for OPA analogues.

AAs produced blue fluorescence using pyridoxal or pyridoxamine and Zn(II) ion in pyridine–methanol solution [176, 177]. The fluorescence reaction mechanism was investigated and the final product exhibiting fluorescence was found to be *N*-pyridoxylidene AA–Zn (II) chelate containing equimolar amounts of AA, pyridoxal, and Zn(II) ion [177, 178]. Pyridine acts as a ligand in the formation of the complex. A post-column labeling technique was developed using pyridoxal–Zn (II) in pyridine–methanol solution [179]. Firstly the formation of *N*-

pyridoxylidene AAs takes place and secondly their chelation reaction with Zn occurs. The reaction is slow and the reagent with the eluent was allowed to react for 10 min in the reaction coil at 65–75 °C before they move to the fluorescence detector [179]. The method is 20–100 times more sensitive than the photometric method using ninhydrin reaction [176]. The sensitivity of this method is comparable to those of fluorescamine and OPA methods [176]. One of the drawbacks of the method is the longer reaction time [179].

A highly selective post-column labeling technique for taurine detection was reported by Yokoyama and Kinoshita [180]. Taurine was converted with hypochlorite into the corresponding *N*-chloramine, which was allowed to react with thiamine to yield fluorescent thiochrome (Table 2, application 21). A two-pump configuration is needed and both reaction coils are maintained at 65 °C. The fluorescence intensity of thiochrome was measured at an excitation wavelength of 370 nm and emission wavelength of 440 nm.

A sensitive fluorescence labeling technique has been developed for detecting underivatized AAs following separation on anion-exchange chromatography. The fluorescence of L-Trp when it is complexed with Cu^{2+} is approximately 95 % quenched [181]. Therefore, a post-column reagent containing the complex Cu(L-Trp)$_2$ is used and a displacement reaction between the eluted AAs and copper(II)-L-tryptophan complex Cu(L-Trp)$_2$, the takes place [181]. The L-Trp released from the displacement reaction is detected fluorometrically and is directly related to the AA concentration.

Reasonably good detection limits could be achieved if the analytes have a comparable affinity to L-Trp for Cu(II). If the analytes have weaker affinities for the Cu(II) then it will result in significantly less L-Trp being displaced from the Cu(L-Trp)$_2$ complex. The conditional stability constants of AAs in the Cu(II) complexes present a measure of the affinity of the AAs for Cu(II). At pH 8.7 the conditional stability constants for the formation of complexes indicate that they are similar to those of L-Trp. Because Cys has two amino groups and two carboxyl groups and a stronger chelating capacity, it has as a result a lower detection limit. The displacement reaction is fast and requires minimal added post-column mixing and less instrumental complexity; this presents an advantage to detection schemes based on a chemiluminescence (CL) reaction, which has a very short lifetime.

Sulfur-containing AAs could be detected using via a ligand-exchange reaction with palladium(II)–calcein complex (non-fluorescent) [182]. When reacted with sulfur-containing AAs, free calcein is released, providing an indirect measure of the analytes via fluorescence detection. The addition of Zn(II) either to the eluent or reagent increases the fluorescent intensity. The palladium(II)–calcein reaction is not specific for sulfur-containing AAs.

Post-column derivatization with chemiluminescence detection

Determination of tyrosine and tryptophan with acidic potassium permanganate has been reported [47]. The oxidation reaction between the AA and permanganate and the conditions producing CL are displayed in Table 3, application 1. A mini reversed-phase column with a mobile phase of 1 % sodium polyphosphate was used for the separation of the two AAs. Optimum responses are generally observed in acidic conditions at pH 2 except the maximum response for tyrosine only is observed at pH 6.75. The limit of detection for tyrosine was two orders of magnitude lower than that of tryptophan.

A CL technique based on the observation that weak CL is emitted during the decomposition of hydrogen peroxide catalyzed by copper(II) in basic media has been developed [183]. The CL is significantly increased upon addition of AA solution into the mixed solution of hydrogen peroxide and copper(II) [183]. This CL emission is dependent on the type of AA added. The reduction of Cu(II) by H_2O_2 in an alkaline medium produces superoxide radical ions (Table 3, application 2); this is followed by the reaction of Cu^{2+} ions with the superoxide ion via an electron transfer reaction in which a micro amount of singlet oxygen with light emission is not sufficient to be used for detection. The addition of low concentration Na_2CO_3 results in an increased emission although with the addition of NaOH there was almost no CL emission observed. This observation indicated that carbonate was not only used as an alkaline medium but that it also takes part in the reaction. In the literature there were several reports [184–189] that support this observation and the mechanism of CL formation by the carbonate including the complexation reactions of AAs are displayed in Table 3, application 2 (reactions 3–8). This CL technique was applied to the determination of AAs, Phe and Trp, after their reversed-phase separation [106]. The reported detection limits were 4.5×10^{-6} M for tryptophan and 1.15×10^{-5} M for phenylalanine and were not sufficient enough to apply this method for AAs determination in body fluids.

The application of the traditional CL reaction using luminol/H_2O_2 catalyzed by a transition metal was reported, too [48, 190]. The method takes advantage of the complexation abilities of underivatized AAs with metal ions. The fact that transition metal ions such as Cu^{2+} and Co^{2+} are necessary catalysts for the luminol CL reaction [48, 190] is used in combination with a displacement reaction between the eluted AAs and these metal cations. When AAs complex the transition metal ions, a much less efficient catalyst for this reaction is formed. Detection is based on adding the CL reagents to the mobile phase post-column. When the separated AAs elute from the column, they form complexes that reduce the catalytic effect of the metal cations, resulting in a loss of the CL signal. At least a dual-pump configuration is required because of the short lifetime of CL. The first

Table 3 Post- column derivatization chemistries and conditions of amino acids analysis using chemiluminescence (CL) detection

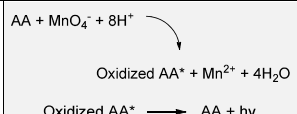
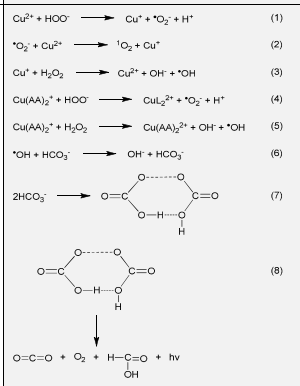
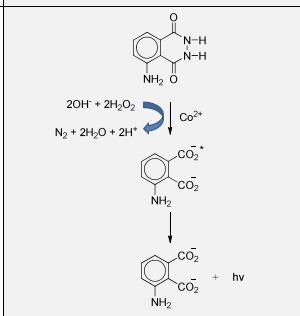
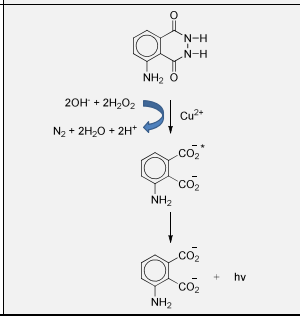
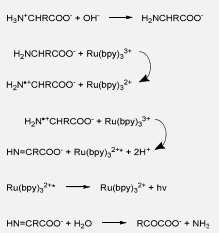
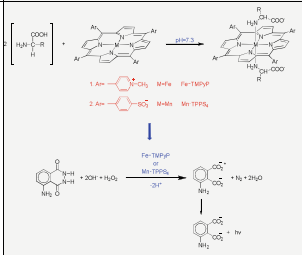
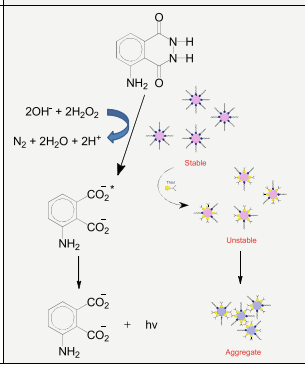
No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
1.	Aromatic AAs Tyr, Trp	Potassium Permanganate R1: $1x10^{-3}$ $KMnO_4$ in 1% (w/v) H_3PO_4 at pH=2 (Tyr, Trp) Flow rate: 1.6mL/min or R1: $3.1x10^{-3}$ $KMnO_4$ in 1% (w/v) H_3PO_4 at pH=6.75 (Optimum conditions for Tyr only) Flow rate: 1.6mL/min		One mixing tee & Ambient Temperature	Using a short reverse phase (C18) column with 1% (w/v) sodium polyphosphate eluent (pH=2), both AAs yield chemiluminescent responses with acidic potassium permanganate. At pH=6.75 only Tyr yields higher CL intesity.	Reverse Phase Chromatography	CL	pH=2 5×10^{-8}M Tyr 3×10^{-8}M Trp pH=6.75 1×10^{-8}M Tyr	[47]
2.	Aromatic AAs Phe, Trp	Cu^{2+}– H_2O_2 – Na_2CO_3 R1: 1.0 M H_2O_2 Flow rate: 1.0 ml/min R2: 0.05M Na_2CO_3 Flow rate: 1.0 ml/min R3: 5 mM $CuSO_4$	 The copper AA complexes are inefficient catalysts because their Cu(I) derivatives are not oxidized by $^•O_2^-$, and reaction (2) will not proceed and the production of $^•OH$ radicals will be favored for strong CL emission through reactions 6-8.	Two mixing tees (one for mixing R1 & R2 solutions) and a coil (for mixing Cu^{2+} with AA) of 40 µL & Ambient Temperature	The reaction of Cu^{2+} and H_2O_2 was carried out in a low concentration of Na_2CO_3 solution results in a relatively high CL emission. The addition of AA such as Phe and Trp to the mixed solution of Cu^{2+}– H_2O_2 – Na_2CO_3, an even stronger CL emission was observed. Carbonate was not only used as alkaline buffer but it takes part in the reaction. There was almost no CL emission observed when fresh NaOH solution was used.	Reverse Phase Chromatography	CL	1.1×10^{-5}M Phe 4.5×10^{-6}M Trp	[47, 183-189]
3.	Primary and Secondary amino group AA	Co^{2+}-Luminol/H_2O_2 R1: 100 nM Co^{2+} Flow rate: 0.1 mL/min R2: 10 mM Luminol/ 20 mM H_2O_2/35 mM KOH/1 µM EDTA (pH=11-12) Flow rate: 0.1 mL/min		Two mixing tees and Detector cell of 85µL & Ambient Temperature	The detection system monitors the CL from a steady flow of Co(II) catalyzing the luminol reaction and hydrogen peroxide in a alkaline solution. When AA is eluted off into the Co(II) stream, some of the Co(II) is complexed and decreased CL light intensity results.	Cation Exchange Chromatography	CL	Acidic 1nmol OH-Pro 20nmol Asp 1nmol Glu Neutral 0.4nmol Gly 0.4nmol α-Ala 2nmol β-Ala 10pmol Cys 1nmol Val 2nmol Phe Basic 0.4nmol Lys 4pmol His 40pmol Arg	[48]
4.	Primary and Secondary amino group AA	Cu^{2+}-Luminol/H_2O_2 R1: 1µM Cu^{2+} Flow rate: 0.7mL/min R2: 5mM Luminol / 10mM H_2O_2 / 100mM Phosphate buffer / 1µM EDTA (pH=11.6) Flow rate: 0.7 mL/min		Two mixing tees and Detector cell of 85µL & Ambient Temperature	The detection system monitors the CL from a steady flow of Cu(II) catalyzing the luminol reaction and hydrogen peroxide in a alkaline solution. When AA is eluted off into the Cu(II) stream, some of the Cu(II) is complexed and decreased CL light intensity results.	Cation Exchange Chromatography	CL	2nmol OH-Pro 0.5nmol Gly 2nmol Val 1pmol His 2nmol Arg	[190]
5.	Primary and Secondary amino group AA (Pro, Asn, Gly, Thr, Cys, Ala, Tyr, Val, Met, Phe, Leu, Lys, His, Trp, Arg)	Electrogenerated chemiluminescence (ECL) R1: 0.05M Borate buffer (pH=12.5) Flow rate: 0.32mL/min R2: 1mM $Ru(bpy)_3(ClO_4)_2$ 0.2M Na_2SO_4 Flow rate: 0.32 mL/min (Working electrode potential : +0.89V)	Electrogenerated chemiluminescence (ECL) 	Two Mixing Tees and 80µL Glass Detection Cell	$Ru(bpy)_3^{3+}$ reagent is electrochemically regenerated post-column prior the detection cell. AAs are oxidized by $Ru(bpy)_3^{3+}$ in alkaline media. Secondary AAs produce higher CL signal.	Cation Exchange Chromatography	CL 600nm	135pmol Ser 6pmol Glu	[191]

Table 3 (continued)

No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
6.	Domoic acid	Electrogenerated chemiluminescence (ECL) R1: 100 mM Phosphate buffer (pH=6.9) Flow rate: 0.1 mL/min R2: 0.25 mM $Ru(bpy)_3(Cl)_2$ 50 mM Na_2SO_4 Flow rate: 0.3 mL/min (Electrolytic Current: 80 μA)	Same as in previous	Two Mixing Tees and a Spiral Detector Cell	$Ru(bpy)_3^{3+}$ reagent is electrochemically regenerated post-column prior the detection cell. Domoic acid containing secondary amine is more reactive than AA with primary amine	Reverse Phase Chromatography	CL	0.4μg/L	[69]
7.	D,L-Trp	Electrogenerated chemiluminescence (ECL) R1: 0.24mM $Ru(bpy)_3(Cl)_2.6H_2O$ 10mM H_2SO_4, 50mM Phosphate buffer (pH=6.9) Flow rate: 0.3 mL/min (Electrolytic Current: 80 μA)	$Cu(D\text{-}Phe)_2^{2+} + D,L\text{-}Trp \longrightarrow Cu(D,L\text{-}Trp)_2^{2+} + D\text{-}Phe$ Electrogenerated chemiluminescence (ECL) reactions are the same as in application 5.	Mixing of the eluent and reagent occurs at the detector cell	Separation of D & L- Trp is achieved using an eluent of methanol-8 mM D-phenylalanine with 4mM $CuSO_4$ (15:85 v/v)	Ligand Exchange Chromatography	CL	0.2pmol	[192]
8.	Aromatic AA Phe, Trp, Tyr	Photochemical combined with Electrogenerated $Ru(bpy)_3^{3+}$ at a potential of +950 V (off-line) R1: Hg Lamp (254nm) R2: 1mM $Ru(bpy)_3(Cl)_2.6H_2O$ 0.05M Na_2SO_4 Flow rate: 0.3mL/min	Photochemical derivatization coupled with Electrogenerated chemiluminescence (ECL) Reactions same as in application 5.	One knitted coil (Photolytic) 890 μL & Ambient Temperature	Aromatic AA upon irradiation with UV light are found to give an increased CL signal on the order of 4–9 times that for nonirradiated compounds.	Reverse Phase Chromatography	Photolytic CL	13 pmol Phe 2 pmol Trp 21 pmol Tyr	[68]
9.	Cys, Tyr, Trp, $(Cys)_2$	Metalloporphyrin-luminol- H_2O_2 R1: 10μg/L Mn-$TPPS_4$, [tetrakis(sulphophenyl) porphyrin] or 10μg/L Fe-TMPyP [tetrakis(N-methylpyridiminiumyl) Porphyrin] in 0.1 M phosphate buffer (pH=7.3) Flow rate: 1.0 mL/min R2: 2.5 mM Luminol, 0.1 mM EDTA in 0 1 M $NaHCO_3$-NaOH buffer (pH= 12) Flow rate: 2.0mL/min R3: 10 mM H_2O_2 Flow rate: 2.0 mL/min		One mixing tee and a delay coil 80 μL for the eluent and metaloporphyrin A mixing tee for Luminol- H_2O_2 reagents A mixing tee for mixing eluent-metaloporphyrin with Luminol- H_2O_2 & Ambient Temperature	In the first reaction the AA is bound to metalloporphyrin through its NH_2 group in anionic form. The AA-metaloporphyrin complex inhibits the catalytic activity of mimetic peroxidase (AA-metalloporphyrin) of the chemiluminescence reaction Luminol- H_2O_2.	Reverse Phase Chromatograph	CL	0.68nM Cys 0.13μM Tyr 8.5μM Trp 22mM $(Cys)_2$	[64]
10.	Sulfur-containing AA (Thiols) Cysteine (Cys), Homocysteine (Hcy), Glutathione (GSH)	Zonyl FSN-capped Gold nanoparticles (GNP)/Luminol/H_2O_2 R1: 0.5 nM colloidal solution of FSN-capped triangular GNP (32 nm), 0.1 M Sodium Phosphate Buffer pH=6.0 Flow rate=1.2 mL/min R2: 0.05 mM Luminol, 30 mM NaOH Flow rate=1.2 mL/min R3: 5.0 mM H_2O_2 Flow rate=1.2 mL/min		Three Mixing Tees and 24μL Detection Cell	Triangular GNPs displayed greater catalytic activity towards luminol CL than spherical GNPs. Upon the addition of a variety of aminothiols CL changes of the triangular GNPs-catalyzed luminol reaction. The detection limits are 100 times lower than the one observed with the GNP aggregation photometric method	Reverse Phase Chromatography	CL	0.016-0.1 pmol	[70]

reagent includes the transition metal cation and the second includes the luminol/H_2O_2 mixture in an alkaline medium. The loss of copper(II) activity resulting from the AA–Cu(II) complexation reaction is related to the AA concentration in the column effluent. Relatively higher detection limits were obtained at the nanomole level, owing to the high CL background and possibly the fact that luminol also complexes transition metal ions.

Brune and Bobbitt [191, 192] used electrogenerated tris(2,2'-bipyridyl)ruthenium(III) as post-column CL reagent for the detection of underivatized AAs. The reaction chemistry and mechanism of the CL emission have been investigated thoroughly and are displayed in Table 3, application 5. It has been demonstrated that the electron-withdrawing/donating character of the R group attached to the α-carbon of the AA influences the CL emission of the reaction. Electron-withdrawing R groups tend to decrease CL (e.g., Ser, Thr), whereas electron-donating R groups tend to enhance CL (e.g., Leu, Val). This would account for the exhibited increase in CL efficiency of secondary amine group AAs such as proline relative to that with primary amine groups. The aforementioned observation was supported by the effect of α-hydrogen on the CL of AAs. Five AAs (glycine, alanine, α-methylalanine, glutamic acid, and α-methylglutamic acid) were compared and it was shown that CL increased from left to right. Consequently it was proven that substitution at the α-

carbon of the AA with an electron-donating group enhances the CL signal. In addition it was observed that phenyl groups or groups capable of delocalizing the radical (reactions 1 and 2) when attached directly to the amine result in decreased CL via a quenching mechanism. The pH-dependence of this reaction has been proven to be the key experimental parameter in applying it as a post-column detection technique. The CL emission is at a maximum when pH values are greater than the N-terminal amino group pK_a of the AA (pH 10) [192]. The stoichiometric mole ruthenium-to-amine ratio of four amines were determined to be 2:1. The detection limits for serine and leucine were determined to be 135 and 3 pmol, respectively, at a signal-to-noise ratio (S/N) of 3 with linearity of two orders of magnitude for Leu [191] and Val (30 pmol) [192]. The post-column CL technique was successfully applied using IEC in the detection of AAs of a protein digest [191] and reversed-phase chromatography in the determination of a secondary amino group AA, domoic acid [69]. Very low detection limits (0.2 pmol) were also achieved by applying the electrogenerated CL technique for the determination of D,L-Trp after ligand-exchange separation using $Cu(D\text{-}Phen)_2^{2+}$ as an eluent [193]. When this technique is combined with a photochemical derivatization using a Hg lamp, the aromatic AAs Phe, Tyr, and Trp were found to increase the CL signal on the order of 4–16 times that of the nonirradiated AAs. Coupling photolysis before the addition of the electrogenerated $Ru(bpy)_3^{3+}$ to a primary amine aromatic AA may result in a photochemical cyclization process, forming a secondary amine which is more CL reactive. CL detection limits of the photolyzed AAs are in the range of 2–20 pmol [68]. The CL detection limits are comparable to those previously reported for aromatic AAs by the photolytic–ECD method [38].

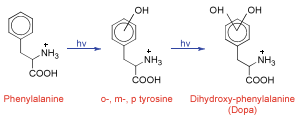
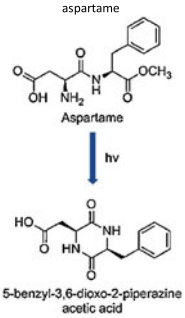
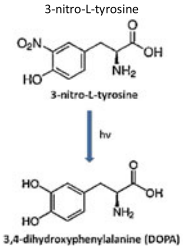
It is well known that AAs can be determined by measuring the amount of H_2O_2 formed during the enzymatic reaction of the AAs with their oxidase [49]. The produced H_2O_2 is then determined via the use of a CL reaction. In the application 9 of Table 3 the use of metalloporphyrins (M-Pr) as a substitute for peroxidase was investigated [64]. Four AAs were determined on the basis that M-Pr exhibit the catalytic activity of mimetic peroxidase in the CL reaction between luminol and H_2O_2 which is inhibited by the presence of an AA. The degree of CL inhibition is related to the AA concentration. The AA interaction with M-Pr results in the formation of mixed ligand complex between the AA and M-Pr. In the complex, the two axial coordination sites of M-Pr were occupied by an AA, and the catalytic activity center for the CL reaction was blocked (Table 3, application 9). A cationic (Fe-TMPyP) and an anionic metalloporphyrin (Mn-$TPPS_4$) were used as the first PCD reagent is mixed with the eluent containing the AA. The complexation with the AAs inhibits the CL between the luminol and H_2O_2 reagents in basic media. All reactions are fast and take place at ambient temperature. More than 20 AAs were tested, and only four, namely L-cysteine, L-tyrosine, L-tryptophan, and L-cystine, quenched the CL intensity.

The last significant application of CL post-column labeling techniques involves the coupling of a triangular GNP reagent in the presence of nonionic fluorosurfactant (FSN) with the CL reaction of luminol and H_2O_2 in basic media [72]. The previous applications of GNPs using photometric detection [112, 113] have some drawbacks: (1) the dynamic range of the analyte-induced colloidal aggregation was very narrow (one order of magnitude) and (2) detection limits were generally in the micromolar range. The triangular GNP reagent having high stability in a wider pH range and salt concentration is the first PCD reagent which is mixed with the eluent containing the sulfhydril AA and the same catalyzes the reaction of luminol and H_2O_2 reagents in basic media. After the interaction with analytes aggregation takes place which destabilizes the reagent and its catalytic activity in the CL reaction. The result is a decrease in CL emission and negative peaks for the analytes. The method exhibits very low detection limits (0.016–0.1 pmol) compared to other CL methods for cysteine, homocysteine, glutathione, cysteinylglycine, and glutamylcysteine. Wider linear ranges were observed (two orders of magnitude) than the one previously reported with the photometric methods using GNPs [112, 113]. Other sulfur-containing AAs such as Met, His, and $(Cys)_2$ do not respond which proves that the reduced aminothiols can form strong Au–S bonds, leading to a decrease in the CL intensity of the triangular GNPs-catalyzed luminol CL [72].

Post-column derivatization with photochemical reaction coupled to electrochemical detection

The application of light as a PCD "reagent" provides a very simple derivatization technique and avoids some drawbacks of chemical derivatizations. The method can be used either on-line, with or without any mixing of chemical reagents and analytes in post-column mode. An extensive review of post-column photochemical reaction systems coupled to electrochemical detection (ECD) in HPLC is provided by Fedorowski and LaCourse [194]. In this technique, analytes eluting from the LC column are photolyzed in a knitted fluoropolymer reaction coil as a function of the wavelength of the light and residence time within the reactor. In several cases, electrochemically inactive analytes are converted into one or more photoproducts having favorable oxidative and/or reductive properties. At other times, analytes already having some EC activity can be converted to new photoproduct(s) with improved EC detection. There is no significant band broadening due to the UV reactor, by using a knitted open tubular design.

Table 4 Post- column derivatization chemistries and conditions of amino acids analysis using photochemical coupled with electrochemical (EC) detection

No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
1.	Aromatic and sulphur-containing AA	Photolytic-Electrochemical Detection R1: Hg Lamp (254nm)	Photooxidation of phenylalanine  	One knitted coil (Photolytic) 2.5 mL & Ambient Temperature	Tyr and Trp showed inherent EC response at the potential of +0.80V without photolysis. They showed no EC responses at +0.60 V. When the lamp was turned on, both Tyr and Trp showed increased EC response, even at +0.60V. Phe was not inherently oxidative active, and did show EC response when the lamp was turned on. The same applies for cystine and methionine. Cysteine which is inherently electrochemically active without being photolyzed. It's peak significantly decreased with on-line, post-column irradiation.	Reverse Phase Chromatography	EC +0.80 V (Aromatic AA) +0.65 V (Sulfur-containing AA)	70 μg/L Tyr 100 μg/L Phe 50 μg/L Trp Low pmol levels for the sulfur-containing AA	[38,42 79]
2.	Phe	Photocatalytic-Electrochemical Detection R1: Hg Lamp (254nm)	Same as in previous	One TiO_2 coated knitted coil (Photocatalytic) 35 μL & Ambient Temperature	Detection limits achieved with photocatalytic reactors were of two orders of magnitude lower than those with a standard photolytic open-tubular reactor	Ion Interaction Chromatography	EC +1.0 V & 0.4 V	29.8 pM (+1.0 V) 113 pM (+0.4 V)	[80]
3.	Aspartame (N-(L-α-Aspartyl)-L-phenylalanine methyl ester)	Photolytic-Electrochemical Detection R1: Hg Lamp (254nm)	Photooxidation of aspartame  	One coil (Photolytic) 1.4 mL & Ambient Temperature	Aspartame is converted into a diketopiperazine, namely 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP), a well known decomposition product of aspartame under certain conditions of temperature and pH.	Reverse Phase Chromatography	EC +0.80 V	0.5 mg/L	[39, 195]
4.	3-nitro-L-tyrosine	Photocatalytic-Electrochemical Detection R1: Hg Lamp (254nm)	Photooxidation of 3-nitro-L-tyrosine  	One TiO_2 coated knitted reaction coil (Photocatalytic) 75 μL & Ambient Temperature	Conversion of NO_2 -Tyr by irradiation, prior to detection, increases the sensitivity and lowers the detection limit. Tyr is electrochemicaly active and can be determined after separation of NO_2-Tyr.	Reverse Phase Chromatography	EC +0.85 V	0.5 nM (10 fmol or 2.3 pg injected)	[77,78]

The use of post-column, on-line photolytic derivatizations in LC with ECD for the analysis of aromatic and sulfur-containing AAs has been reported by Dou and Krull [38]. The separation of methionine and cystine was achieved by using a reversed-phase column and a post-column photolytic EC detector. Both AAs had no oxidative EC responses under mild potential conditions (+0.65 V vs Ag/AgCl) without irradiation, and could be detected at the same potential after irradiation with light at 256 nm. Instead, the photooxidation of cysteine results in a disulfide that could not be detected oxidatively. In the reverse process, the photodissociation of the sulfur–sulfur bond of cystine, which yields an electroactive thiol compound, may be considered as the main mechanism of photolytic EC detection of cystine. The aromatic AAs Tyr and Trp are electrochemically active at a higher potential of +0.80 V without photolysis. They had no EC responses at +0.60 V. When the UV lamp was turned on, both Tyr and Trp EC responses were increased, even at +0.60 V potential. Phe was not inherently oxidatively active, and an EC response was observed when the lamp was turned on. For Phe there were no EC signals without irradiation, even when the applied potential reached +1.20 V. Upon irradiation sensitive EC signals were observed at both +0.80 and +0.60 V. The mechanism of L-Phe photochemical derivatization is shown in Table 4, application 1, resulting in a mixture of known hydrolytic products of Tyr isomers (*o*-, *m*-, and *p*-) and L-dopa [79]. The detection limits for AAs and peptides studied are low picomole levels. The method has been applied to the determination of phenylalanine in human urine [42].

Kaufman et al. [80] using a photocatalytic reactor with a TiO_2 coated knitted coil achieved detection limits of two orders of magnitude lower than those with a standard photolytic open tubular reactor.

Galletti et al. [39] applied this method to the determination of aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester). Aspartame is converted into a diketopiperazine, namely 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP), a well-known decomposition product of aspartame under certain conditions of temperature and pH. Aspartame, which is electrochemically inactive, was oxidized by applying a voltage in the range 0.1–1.1 V after post-column irradiation at 254 nm [195]. A detection limit of 0.5 mg/L (signal-to-noise ratio 3:1) was obtained. An improved method using a TiO_2 coated knitted reaction coil increased the detection of phenylalanine by twofold at the picomole level [80] and 3-nitro-L-tyrosine at the femtomole level [77]. 3-Nitro-L-tyrosine is converted photochemically to L-Dopa which is electrochemically active. Comparison of different detection methods, including UV, oxidative, and redox electrochemistry, and post-column photolysis followed by electrochemical detection [80] demonstrated that the HPLC-photolysis-ECD with post-column photolysis followed by electrochemical detection is the most effective method with the lowest detection limit of 0.01 pmol, and a linear dynamic range from 2 nM to 100 mM [78].

Post-column derivatization using immobilized enzyme reactors and solid-phase reactors

Liquid chromatography can be coupled to various selective post-column reaction detection devices which can use either homogeneous solutions or heterogeneous solid-phase reagents. Such solid-phase reactors (SPR) are immobilized enzymes on a solid support contained in an on-line packed-bed immobilized enzyme reactor (IMER). IMERs are a subcategory of solid-phase reactors where other compounds can be immobilized and are considered as catalytic reactors. After separation of the AAs, the column effluent is mixed with a make-up flow containing the substrates necessary for the enzymatic conversion in the IMER.

The stereoselective enzymatic detection of AAs can readily be made by using L- or D-AA oxidases (LAAO and DAAO). Kiba and Kaneko [49] used an IMER with two co-immobilized enzymes L-AA oxidase (EC. 1.4.3.2) (AAO) and peroxidase (E.C. 1.11.1.7) (PO). First the enzyme, L-AA oxidase, in the presence of molecular oxygen oxidizes the L-AAs forming H_2O_2 and a 2-keto-acid (Table 5, application 1). Homovanilic acid, a non-fluorescent compound, is the substrate reagent for the second immobilized enzyme peroxidase and is mixed with the eluent before the IMER. The produced H_2O_2 oxidizes homovanilic acid in the presence of peroxidase to form to the highly fluorescent 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid that emits light at 425 nm [196] when it is excited at 315 nm. The reagent homovanillic acid is very stable and can be used for several months [197]. The simultaneous determination of tyrosine, phenylalanine, tryptophan, and methionine was achieved with detection limits of 5–10 μM [49]. Other AAs respond to the post-column IMER detection system such as Lys, His, CySH, Arg, Leu, and Ileu. No activity was found for the D-AAs. Alternatively the produced H_2O_2 in the AAO reactor is reduced in a second IMER containing horseradish peroxidase (HRP) using 4-aminoantipyrine (4-AP) and dichlorophenolsulfonate (DCPS) as the reducing agent to form a red colored complex (quinoneimine) that is measured at 514 nm [198].

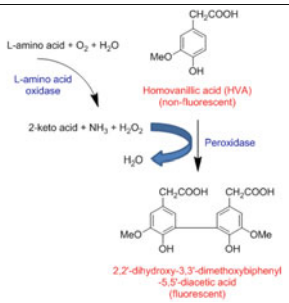
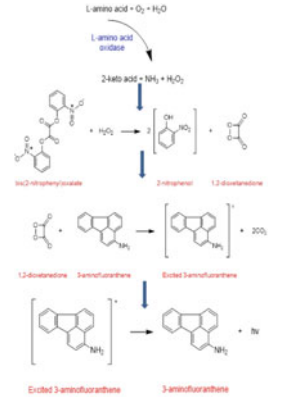
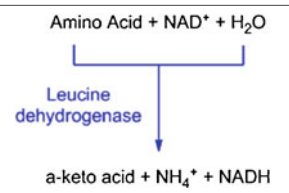
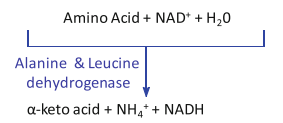
Jansen et al. [50] used the same enzyme as before (L-AA oxidase) immobilized on controlled-pore glass beads. The produced H_2O_2 oxidizes bis(2-nitrophenyl)oxalate (2-NPO) to produce 2-nitrophenol and a cyclic peroxide (1,2-dioxetanedione). The peroxide reacts with a 3-aminofluoranthene immobilized on glass beads in the CL cell to give two molecules of CO_2 and, in the process, an electron in the 3-aminofluoranthene molecule which is promoted to an excited state. When the excited 3-aminofluoranthene molecule returns to its ground state, CL is emitted. This IMER method uses an immobilized fluorophore in the detector cell because CL is a process with short lifetime. Detection limits of 0.35–3.0 μM are obtained for several AAs. The method is stereoselective only for certain L-AAs (see Table 5).

The use of immobilized leucine dehydrogenase (LeuDH) in a post-column reactor for the sensitive detection of branched-chain AAs (BCAAs) such as L-leucine, L-isoleucine, and L-valine was reported [63]. In the IMER, LeuDH catalyzes the deamination of the BCAAs in the presence of nicotinamide adenine dinucleotide (NAD^+) and the NADH produced is detected fluorometrically. BCAAs are separated on a reversed-phase column. A dual-pump configuration PCD system is needed for the introduction of NAD^+ reagent and the buffer before the IMER.

A selective technique was introduced for the determination of aliphatic AAs such as L-alanine, L-valine, L-isoleucine, and L-leucine in serum by HPLC using IMER with co-immobilized enzyme alanine dehydrogenase (AlaDH)/leucine dehydrogenase (LeuDH) and fluorescence detection [65]. The enzymes were simultaneously immobilized on chitosan beads. The separation was achieved by reversed-phase chromatography with phosphate buffer (pH 7.0) as an eluent. In the packed-bed reactor, the two enzymes AlaDH/LeuDH catalyze the deamination of the AAs in the presence of NAD^+ and the NADH produced is detected fluorometrically (Table 5, application 4). The linear range of the HPLC method was over two orders of magnitude and detection limits of 1–2 μM were obtained.

A simultaneous determination of BCAAs and Phe using co-immobilized phenylalanine dehydrogenase (PheDH)/LeuDH on tresylate-containing poly(vinyl alcohol) beads has been proposed [41]. No reagent in the PCD system is

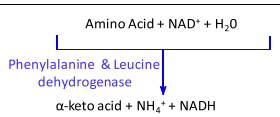
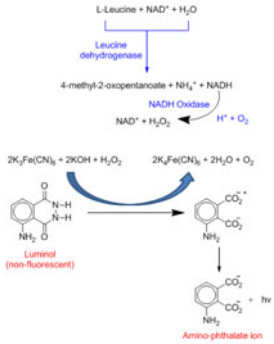
Table 5 Post- column derivatization chemistries and conditions of amino acids analysis using immobilized enzyme reactors (IMER)

No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
1.	Lys, His, Cys, Arg, Met, Leu, Ileu, Tyr, Phe and Trp	L-amino acid oxidase (EC. 1.4.3.2) (AAO) / peroxidase (E.C. 1.11.1.7) (PO) R1: 1 mM Homovanillic acid (HVA), 0.4 M tris(hydroxymethyl)amino-methane (Tris) Flow rate: 0.2mL/min		Packed-bed reactor (250x4.0mm I.D.) with co-immobilized enzymes (AAO)/(PO) on controlled-pore glass beads & 40°C	L-amino acid oxidase catalyses the deamination of L-amino acids and H_2O_2 is produced. The H_2O_2 produced oxidizes HVA with substrate peroxidase enzyme to a fluorescent product. No reaction is observed with Pro, Thr, Asn, Ala, Ser, Val, Gln and D-amino acids	Reverse Phase Chromatography	FL Ex. 315nm Em.425nm	5 μM Phe, Trp and Met 10 μM Tyr	[49, 196, 197]
2.	Lys, His, Cys, Arg, Met, Leu, Ileu, Tyr, Phe and Trp	L-amino acid oxidase (EC. 1.4.3.2) (AAO) R1: 0.4 g/L bis(2-nitrophenyl) oxalate(2-NPO) in acetonitrile Flow rate: 1.5mL/min The fluorophore (3-aminolluoranthene) was immobilized on glass beads in the CL cell (30mmX2.5 mmi.d.)		Packed-bed reactor (250x4.0mm I.D.) with immobilized enzyme (AAO) on controlled-pore glass beads & Ambient Temperature	L-amino acid oxidase catalyses the deamination of L-amino acids and H_2O_2 is produced. The H_2O_2 produced oxidizes 2-NPO to produce 2-nitrophenol and a cyclic peroxide (1,2-dioxetanedione). The peroxide reacts with a 3-aminofluoranthene to give two molecules CO_2 and in the process, an electron in the 3-aminofluoranthene molecule which is promoted to an excited state. When the excited 3-aminofluoranthene molecule returns to its ground state, a photon of light is released. No activity was found for the other L-amino acids and D-amino acids	Reverse Phase Chromatography	CL	0.35-3.0 μM	[50]
3.	Branched-chain Amino Acids (BCAAs) (Leu, Ileu, Val)	Leucine dehydrogenase (E.C. 1.4.1.9) (LeuDH) R1: 0.4 M glycine-KCl/KOH buffer pH=10.8 Flow rate: 0.25 mL/min R2: 35 mM NAD^+ Flow rate: 0.25 mL/min		Packed-bed reactor (10x4.0mm I.D.) with immobilized enzymes leucine dehydrogenase (LeuDH) on epoxy activated and aminated beads & 40°C	Immobilized LeuDH enzyme in a post-column reactor is used for the sensitive detection of BCAA after separation on a reverse phase column. In the packed-bed reactor, the LeuDH catalyses the deamination of the BCAAs in the presence of nicotinamide adenine dinucleotide (NAD +) and the NADH produced is detected fluorometrically. Interference from the metal ions was avoided by addition of EDTA to the flow stream.	Reverse Phase Chromatography	FL Ex. 340 nm Em.465 nm	0.5 μM each	[63]
4.	Aliphatic AA (Ala, Val, Ile, Leu)	Alanine dehydrogenase (E.C. 1.4.1.1) (AlaDH)/ Leucine dehydrogenase (E.C. 1.4.1.9) (LeuDH) R1: 0.3 M Carbonate buffer pH=9.5 Flow rate: 0.2 mL/min R2: 16 mM NAD^+ Flow rate: 0.2 mL/min		Packed-bed reactor (50x4.0mm I.D.) with co-immobilized enzymes alanine dehydrogenase (AlaDH)-leucine dehydrogenase (LeuDH) on chitosan beads (because of their stability in alkaline solution & hydrophilicity) & 40°C	Since AlaDH and LeuDH have their highest activities in the pH range 9-10, glass beads are unsuitable as the support for the enzymes because of their poor chemical stability in alkaline solution. In the packed-bed reactor, the AlaDH/LeuDH catalyses the deamination of the AA in the presence of nicotinamide adenine dinucleotide (NAD^+) and the NADH produced is detected fluorometrically. D-amino acids did not produce any response.	Reverse Phase Chromatography	FL Ex. 340 nm Em.465 nm	2 μM Ala (30pmol in a 30μL injection) 1 μM (Val, Ile, Leu) (30pmol in a 30 μL injection)	[65]

needed because NAD^+ is added to the mobile phase (2.5 mM NAD^+ in 0.1 M carbonate buffer, pH 9.5). The same reaction scheme is applied as the one before. Detection limits of 0.3 μM Ile and 0.8 μM Phe were obtained.

A liquid chromatographic system with co-immobilized LeuDH and NADH oxidase (NAOD) reactor was reported [40] for the determination of branched-chain AAs (BCAAs) such as L-leucine, L-isoleucine and L-valine. The enzymes were simultaneously immobilized on tresylate-containing poly(vinyl alcohol) beads. The separation was achieved by reversed-phase column using phosphate buffer (pH 7.5) as an eluent. In the packed-bed reactor with the co-immobilized enzymes, the LeuDH catalyzes the deamination of the BCAA in the presence of NAD^+ and the resulting NADH is removed

Table 5 (continued)

No.	Amino Acids	Derivatization reagent(s)	Chemistry & Derivative(s)	Reaction Coil(s) & Temperature	Comments	Chromatography	Detector	Detection Limits	References
5.	Branched-chain Amino Acids (BCAAs) (Leu, Ileu, Val) & Phe	Phenylalanine dehydrogenase (E.C. 1.4.1) (PheDH)/ Leucine dehydrogenase (E.C. 1.4.1.9) (LeuDH)		Packed-bed reactor (40x4.0mm I.D.) with co-immobilized enzymes Phenylalanine dehydrogenase (PheDH)-leucine dehydrogenase (LeuDH) on tresylate-containing poly (vinyl alcohol) beads & 35°C	NAD^+ is added to the mobile phase (2.5mM NAD^+ in 0.1M Carbonate buffer pH=9.5). The reactor had activities for Phe, Leu, Ileu, Val, Met & Tyr.	Reverse Phase Chromatograp	FL Ex. 340 nm Em.465 nm	0.3μM Ile 0.8μM Phe	[41]
6.	Branched-chain Amino Acids (BCAAs) (Leu, Ileu, Val)	Leucine dehydrogenase (E.C. 1.4.1.9) (LDH)/ NADH oxidase (NAOD) R1: 5 mM NAD^+ in 0.02 M phosphate buffer (pH=7.0) Flow rate: 0.15mL/min R2: 5 mM luminol in 0.4 M carbonate/0.4M hydrogen carbonate buffer (pH=10.5) Flow rate: 0.15 mL/min R3: 20 mM $K_3Fe(CN)_6$ Flow rate: 0.4mL/min		Packed-bed reactor (50x4.0mm I.D.) with co-immobilized enzymes leucine dehydrogenase (LeuDH)-NADH oxidase (NAOD) on tresylate-containing poly (vinyl alcohol) beads & 50°C Reaction Coil 180μL & Ambient temperature	The eluent from the column was combined with the NAD^+ solution and the luminol solution at a four-way valve prior to elution through the reactor. Co-immobilized LeuDH-NADH oxidase (NAOD) enzymes in a post-column reactor are used for the sensitive detection of BCAA in plasma after separation on a reverse phase column. In the packed-bed reactor, the LeuDH catalyses the deamination of the BCAA in the presence of nicotinamide adenine dinucleotide (NAD +) and the resulting NADH is removed by the NADH oxidase with formation of H_2O_2. The H_2O_2 produced in the reactor was detected by measuring the chemiluninescence emitted when mixed with luminol and $K_3Fe(CN)_6$	Reverse Phase Chromatography	CL (100 μL flow cell)	0.1 μM each	[40]

by the NADH oxidase with formation of H_2O_2. The H_2O_2 produced was detected via a luminol-hexacyanoferrate(III) reaction producing CL (Table 5, application 6). A three-pump PCD configuration was used for the three reagents. The NAD^+ reagent is mixed with the eluent before the IMER and the luminol reagent is added next in series with the $K_3Fe(CN)_6$ reagent solution. The system shows a linear range from 0.3 to 300 μM for each AA and the detection limit was 0.1 μM.

The other type of solid-phase reactors is considered as stoichiometric (the support material participates in the reaction with the analyte). Solid-phase reagents can be prepared on different types of supports, such as glass beads, silica, Sepharose, alumina, ion-exchange resins, and organic polymers [199–203]. An early work in the field of solid-phase reactors involves detection of thiols and disulfides in which the analytes release a chromophore from the packing material in a column beyond the analytical column, and the absorbance of the chromophore is measured [199, 200]. In the first technique [199] a Sepharose-bound thiol reduces disulfides to thiols, which can be detected by reacting with a solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB-Ellman's reagent) previously reported [102], a reagent which generates a dianion (NTB^{2-}) absorbing at 412 nm. Disulfides must be reduced to thiols in the SPR prior to the detection reaction. The reduction step creates a problem because another reagent must be added in order to complex the released reducing agent to prevent the production of the dianion itself. In the second technique [200] the homogeneous solution of the post-column reagent DTNB is replaced with an SPR containing a chromophoric reagent which would be released into the stream after the reaction of a thiol in the SPR. A strong chromophore, *N*-dinitrophenylcysteine (DNP-cysteine), was covalently bound on a Sepharose column. The thiols which are eluted off the analytical column undergo a disulfide interchange reaction with the Sepharose-bound disulfide and the DNP-cysteine or its mixed disulfide with the thiol exits the SPR column and can be detected at 412 nm. The SPR post-column system response is linear from 0.15 to 2.5 μg for cystine [199] and from 0.15 to 1.25 μg for cysteine [200]. If a fluorescent reagent such as di-Dns-cystine is covalently bound to the SPR column instead of di-DNP-cystine, a fluorescence detector can be used in pIace of the UV–Vis detector [200]. In a subsequent application 2,2'-dipyridyl disulfide (2PDS) which is covalently bound either to silica [201] or an organic polymer [202] interacts with a thiol and the released 2-thiopyridone (2-TP) is detected at 345 nm.

Idowu and Adewuyi [203] reported the use of a non-fluorescing reagent, sodium benzoxazole-2-sulfonate, as a derivatization reagent as both a solution and solid-phase reagent for amines and AAs. The sulfonate reagent was ionically attached to an anion-exchange support. Sodium benzoxazole-2-sulfonate is water-soluble and its derivatives

with AAs exhibit a blue fluorescence of the 2-(*N*,*N*-dialkylamino)benzoxazoles.

A PCD of AAs uses SPR filled with copper(II) oxide powder [204]. AAs are mainly non-absorbing in the UV region and can be detected only at very low wavelengths (190–210 nm) with rather low absorptivities. However, their Cu(II) complexes show significant absorbance in the UV region (220–240 nm) owing to intensive charge-transfer bands and also weaker absorbance in visible region due to *d*–*d* transitions. As AAs are eluted off the column and have a high affinity for complex Cu(II) ions, they have the ability to extract copper atoms from insoluble compounds. The interaction of AAs with CuO leads to the formation of corresponding complexes which results in increase of UV–VIS absorbance. Although the strong coordination ability of AAs is at high pHs (>7.0), a lower pH of 5.5 provides better sensitivity owing to different distributions of the complex species. The yield of the derivatization is dictated mainly by the flow rate. Detection limits are in the nanomole and picomole range. The solid-phase derivatization method is simple, efficient, and economical for detecting a wide variety of non-absorbing AAs by UV–VIS.

The solid-phase reagents do not require an organic reaction solvent, or pumping and mixing units. They have hydrophobic extraction properties with increased analyte derivatization selectivity. Simple, faster, milder, and more efficient reactions take place with less contamination and/or background due to excess derivatization reagent. They have improved chemical stability over time. Higher reaction capacities are observed because of the high concentration of the immobilized reagent. The solid-phase reagent is usually consumed in the reaction and must therefore be replenished when depleted. There is the possibility to use mixed-bed reagents with different derivatization tags for the labeling and confirmation of various analytes.

Post-column addition in LC/MS

Liquid chromatography–mass spectrometry (LC/MS) using atmospheric pressure ionization (API) is completely different from the hitherto available analytical methods used to separate and detect AAs. Several LC/MS/MS methods [205–207] have been reported using reversed-phase chromatography of derivatized AAs and compared well with the cation-exchange/ninhydrin procedure that has been the state of the art for decades. Free AAs in biological fluids have been also demonstrated in several reports [36, 37, 58–62, 208, 209] to be separable and detectable using LC/ESI-MS with perfluorinated acids as ion-pairing agents. ESI is considered to be useful for AAs because they form ionic species in solution. However, one major obstacle is the weak ionization of some AAs during ESI techniques under certain pH conditions and in the presence of a strong ion-pair agent. The chemical and physical properties of an AA are perhaps the most critical parameters for obtaining enhanced sensitivity in various ionization modes. Ionization state and surface activity that are directly related to the properties of the AAs will determine the ionization efficiency which is expected to improve detection sensitivity. Therefore a post-column infusion/addition should be performed in order to induce chemical derivatization or change ionization state and surface activity in a target functional group before analysis by MS for enhancement of ionization.

The major advantage of post-column infusion/addition is that ionization conditions for the AAs could be optimized without changing the chromatographic separation. The application of post-column infusion/addition a reagent has involved (1) pH adjustment [59]; (2) decrease of ion-suppression (e.g., the “TFA fix”) [210]; and (3) improvement of nebulization and desolvation or the ionization efficiency through the formation of an adduct ion.

The MS response of the AAs studied (Phe, Ile, Gln, and Pro) under optimum chromatographic conditions (ACN–water, 75:25) was rather low as the overall detection limits were about 1 mg/mL [59]. In order to increase the MS response of the AAs, an aqueous liquid that contained 500 mM formic acid was added post-column after the splitting of effluent (split ratio 1:20). Formic acid was preferred to trifluoroacetic acid (TFA) or its higher homologues because of signal suppression caused by strong ion-pair agents. These results confirm previous observations by the same researchers [211] that an acid mobile phase improves MS response for AAs because all AAs are protonated under these conditions. In addition to these results, Hiraoka et al. [212] has shown that the AAs can be detected with higher sensitivities in acid solutions in the positive mode than in the negative mode.

Signal suppression with strong acid-containing mobile phases such as TFA or perfluorinated carboxylic acids has led to decreased sensitivity in the MS detection of underivatized AAs. Since a strong ion-pair formed between AAs and the perfluorinated carboxylic acids, the poor dissociation of the ion-pair may decrease the formation of protonated sample cations and may reduce their ionization efficiencies. Post-column addition of a weak acid could be used to solve this problem, which has been referred to as the TFA fix. Post-column addition of a mixture of propionic acid–2-propanol (75:25, v/v) overcame the ion suppression of TFA and heptafluorobutyric acid (HFBA) [196], resulting in a 10–100-fold improvement in detection. Petritis and colleagues [209] compared HFBA, nonafluoropentanoic acid (NFPA), tridecafluoroheptanoic acid (TDFHA), and pentadecafluorooctanoic acid (PDFOA) and found that 10 mM perfluorocarboxylic acids with ESI-MS created an ionization suppression of 1.5–2-fold in underivatized AAs. Apffel et al. [210] proposed the post-column addition of a highly concentrated weak acid (RCOOH) which would compete with TFA (strong acid) or perfluorinated carboxylic acids to pair

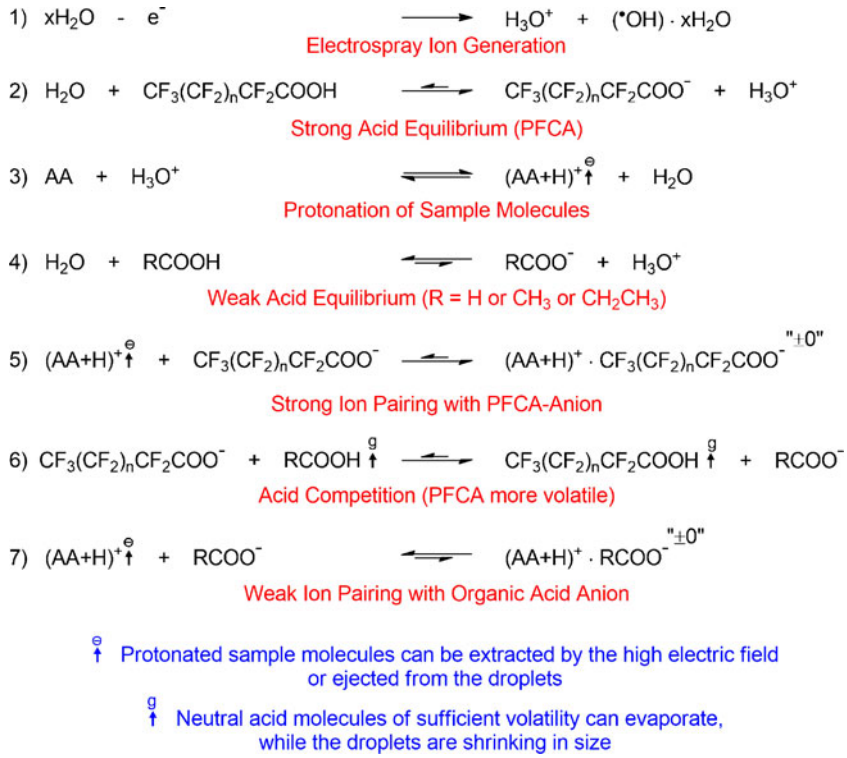


Fig. 4 Proposed mechanism for perfluorocarboxylic acid signal suppression [210]

with the analyte. The mechanism of signal suppression is illustrated in Fig. 4.

If perfluorocarboxylic acid is used in the mobile phase as the ion-interaction agent, the dissociation of a strong acid takes place in equilibrium 2 (Fig. 4). Under normal electrospray conditions the protonation of AAs takes place during equilibrium 3 in which protonated AA molecules are released into the gas phase (ion evaporation process). If a weak acid additive, such as formic, acetic, or propionic acid is mixed with the effluent via post-column addition, the weak acid equilibrium 4 occurs. Ion-pair association between the perfluorinated carboxylate anion and the protonated AA molecule occurs in equilibrium 5. This process is primarily responsible for signal suppression. In the presence of high concentrations of a weak acid (RCOOH), the competition between perfluorocarboxylate anion and the weak acid would be driven towards the deprotonation of the weak acid (equilibrium 6). The resulting perfluorocarboxylic acid could be evaporated from the droplet and during equilibrium 7 the protonated AA cation would form an ion pair with the new anion of the weak acid ($RCOO^-$). The weak ion pairing between the AA and the weak acid favors the protonated analyte which can be ion evaporated and ejected from the droplet and subsequently extracted by the electric field for mass analysis. The proposed ion-pair mechanism (Fig. 4) is also supported by the observation that weakly basic analytes show an increase in signal with TFA relative to acetic acid-containing mobile phase. In such cases protonation of the analyte is enhanced by the strong acid which does not form ion pairs [210].

Optimization in post-column labeling techniques

Chromatographic separation of AAs and the post-column labeling technique are two separate and distinct events. Optimization of each event more often is investigated independently. However chemical compatibility is needed in order to have a successful coupling. Pickering [213] described in detail the chemical requirements needed for a successful marriage between IEC of AAs and post-column labeling techniques. Each post-column reaction system is guided accordingly by a set of considerations described below in order to optimize AA reactivity and enhance signal strength without compromising chromatographic efficiency or reproducibility.

Reaction rate–derivative stability

Only a few post-column reactions of interest are instantaneously completed; the reaction time needed to obtain an appreciable conversion may range from several seconds to minutes. Completeness of the reaction is not an important issue in PCD application. It may be further needed to heat the reaction in order to accelerate the desired reaction. The kinetics of a post-column labeling reaction plays an important role. In the three classes: (I) open tubular reactor, (2) packed-bed reactor, (3) air or immiscible

liquid segmented reactor, reaction times increase in the order cited. The choice of a specific reactor is not only dependent on the desired reaction time but also on the maximum dispersion that could be afforded. The theory of band broadening of various designs of post-column reaction systems and their application in post-column detection of amines and AAs with Fluram (fluorescamine), NBD-Cl, and OPA were described by Frei [214]. As a rule, one could say that for very short reaction times of a few seconds a non-segmented tubular reactor system may be used. For reaction periods above this the segmentation principle could be recommended [215]. Post-column dansylation with slow kinetics was made possible with a dynamic two-phase reaction system based on solvent segmentation [215]. Therefore, in order to minimize band spreading, it is important to construct PCD systems having minimal reaction volume for an effective detection. The requirement of fast kinetics is not enough for a successful labeling reaction. The reaction product(s) between the AAs and reagent need(s) also to be stable through the PCD system till it reaches the detector cell.

Reagent stability

Reagent must be stable for at least 2 days. A reproducible signal and a stable baseline are the two key requirements. In case of reagent oxidation upon standing in the reservoir, purging is recommended with an inert gas to maintain an inert blanket atmosphere above the reagent. Owing to the instability of hydrindantin in the ninhydrin reagent, purging with inert gas is always required [14].

Reproducibility

The derivatization reaction must be reproducible and produce the same peak area for any given AA species over the time. For maximum reproducibility constant and precise flow of reagents is important. Using hollow fiber ion-exchange membranes for introduction of NaClO and OPA reagents without any pneumatic or mechanical pumping and minimum volumetric dilution leads to sensitive and reproducible assays of AAs [120, 121]. Reaction coil thermostating is always required for good reproducibility. Completeness of the reaction is not needed but reproducibility is. Maximum sensitivity is achieved when the reaction is complete.

Background (noise)

The reagent's detector response (background) must be minimal. Its background absorbance or fluorescence must be low. Because conventional LC detectors monitor the difference of the analyte signal from background; low reagent background noise improves the S/N and the limits of detection (LOD). Therefore, the usage of non-absorbing or non-fluorescent reagents is a requirement for high sensitivity analysis. Most reciprocating pumps produce pulses which contribute significantly to the baseline noise. A pulse dampener connected on-line with the reagent(s) completely suppresses short-term pump pulses and they have little effect on pump variations which produce long-term noise. Syringe pumps with pulseless flow are ideal and generate very low noise baselines. Gas pressurized reagent pumping provides noise characteristics superior to syringe pumps [94]. If helium is used as the pressurizing gas, there is no bubble formation in the detector cell if a suitable amount of backpressure is applied at the detector exit. The electronic reduction of noise is accomplished by choosing either the level of time constant or rise time suppressing fast or short-term noise. Application of software techniques in real time or post-run can suppress noise. In real time this can be achieved by digital sampling of the noise followed by baseline averaging for smoothing. Baseline noise suppression in post-run can be done by using special algorithms (e.g., moving average, Savitsky Golay) involving averaging or filtering protocols. A novel dual-wavelength procedure is described by Jones [216] for the elimination of baseline pump noise in LC-post-column reaction derivatization systems with photometric detection. The method is applicable when there is some overlapping between the spectrum of the analyte derivative and the spectrum of the excess reagent. One wavelength is chosen to monitor the analyte derivative and the other wavelength is set close to the isosbestic point. After mathematical manipulation of the data using commonly available computer software, the pump noise can be subtracted from the analyte baseline, achieving higher S/N ratios. The same can be applied when fluorescence detection is used with the capability of monitoring at least two emission wavelengths.

Solubility

All compounds (reactants and products) must remain in solution. Precipitates may clog up PCD tubing and have the strong potential to burst reaction coils and detector cells.

Compatibility

Successful coupling of a PCD post-column labeling reaction with an HPLC system is dictated by the PCD reaction phase which must be compatible with the HPLC mobile phase. Mixing of an aqueous mobile phase having buffers with a post-column reagent dissolved in an organic solvent always present a problem. Improper mixing will cause refractive index discontinuities resulting in increased UV–Vis detector noise. The formation of precipitate due to the incompatibility of the two phases may result in blocking of tubing and reaction coil or the detector cell bursting.

Optimization of the PCD conditions involves the study of several PCD variables which all will lead to the highest sensitivity, accuracy, and reproducibility of the PCD system. Various post-column labeling techniques in this review [49, 106, 120, 163] examined in detail all the variables necessary

to achieve the optimum conditions for the labeling reactions with AAs. The chemical variables of the post-column labeling reaction are the pH of the reagent(s) mixture, the concentration of the reagent(s), the type of buffer and its concentration, and the type and percentage of reagent solvent; and the instrumental parameters that may affect the reaction between the AA and the reagent are the temperature of the reaction coil(s), the reaction coil volume, the mixing tee volume, and the flow rate of the reagent(s). Both types of variables will affect the optimal conditions of a post-column labeling reaction between AAs and reagent. In addition the various surfactants added to the post-column labeling reagent mixture may have several effects such as (1) solubilization of organic reagent; (2) micelle formation which can enhance the analytical response of the photometric/fluorescent labeling reaction; (3) prevention of quenching; and (4) modification of the internal surface of the tubing and other parts of the reactor to minimize the possible interaction of AA derivatives with possible peak tailing. The presence of cationic micelles of HTAB in the reaction mixture enhanced the sensitivity of the detection by nearly twofold [102]. The addition of Brij-35 [117] or SDS [132] to the labeling agent improves the stability and response of AAs derivatives and prevents their fluorescence quenching. The careful study of all these variables together with a compatibility evaluation of an eluent and post-column reagent conditions will lead to full optimization of an LC-post-column labeling of AA analysis.

Conclusion

Among the various post-column labeling techniques, those that enable photometric and fluorescent detection are the most widespread because they cover the detection of a large number of AAs. The separation techniques most successfully coupled with the photometric and fluorescent detection are either ion-exchange or ion-interaction chromatography. The CL post-column labeling techniques provide very sensitive detection of a limited number of AAs. The mobile phase is very crucial for the HPLC-CL system and, therefore, more compatibility studies are needed because what is suitable for the separation of analytes is not always compatible with the CL reaction. The immobilized enzyme reaction techniques are very specific and owing to their specific enzymatic activity they are limited to the analysis of certain AAs. The IMER reactors are usually combined with reversed-phase chromatography which is not capable of separating large numbers of AAs with ion exchange or IIC. The application of NP reagents as novel PCD reagents provides more stable, less noisy baselines and a chromatography free of interferences because photometric detection is accomplished at longer wavelengths.

Post-column labeling techniques have several advantages over pre-column techniques. First, the post-column labeling can be automated, and thus the influences of performance variation of the assay are usually less; secondly, less sample pretreatment before HPLC separation is required in PCD, avoiding the changes in the conditions of the chromatographic separation of the analytes when pre-column derivatization is applied. However, the successful post-column reaction system requires fast kinetics of the labeling reaction and high selectivity of the post-column reagents towards the analytes. As a result, the post-column reagents reported in the literature for the determination of AAs are less numerous than the pre-column ones. Therefore, there is great interest in exploring new post-column reagents for HPLC assay of AAs. Another aspect is the need to study the optimum conditions (compatibility variables) when coupling various types of chromatographic separations with PCD techniques. Several potential reactions have not been applied in post-column analysis because of compatibility problems and further investigation is required. Finally the detailed study of kinetics and mechanism of chemical reactions in post-column labeling techniques in AA analysis by LC will lead to the development of the optimum conditions of analysis.

Future research should be focused on new reagents providing higher selectivity and sensitivity in the detection of AAs, development of new SPRs with fast kinetics, new PCD instrumentation with cooling and reagent mixing capabilities, the improvement of the separation of the underivatized AAs using IEC (fast separations), and finally on the applications of PCD with various ionization MS detector techniques.

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