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A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids

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Abstract

This study compares the main commercial detectors that can detect amino acids in their underivatized form. The detectors tested are: the chemiluminescent nitrogen detector (CLND), the evaporative light scattering detector (ELSD), the nuclear magnetic resonance spectrometer, conductivity detector, refractive index, UV, and electrospray quadrupole mass spectrometry (in simple and tandem MS mode). As ELSD, CLND and MS require a volatile mobile phase, an ion-pair reversed-phase liquid chromatographic system was selected, consisting of an octadecyl column and an aqueous mobile phase containing pentadecafluorooctanoic acid as volatile ion-pairing reagent. Underivatized taurine, hypotaurine, aspartic acid, hydroxyproline, asparagine, serine, glycine, glutamine, cysteine, glutamic acid, threonine and alanine were simultaneously analysed with each detector. In order to test the applicability of these detectors to ''real world'' samples, the amino acid stoichiometry of the tetrapeptide Gly–Gly–Asp–Ala was determined with each detector after acid hydrolysis. The detectors were compared in terms of linearity, limit of detection, advantages and disadvantages as well as special features (capacity to provide structural information, specificity, quantification with single calibration curve, etc.).

Keywords: Detection, LC; Evaporative light scattering detection; Nuclear magnetic resonance; Mass spectrometry; Chemiluminescent nitrogen detection; Refractive index detection; UV detection; Conductivity detection; Ion-pairing reagents; Amino acids

1. Introduction

Amino acids are arguably the most important compounds from a biological point of view. As a result, numerous publications deal with their analysis, and several manufacturers have developed specialized analytical instruments for their analysis, the so-called ''amino acid analysers''. The high polarity of amino acids, their low volatility and their lack of a strong chromophore group makes their separation and detection difficult. After 50 years of research, no analytical method has been developed which is superior to all the others and free of drawbacks. As a result, any new analytical instrumentation concept invented is almost always applied first to amino acid analysis. It is now widely accepted that the most difficult part of amino acid analysis is not amino acid separation but amino acid detection.

In order to solve this problem, derivatization

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approaches were developed with the purpose of increasing analyte volatility (for GC–flame ionization detection/MS analysis) or creating amino acid derivatives with strong chromophore/fluorophore groups (for LC or CE–UV/fluorescence analysis). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [1,2], 9-fluorenylmethylchloroformate (FMOC) [3], *o*-phthalaldehyde (OPA) [4] and phenylisothiocyanate (PITC) [5] are the most widely used amino acid derivatization reagents. However, all of the existing derivatization methods present one or more of the following drawbacks: derivative instability, reagent interferences, long preparation time, inability to derivatize the secondary amino groups, increased void volume for the post-column derivatization methods, laborious derivatization hindering automation, long chromatographic separation of certain amino acid derivatives, and problems with derivatization towards specific amino acids [6–12].

An alternative to derivatization methods is indirect amino acid detection. Ion-pair reversed-phase liquid chromatography (RP-IPC) with indirect UV [13–19] or fluorescence detection [19] has been used by employing tryptophan, salicylate, nicotinamine or α-napthylamine as the optically detectable component. Furthermore, sodium salicylate, *p*-aminosalicylic acid or 4-(*N*,*N*′-dimethylamino)benzoic acid have been used as the optically detectable component in capillary electrophoresis/electrophoretic microchips with indirect UV [20–23] or fluorescence detection [24–26]. The indirect mode of detection for amino acid analysis has been used with other than UV/fluorescence detectors, such as the amperometric [27], thermooptical [28], chemiluminescence [29] and conductivity detectors [30,31].

Amino acids can be further detected by UV after complexation with transition metal ions (e.g. complexation with Cu(II) and detection at 250 nm) [32–35]. A method which uses the principle of the above methods (complexation and indirect fluorescence detection) has been recently described by Yang and Tomellini [36]. This detection is based on a displacement reaction between the eluted amino acids and a copper(II)–L-tryptophan complex Cu(L-Trp)$_2$. In the complex Cu(L-Trp)$_2$, the fluorescence of L-Trp is ~95% quenched; with the addition of analytes with strong affinity for Cu(II) such as natural amino acids, L-Trp is released from the complex and fluorescence signal intensity is recovered [36].

Bobbitt and co-workers [37–41] detected underivatized amino acids by chemiluminescence (CL) based on the chemiluminescent reaction between in situ generated $Ru(bpy)_3^{3+}$ and the amino acids. Moreover, our group [42] described direct amino acid analysis by the chemiluminescent nitrogen detector (CLND). The weak absorbance of amino acids at low UV wavelengths has been used for direct UV detection (185–214 nm) when high sensitivity was not necessary [43–48].

The detector that has been investigated the most for underivatized amino acid analysis is the amperometric/electrochemical detector [49–84]. The number of amino acids that can be detected by classical carbon electrodes is limited [57–60]. As an alternative, several transition metal-based electrodes such as copper [61–71] and nickel [72–74], as well as noble metal-based electrodes such as gold [75–77] and platinum [78], have been proposed as amperometric sensors for the direct determination of amino acids. Chemical modifications of some of the above electrodes have been investigated in order to increase the number of amino acids analysed and/or increase the resistance of the composite electrode to fouling [79–84]. Recently Brazill et al. [85] demonstrated direct amino acid detection utilising sinusoidal voltametry at a copper electrode. The authors reported that this method is sensitive, selective (allowing three-dimensional data for all analytes detected) with reduced problems of electrode fouling [85]. Direct detection of amino acids has been further achieved by the refractive index detector (RID) [86] and the evaporative light scattering detector (ELSD) [87–96]. The detection of these compounds is straightforward thanks to the universal character of these detectors. Nuclear magnetic resonance (NMR) has also been used for the direct detection of amino acids with [97,98] or without [99] previous chromatographic separation.

Finally the ionisable, thermolabile, and polar character of underivatized amino acids makes them good candidates for analysis by electrospray ionization mass spectrometry. Indeed, recently we introduced the analysis of 20 underivatized proteinogenic amino acids by ion-pairing RP-IPC–electrospray

ionization mass spectrometry (ESI-MS) [96]. This was followed by Soga and Heiger [100] and Martin-Girardeau et al. [101] who achieved the CE–ESI-MS of the above compounds. More recently, we demonstrated the LC–ESI-MS–MS analysis of 20 native proteinogenic amino acids [102]. The analysis of amino acids other than proteinogenic amino acids is also possible [103–105]; indeed, the simultaneous analysis of 77 underivatized amino acids has been recently achieved by RP-IPC–ESI-MS–MS [104,105]. Finally, LC–ESI-MS–MS has been used for the direct analysis of small peptides [106], as well as chiral amino acids [107].

In this study seven commercial liquid chromatographic detectors which can detect amino acids in their underivatized form are tested in relation to sensitivity, linearity, quantitation, etc. The detectors tested are: the ELSD, CLND, refractive index detector (RID), conductivity detector (CD), UV, NMR and triple quadrupole ESI-MS(–MS). Other liquid chromatographic detectors that may potentially allow direct amino acid detection such as the density detector [108] and the oscillometric detector [109], have not been tested as they are not commercially available and/or widespread. The amperometric detector commercialised by Dionex has not been tested as it is not available in our laboratory and a free loan was not possible.

2. Experimental

2.1. Reagents

HPLC-grade methanol (MeOH), acetonitrile (CH_3CN) and tetrahydrofuran (THF) were obtained from J.T. Baker (Noisy le Sec, France). Taurine (Tau), hypotaurine (Hpt), aspartic acid (Asp), hydroxyproline (Hyp), asparagine (Asn), serine (Ser), glycine (Gly), glutamine (Gln), cysteine (Cys), glutamic acid (Glu), threonine (Thr), and alanine (Ala) were purchased from Sigma or Aldrich (St. Quentin Fallavier, France). Pentadecafluorooctanoic acid (PDFOA) was purchased from Aldrich. The tetrapeptide Gly–Gly–Asp–Ala was purchased from Bachem (Torrence, CA, USA). Deuterium oxide (99.9 atom%) was obtained from Aldrich and used as the mobile phase eluent and for the preparation of amino acid solutions for NMR experiments, and 18-MΩ deionized water from an Elgastat UHQ II system (Elga, Antony, France) was used as HPLC-grade water and for the preparation of amino acid solutions.

2.2. Apparatus

The HPLC equipment consisted of a Merck–Hitachi LaChrom system equipped with an L7100 quaternary pump, a L7200 automatic injector, and a D7000 interface (Merck, Darmstadt, Germany). Instrumental control, data acquisition and data processing were provided by HSM software. The ELSD was a Sedere (Vitry-sur-Seine, France) model Sedex 55 set as follows: drift tube: 60 °C, nebulizer gas pressure: 2.3 bar, gain: 9. The RID was a Thermo Separation Products (Les Ulis, France) model RI-150. The conductivity detector was a Metrohm (Herisau, Switzerland) model 732 IC operating in the negative polarity mode. Conductivity meter cell temperature was 35 °C. The CLND was an Antek (Alytech, Juvisy sur Orge, France) model 8060 set as follows: O_2: 202 ml min^{-1}, He: 99 ml min^{-1}, make up: 50 ml min^{-1}, photomultiplier tube (PMT) voltage: −750 V.

The LC–NMR chromatographic system consisted of a Bruker model LC22 pump (Bruker, Karlsruhe, Germany), a Rheodyne injection valve (Cotati, CA, USA) with a 20-μl sample loop and a Bischoff model Lambda 1010 UV–Vis HPLC detector (Bischoff, Germany) operating at 210 nm. The LC system was controlled by Bruker Hystar software. The ELSD was used for amino acid detection in the same way as described previously [98]. ^{1}H-NMR spectrometry was performed on a Bruker Avance 400-MHz NMR spectrometer (Bruker, Wissembourg, France), operated by XWinNMR software on a Silicon Graphics Indy Workstation under the IRIX operating system. Spectra were obtained in the stopped-flow mode at 27 °C, using a 120-μl flow cell LC–NMR probe. A total of 128 free induction decays (FIDs) were recorded for each chromatographic peak, using an acquisition time of 2.01 s and 16 000 points. No presaturation of the residual water

signal was applied to avoid distortion of product signals integration.

LC–ESI-MS(–MS) was carried out using a Perkin-Elmer (Toronto, Canada) model LC-200 binary pump and a Perkin-Elmer Sciex (Forster City, CA, USA) API 300 mass spectrometer triple quadrupole with IonSpray as ion source. The mass spectrometer was operated in positive ion mode. Nitrogen was used as curtain and collision gas. State file was as follows; NEB=9, CUR=7, CAD=1, IS=5000, OR=20, RNG=200, Q0=−5, IQ1=−6, ST=−10, RO1=−6, IQ2=−15, RO2=−20, IQ3=−35, RO3=−25, DF=−400, CEM=2100. Quad 1: 30 (0.010), 100 (0.050), 1000 (0.400), 2000 (0.742). Quad 3: 10 (0.008), 100 (0.035), 1000 (0.285), 2000 (0.530). The NEB=9 (nebulizer gas) corresponds to a flow rate of 1.08 l min^{-1} and the CUR=7 (curtain gas) corresponds to a flow rate of 1.02 l min^{-1}. The selected ion monitoring (SIM) and the selective reaction monitoring (SRM) modes were used to monitor amino acids in simple and tandem MS, respectively. The dwell time was set at 100 ms and the pause time was 5.0 ms. Injections were done by a Perkin-Elmer series 200 autosampler fitted with a 10-μl loop. The protonated molecule $[M+H]^+$ was used as the selected ion in simple MS and as the parent ion in tandem MS. Amino acid chosen ion transitions for MS–MS analysis were as follows: Tau (126→108), Hpt (110→92), Asp (134→88), Hyp (132→86), Asn (133→87), Ser (106→60), Gly (76→30), Gln (147→130), Cys (122→76), Glu (148→130), Thr (120→74), and Ala (90→44).

HPLC was carried out isocratically under ambient temperature at a flow rate of 1 ml min^{-1} using a 125×4 mm stainless steel column, packed with 5 μm Purospher RP-18e (Merck, Darmstadt, Germany). For LC–CLND experiments, a 125×3 mm column of the same brand was used with a flow rate of 0.6 ml min^{-1}. A split of 1/30 and 1/4 was used to avoid too high a flow rate in the ion source and the CLND detector, respectively. Mobile phase was 0.5 m*M* pentadecafluorooctanoic acid in water. The concentration of amino acid solution was as follows: for LC–NMR 2000 mg l^{-1} for each amino acid (injection loop: 20 μl); for all the other detectors: 50 mg l^{-1} for Tau and Hpt, 100 mg l^{-1} for all the other amino acids (injection loop: 10 μl). Before each re-equilibration of the column with PDFOA, a regeneration procedure was applied as follows: methanol (30 ml), acetonitrile (30 ml), tetrahydrofuran (30 ml) and then methanol (30 ml).

2.3. Sample preparation

A slightly modified standard conventional acidic hydrolysis procedure [110] was used to hydrolyse Gly–Gly–Asp–Ala into its free amino acids. A 1.3-mg sample of the tetrapeptide was digested for 24 h in 2 ml of 6 *M* HCl containing 0.5% phenol, heated at 110 °C. The sample was then lyophilised and reconstituted with 2 ml of mobile phase (0.5 m*M* PDFOA in water). The same hydrolysate was used for all detectors.

3. Results and discussion

Comparison of different analytical instruments or chromatographic supports [111] is very useful as it helps scientists to choose among them, in relation to their analytical requirements. Concerning analytical instruments, McCrossen et al. [112] compared the ability of different chromatographic detectors to determine organic impurities in a drug substance. In the case of underivatized amino acid analysis, Welch et al. [30,75] have compared performances between pulsed and potential-sweep pulsed coulometric detection [75] as well as between pulsed amperometric and indirect conductivity detection [30]. Anion-exchange chromatography was used for amino acid separation. A few years later, Simonson and Pietrzyk [19] compared indirect photometric, fluorometric and electrochemical detection for amino acid analysis after ion-pair chromatographic separation with tetraalkylammonium and salicylate as the detection active counter-ion.

3.1. Choice of the chromatographic conditions

In order to compare all the detectors tested in this study, the same chromatographic conditions should be used for amino acid separations. This is particularly difficult due to the different chromatograph-

ic requirements of the tested detectors. The ELSD, CLND and MS need a volatile mobile phase, the CLND requires the total absence of any nitrogen-containing solvents or additives (CH_3CN, NH_3, alkylamines, etc.), the UV needs solvents and additives which are transparent at low UV wavelengths, the CD needs mobile phases with low conductivity for maximum solute sensitivity, the ^{1}H-NMR needs the absence of H in the mobile phase and the RID is compatible only with isocratic separations. Finally, the mobile phase should be as simple as possible (with a minimum number of solvents/additives) in order to avoid the system peaks observed with some detectors [113–115]. Among all the separation techniques proposed up to now for underivatized amino acid separations, the only separation system which fulfils the above requirements is that recently proposed by our group [93]. This chromatographic system consists of an aqueous mobile phase containing a long *n*-alkyl chain perfluorinated carboxylic acid as volatile ion-pairing reagent. Indeed, a Purospher RP-18e octadecyl column and a mobile phase consisting of 0.5 m*M* PDFOA in water gave base line separations for 10 of the more polar proteinogenic amino acids: Asp, Asn, Ser, Gly, Gln, Cys, Glu, Thr, Ala and Pro [93]. In order to decrease the analysis time the last eluted amino acid proline was replaced by the early eluted Tau, Hpt and Hyp. PDFOA was a suitable ion-pairing reagent for NMR experiments due to the absence of proton in its structure and because only a low concentration (0.5 mM) of this reagent is required in mobile phase to obtain satisfactory LC separations.

It must be pointed out here that compatible conditions for all the detectors tested do not necessarily mean optimum conditions. For example the ELSD and MS, whose detection limits depend on the spray stability [116,117], are the most penalised by a 100% aqueous phase.

3.2. Underivatized amino acid detection by ELSD, UV, CD, RID, CLND, MS(–MS), and NMR

3.2.1. ELSD

This type of detector can detect all solutes that are less volatile than the mobile phase. Amino acids are non-volatile compounds and are therefore suitable analytes for this type of detection. The SFC–ELSD analysis of these compounds was introduced by our laboratory in 1992 [87]. Fig. 1a depicts the simultaneous chromatographic analysis of the 12 native amino acid standard mixture by ELSD. Fig. 2a shows the chromatographic analysis of the tetrapeptide hydrolysate using ELSD. Limits of detection varied from 1 mg l^{-1} for Tau to 10 mg l^{-1} for Cys. The low ELSD response for Cys has been already observed by Peterson et al. [92].

3.2.2. UV

Most amino acids absorb at low UV wavelength thanks to their carboxylic function. Signal-to-noise ratios (S/N) of the amino acids analysed were measured at 190, 195, 200, 210 and 214 nm. The 210-nm wavelength provided the best S/N values for the mobile phase used in this study. Taurine and hypotaurine do not absorb at 210 nm as their acid function is not a carboxylic one. As shown by Fig. 1b, all the amino acids are detected except for Tau and Hpt. An unknown peak was eluted just after Ala in the peptide hydrolysate sample (Fig. 2b). Limits of detection varied from 0.9 mg l^{-1} for Asn to 4.5 mg l^{-1} for Hyp.

3.2.3. CD

Ion-pair chromatography coupled with non-suppressed conductivity detection for the analysis of underivatized amino acids has been recently investigated by our group [31]. In spite of the low concentration of perfluorinated carboxylic acids used in this study, amino acids were detected in the indirect conductivity mode due to the high difference in limiting equivalent ionic conductance between the hydrogen cation of the mobile phase and the ammonium cation of the amino acids. As can be seen from Fig. 1c, amino acids give positive or negative response deviations. Briefly, amino acid response deviations relative to the baseline depended on their apparent charge; with PDFOA as ion pairing reagent, negatively charged amino acids give (in positive conductance mode) positive peaks, while positively charged amino acids give negative peaks. Limits of detection varied from 1 mg l^{-1} for Asp to 25 mg l^{-1} for Glu. The low sensitivity of Glu is justified as the

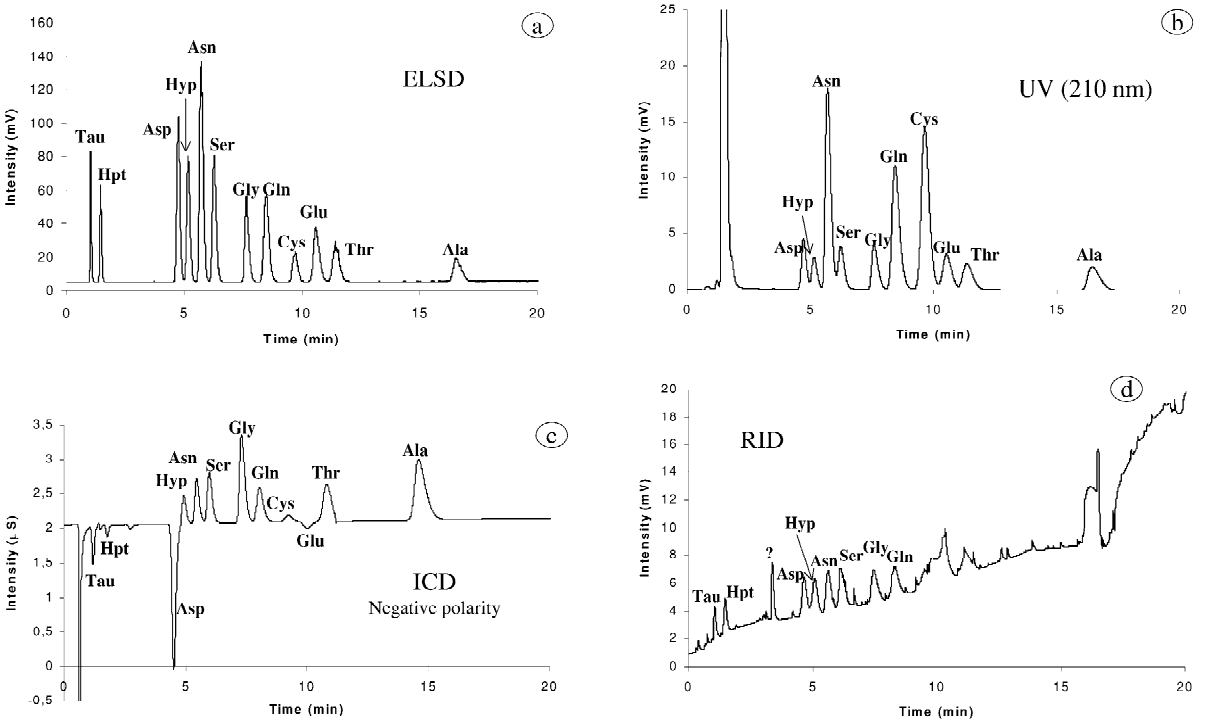

Fig. 1. Liquid chromatographic isocratic simultaneous analysis mixtures of 12 (six for NMR) underivatized amino acids. Column: Merck, Purospher RP-18e (125×4 mm). Mobile phase: 0.5 m*M* pentadecaflurooctanoic acid in water (in deuteriumoxide for NMR). Detection by: (a) evaporative light scattering, (b) UV at 210 nm, (c) indirect conductivity, (d) refractive index, (e) chemiluminescence nitrogen, (f) simple MS, (g) tandem MS, and (h) ELSD when used as monitor detector for NMR. Asterisks: negative peaks which correspond to the time needed for the mobile phase to reach the ELSD once the LC restarts. Injected concentrations: 50 mg l^{-1} for Tau and Hpt, 100 mg l^{-1} for the other amino acids; for NMR only: 2000 mg l^{-1}. Injection loop: 20 μl for NMR, 10 μl for all the other detectors.

pH of the mobile phase is very close to its isoelectric point and partially annihilates its conductivity response.

3.2.4. RID

As can be seen from Fig. 1d, the mobile phase was not ideal for the RID. Several system peaks were induced, preventing the analysis of some of the amino acids. Limits of detection were only ~50 mg l^{-1} probably due to the low difference between the refractive index of the mobile phase and the amino acids analysed. The amino acid stoichiometry of the tetrapeptide could not be calculated by this method (Fig. 2d).

3.2.5. CLND

Underivatized amino acid analysis by LC–CLND was introduced recently by our group [42]. This detector can detect any nitrogen-containing compound (except N_2) and its sensitivity is directly related to the percentage of nitrogen in a molecule. Furthermore, as we have shown recently [42], the equimolarity of this detector allows amino acid quantification using a single calibration curve; even in the case of co-elution with nitrogen-free containing compounds the quantification is not perturbed. Moreover, it is even possible to quantify the amino acid stoichiometry of peptide hydrolysates without the need for calibration curves if the molecular mass of the peptide is known [42]. Limits of detection varied from 0.33 mg l^{-1} for Asn to 0.7 mg l^{-1} for Thr.

3.2.6. MS

Simple and tandem MS of the 20 proteinogenic amino acids has been recently investigated

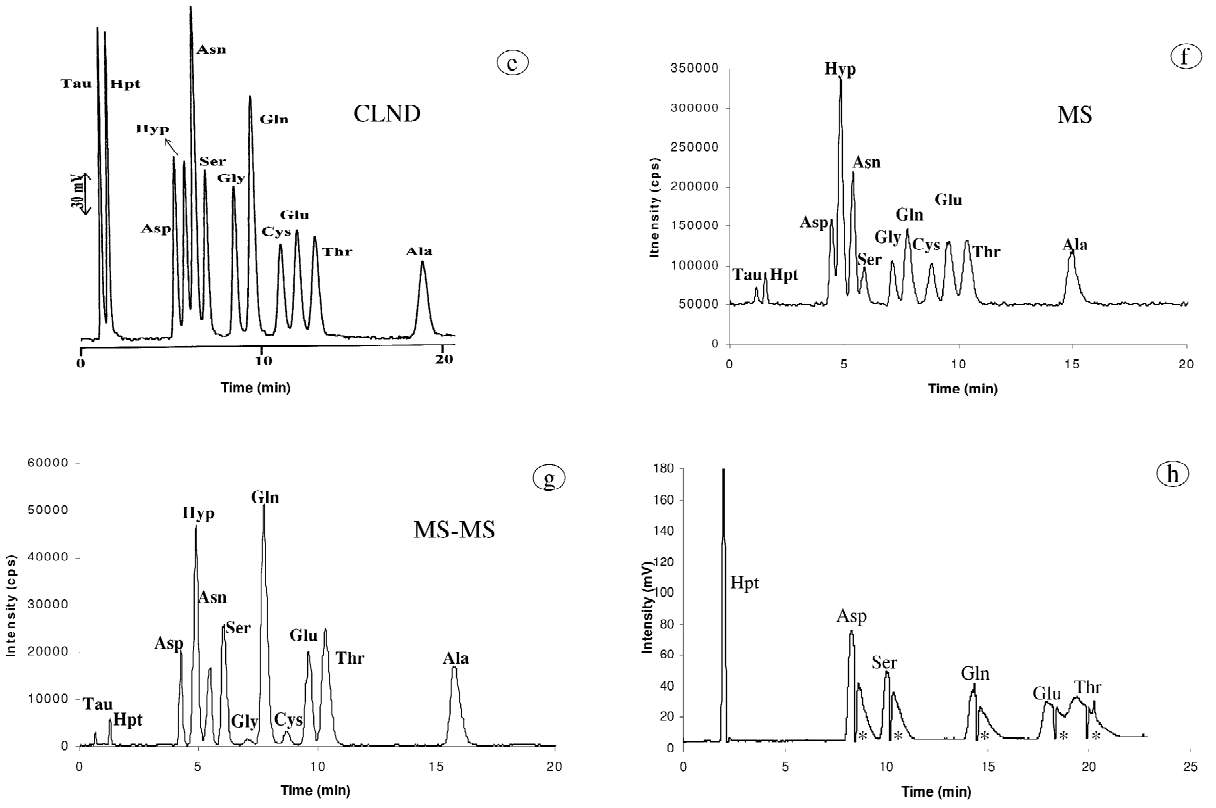

Fig. 1. (*continued*)

[96,102,117]. The high specificity of this mode of detection even allows the co-elution of several amino acids. The limitations of this method have been listed and relate to the intrinsic problems of MS when dealing with closely related chemical structures (isomers and isobars, collisionally induced dissociation fragments, etc.) [96,102]. Limits of detection varied from 0.2 to 5 mg l^{-1} for simple MS and from 0.08 to 0.8 mg l^{-1} for tandem MS.

3.2.7. NMR

The most sensitive mode of NMR is the stop-flow mode. However, this mode is dependent on a monitor detector to trace the analytes of interest and to instruct the LC to stop for NMR spectrum acquisitions. UV is usually used as a monitor detector, but its use is problematic with molecules which do not contain chromophore groups. Recently, we demonstrated that the ELSD can be a universal monitor detector for NMR and presents several advantages over UV, refractive index, and mass spectrometry [98]. Fig. 1h presents the LC–ELSD of a standard mixture of six amino acids. In fact, while PDFOA is a fully compatible mobile phase additive for NMR (no interferences were observed), the use of $^{2}H_{2}O$ caused changes in amino acid retention times due to its physicochemical differences with $H_{2}O$. In order to achieve satisfactory separations the 12 amino acids were injected in two different mixtures. Limits of detection (LODs) varied from 100 to 500 mg l^{-1} but high NMR frequency as well as new probe technology could improve these LODs. NMR was not used for determination of the amino acid stoichiometry of the tetrapeptide.

3.3. Comparison of the investigated detectors

Table 1 summarises the limits of detection ob-

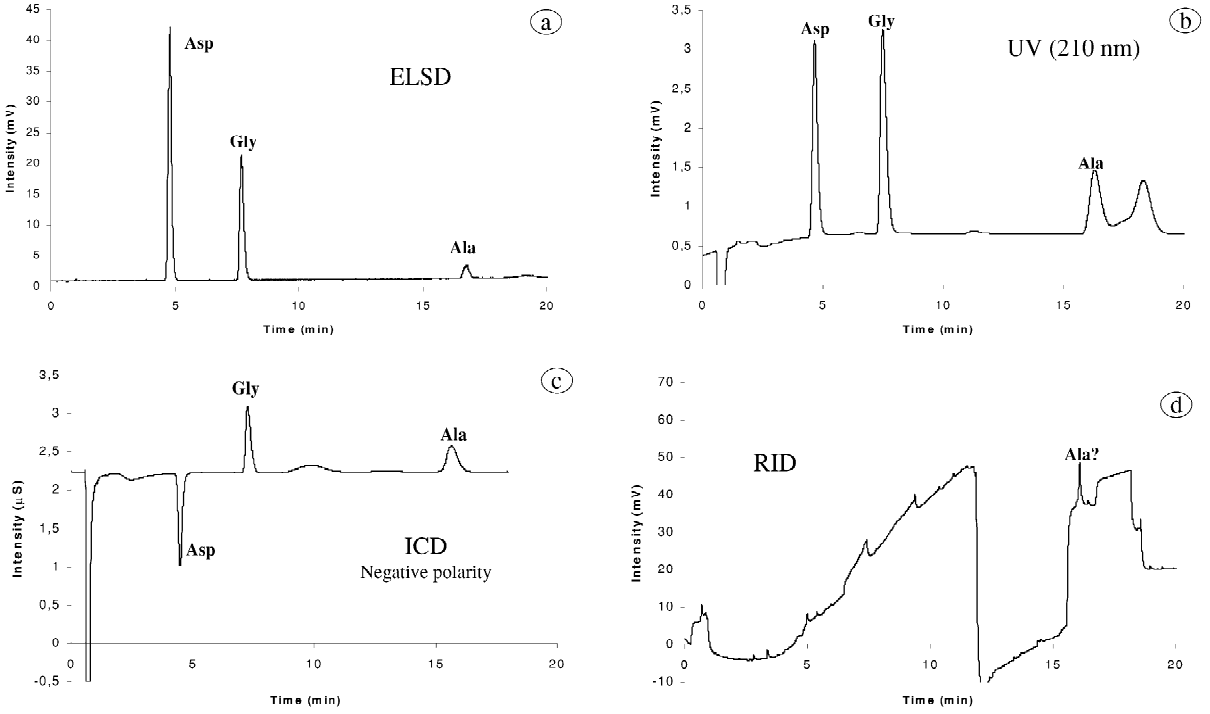

Fig. 2. Liquid chromatographic isocratic amino acid analysis of the tetrapeptide hydrolysate. Same chromatographic conditions as in Fig. 1. Detection by: (a) evaporative light scattering, (b) UV at 210 nm, (c) indirect conductivity, and (d) refractive index.

tained for each amino acid with the different detectors. CLND and MS gave LODs in the high ppb range, ELSD, UV and CD LODs were in the low ppm range, whereas RID and NMR were in the middle-high ppm zone. Overall, the best LODs are obtained with tandem mass spectrometry. As already mentioned, an aqueous mobile phase hinders MS sensitivity due to spray instability, decreased ion

Table 1
Summary of the amino acid limits of detection obtained with the different detectors

Amino acids	Limits of detection (mg l^{-1})							
	ELSD	UV	CD	RID	CLND	MS	MS–MS	NMR
Tau	1	NA[a]	5	50	0.6	0.4	0.5	100
Hpt	2	NA	5	50	0.5	0.5	0.4	150
Asp	2.5	3	1	50	0.6	0.4	0.2	250
Hyp	2.5	4.5	4	50	0.6	0.2	0.08	300
Asn	2.5	0.9	3	50	0.33	1	0.8	300
Ser	2.5	3	3	50	0.5	0.5	0.2	300
Gly	5	2	1.5	50	0.4	0.5	0.5	200
Gln	5	2.7	4	50	0.6	0.3	0.1	400
Cys	10	3	5	NA	0.3	1	0.8	500
Glu	5	3	25	NA	0.6	5	0.15	500
Thr	5	3	3	NA	0.7	0.3	0.1	500
Ala	7.5	3	1.5	NA	0.6	1	0.3	500

[a] Detection of the analyte can not be achieved by this detector or was disturbed by system interferences.

Table 2
Summary of the LC determination of the amino acid stoichiometry by the different detectors

Detector	Amino acids	Equation	r^2	Theory	Calculated
UV	Asp	$y = 82x + 418$	1	1	1.05
	Gly	$y = 53x + 1134$	0.998	2	2.03
	Ala	$y = 73x - 262$	0.997	1	0.87
ELSD	Asp	$y = 1.3785x + 1.0545$	0.9986	1	1.1
	Gly	$y = 1.4091x - 0.2532$	0.994	2	2.02
	Ala	$y = 1.4326x - 0.3349$	0.9869	1	0.96
CD	Asp	$y = 3300x - 35\,760$	0.9996	1	0.99
	Gly	$y = 1657x - 59\,687$	0.9988	2	2.01
	Ala	$y = 2548x - 51\,603$	0.9977	1	1.03
MS	Asp	$y = 2955x + 56\,919$	0.9985	1	1.04
	Gly	$y = 1367x + 51\,822$	0.9997	2	1.98
	Ala	$y = 2178x + 61\,280$	0.999	1	0.95
Tandem MS	Asp	$y = 369x + 10\,444$	0.9986	1	1.10
	Gly	$y = 22.7x + 1171$	0.9994	2	2.05
	Ala	$y = 454x + 15\,218$	0.9984	1	0.96
CLND	Asp	$y = 14\,601\,937x - 34\,216$	1	1	1.07
	Gly	$y = 14\,570\,887x - 29\,024$	1	2	2.04
	Ala	$y = 14\,675\,371x - 48\,119$	0.9999	1	0.94

Linearity equations and corresponding regression coefficients are shown. Linearity was tested for 2 orders of magnitude: 1–100 mg l^{-1} for MS(–MS), 0.005–0.5 m*M* for the CLND and 5–500 mg l^{-1} for the rest of the detectors. For ELSD, the equations have been obtained by using double logarithmic co-ordinates.

extraction, etc. Indeed, at least an order of magnitude better LODs are obtained by using hydrophilic interaction chromatography (HILIC) [106] where acetonitrile–water (75:25) was used as mobile phase. Finally, recent instrumentation advances lead to new more sensitive ESI-MS and NMR instruments. Improvements in the ion sampling region, ion transfer region and in collision cell design for triple quadrupole mass spectrometers [118] as well as the use of higher magnetic fields and cryogenically cooled probes for NMR [119] have considerably increased their sensitivity (by at least an order of magnitude).

Table 2 summarises the quantification results obtained for the tetrapeptide Gly–Gly–Asp–Ala hydrolysate. Satisfactory linear regressions were obtained for all the detectors (except the ELSD) with good correlation coefficients for 2 orders of magnitude. ELSD gave linear regression by using double logarithmic co-ordinates. Most of the detectors tested in this study have a linear range of 3 or even more orders of magnitude, however in this study they were tested only up to 2 orders of magnitude. The calculated amino acid stoichiometry was in good agreement with theoretical results. The Ala value calculated by UV was the least accurate but quantification may have been perturbed by the unknown peak eluted at the end of Ala peak (Fig. 2b). The Bachem certificate of analysis gives: Gly: 2, Ala: 1, Asp: 0.98. The best correlation between theoretical and experimental values were obtained with the conductivity detector.

Table 3 summarises the comparison of the detectors investigated when applied for amino acid analysis. MS combines the highest specificity and sensitivity, while NMR and MS are the most expensive methods, followed by CLND. The ELSD response is proportional to the injected mass of a compound (equimass) [116], while the CLND response is directly proportional to the percentage of nitrogen in the molecule (equimolar to the nitrogen content) [120]. Thanks to these features both ELSD and CLND are used in combinatorial chemistry for the quantification of unknown compounds by using single calibration curves, with overall %RSD of 20

Table 3
General comparison of the detectors investigated for amino acid analysis

	Detector						
	ELSD	UV (210)	CD	RID	CLND	MS(–MS)	NMR
LOD (for amino acids)	++	++	++	+	+++	+++	−
Linearity	Non-linear	Linear	Linear	Linear	Linear	Linear	NI
Specificity	−	−	−	−	+	+++	−
Equimass or equimolar	20% [121]	−	−	−	10% [122]	−	−
Price	−	−	−	−	+	++	+++
Structural information	−	−	−	−	−	++	+++
Detector range (orders of magnitude)	2	2	2	2	2	2	NI
Easy to use	+++	+++	++	+++	++	+	+
Universal	Yes	No	No	Yes	No	Yes	Yes
Gradient compatible (with the system used)	Yes	No	No	No	Yes	Yes	Yes
Mobile phase requirements	Volatility	Low absorptivity	Low conductivity	None	Volatility, absence of nitrogen containing compounds	Volatility	Low concentrations of proton containing compounds
Chromatographic system stability	+++	+	+	−	+++	+++	+
Applicability in complex matrices	−	−	−	−	+	+++	++

NI, not investigated.

[121] and 10 [122], respectively. ELSD, CLND, MS and NMR can also be used with gradient elution, whereas the other detectors will be perturbed by concentration changes of PDFOA in the mobile phase. MS seems to be the most adequate detector for complex matrices due to its high specificity; NMR can also be used in complex matrices as interfering compounds can be suppressed with adequate sequences [123].

It must be pointed out that the aim of this study was to test the different detectors that can detect amino acids in their underivatized form in order to give scientists the possibility of choosing the right one in relation to their analytical needs. Each method has its advantages and drawbacks and depending on the matrices investigated as well as their amino acid contents (absolute quantities, existence of low concentrations of amino acids in the presence of high concentrations of others, etc.), certain detectors can successfully determine the amino acids while others may fail to do so.

4. Conclusion

In this study several detectors were tested regarding their ability to detect underivatized amino acids. The UV and RI detectors failed to detect all the amino acids investigated either due to the absence of a chromophore group in their structure or due to system interferences. The ELSD was very easy to use with fairly good LOD. The CD was operated in indirect mode and gave good LOD for all amino acids except for glutamic acid. With the exception of the refractive index detector, all the other detectors were compatible with the chromatographic conditions used, gave satisfactory results concerning linearity and good agreement between theoretical and experimental values of the amino acid stoichiometry of the tetrapeptide hydrolysate. The most promising of the detectors tested in this study seems to be tandem MS and the CLND due to their higher sensitivity and specificity. Furthermore, the CLND equimolarity will be very useful for the determi-

nation of amino acid stoichiometry in peptide–protein hydrolysates. This study demonstrates that there are now several chromatographic detectors which allow the direct detection of amino acids. The combination of the above detectors with a more powerful chromatographic system than that used in this study could partially replace amino acid analysis by derivatization methods.

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