224 nm deep-UV laser for native fluorescence, a new opportunity for biomolecules detection

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Abstract

A new highly sensitive and compact 224 nm laser-induced native fluorescence (LINF) detector was developed using a new generation of deep-UV laser and an innovating elliptical flow cell. The use of deep-UV excitation at 224 nm allows to achieve fluorescence detection of an important range of molecules containing a single aromatic ring. The LINF detector was first evaluated in liquid chromatography. An improvement of a factor 500 over a conventional fluorimeter is reached with a limit of detection (LOD) of 1.5 pmole for ibuprofen. LODs were in the nanomole range for phenylalanine and in the picomole range for tyrosine and tryptophan. The LINF detector is able to detect the same levels of peptides concentrations as an ESI-ion trap spectrometer used in scan mode. In this application, LINF outperforms the UV detection at 214 or 254 nm and could be used with different additives with no noticeable effect on the detection.
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Keywords: HPLC; Native fluorescence; Deep-UV laser; Laser-induced fluorescence

1. Introduction

The interest in biomolecules, such as peptides or proteins, has increased the demands in instrumental tools. For separation of such molecules, high-performance liquid chromatography (HPLC) is widely used and still evolving towards innovative solutions [1]. A variety of methods to detect biomolecules present in samples of interest have been developed. UV-absorbance is the major technique of detection used for peptides and proteins with preferred spectral bands at 214 nm associated with absorption of peptide bond, 254 nm associated with π electrons, or less often at 280 nm associated with absorption of the aromatic amino acids such as phenylalanine, tyrosine and tryptophan [2]. As UV detection presents a limited sensitivity, other methods can be used. Electrochemical detection (ED) has increasingly become an important tool because of ultra low limits of detection [2,3]. Nevertheless, ED is quite a delicate technique because of short lifetime of the electrodes

and problems of maintenance which cause variability of sensitivity [4]. Mass spectrometry can detect biological samples in very small amounts and has become an important technique for identification of proteins [2]. However, when coupling this detection method with liquid chromatography, incompatibilities may occur: for example, trifluoroacetic acid (TFA) is a very suitable additive for separation of protein by HPLC but forms very strong ion pairs that prevents the ionization of the protein in electrospray mass spectrometry (ESI-MS) whereas formic acid is better for MS detection but resolution and recoveries obtained with this additive are lower than with TFA [5,6].

Fluorescence is a very sensitive method in biomolecule analysis and can perform simultaneous detection of low-abundant and high-abundant species [2]. Lasers can be used as excitation source because of their high power, their monochromaticity and their directivity, which increase the efficiency of molecule excitation and the intensity of fluorescence emission. Laser-induced fluorescence (LIF) is often used in capillary electrophoresis (CE) but scarcely used with HPLC. Most often, lasers operate in the visible or near-UV region, which imply a use of dyes [7–9]. Derivatization methods enhance the detectability but complicate the chromatogram because derivatization agents are not

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specific and involve additional procedures, which lengthen the time of analysis [4,7]. Exploiting the native fluorescence of molecules using lasers operating in the deep-UV (wavelengths under 300 nm) has become a new alternative for detection; it has been successfully employed with catecholamines [10–12], aromatic amino acids [10–14], proteins [14,15], morphine and derivates [16,17], or other drugs containing an aromatic ring [18,19]. However, these kind of lasers tend to be expensive and quite voluminous. The routine use of deep-UV excitation for analysis of biomolecules has been dependent on the development of new lightweight lasers emitting in the 200–250 nm range. Hollow cathode ion lasers are an efficient source of deep-UV light for low cost and compact. Two major lasers are available with wavelengths in deep-UV: a helium charge exchange pump sputtering silver hollow cathode laser operating at 224.3 nm and a neon charge exchange pump sputtering copper hollow cathode operating at 248.6, 260.0 or 270.3 nm. A small 266 nm high speed pulsed laser also exists, which is less expensive than doubled or tripled lasers but it has only been tested in CE [20]. Excitation at 224.3 nm falls within the absorption band for aromatic amino acids [21]. Therefore, it can be used as excitation source for analysis of aromatic amino acids or any biomolecule derived from them.

In this report, a new detector using this laser and an innovating elliptical flow cell is studied. First, an evaluation of its performance is realized with ibuprofen, which has a maximum of excitation at 224 nm and thus allows a direct comparison with a classical HPLC fluorimetric detector. Then, this detector is assessed for amino acids, peptides and proteins analysis.

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN) was HPLC grade and was purchased from VWR International (Fontenay/s Bois, France). Ultra pure water (Milli-Q plus 185 filtration system; Millipore, Molsheim, France) was used in all experiments. Samples and running phosphate buffer (25 mM, pH 7.0) were prepared using sodium hydrogenophosphate and dihydrogenophosphate purchased from Merck (Darmstadt, Germany).

Phenylalanine (Phe) and tyrosine (Tyr) were purchased from Fluka (Buchs, Switzerland). Ibuprofen, tryptophan (Trp), a mixture of five peptides (Gly-Tyr, Val-Tyr-Val, Met-enkephalin, Leu-enkephalin and angiotensin II) and a mixture of four proteins (ribonuclease A, cytochrome *c*, holotransferrin and apomyoglobin) were purchased from Sigma–Aldrich (St. Quentin Fallavier, France).

2.2. Instrumentation

Fluorescence spectra and measurement of fluorescence quantum yield were performed with a Perkin-Elmer LS 50 B spectrofluorimeter (Perkin-Elmer, Boston, MA, USA).

The HPLC system for LINF detection comprised a HP 1050 Series quaternary pump and degasser (Hewlett-Packard, Palo Alto, CA, USA), and a 20 μl Rheodyne manual injection valve

(Rheodyne, Rohnert Park, CA, USA). For conventional spectrofluorimeter detection, the HPLC system comprised a Kontron 422 pump, a Kontron 465 Autosampler (Kontron Instruments, Montigny Le Bretonneux, France), a Uniflows Degasys DG 1310 degasser (Uniflows, Tokyo, Japan) and a Shimadzu RF-551 spectrofluorimeter (Shimadzu, Kyoto, Japan) with the following settings: high sensitivity ($\times 1024$), gain $\times 16$ and response filter of 1.5 s. A HP 1100 Series binary pump and UV detector at 214 nm with a 20 μl Rheodyne manual injection valve and an Esquire LC ion trap mass spectrometer equipped with an electrospray ionization interface (Bruker Daltonics, Billerica, MA, USA) were used for analysis of peptides and proteins. The instrument was tuned to get the MS conditions enabling the highest response for each compound. For peptides, the source settings are: temperature of 350 °C, flow rate of 8 l/min, nebuliser pressure of 50 psi and capillary tension of 4500 V. For proteins, the source settings are: temperature of 280 °C, flow rate of 6 l/min, nebuliser pressure of 15 psi and capillary tension of 5000 V. For both peptides and proteins, the ion trap settings are −5 V for the first lens and −60 V for the second lens.

2.3. Chromatography

Samples were all analyzed at room temperature. The mobile phases were prepared as needed and degassed by ultrasound prior to use. Injection volumes of all samples were 20 μl.

Ibuprofen and aromatic amino acids were analyzed using a 5 μm Hypersil BDS C18 (50 mm × 4.6 mm I.D.) column (Thermo Electron Corporation, Waltham, MA, USA). Different mobile phases were used consisting of isocratic ACN—phosphate buffer pH 7 mixing, at a flow rate of 1 ml/min, in proportion depending on the compound: 30/70 (v/v) for ibuprofen, 5/95 for phenylalanine, 4/96 for tryptophan and 1/99 for tyrosine.

Peptides were separated on a 4 μm Synergi Hydro-RP (30 mm × 2 mm I.D.) column (Phenomenex, Le Pecq, France) and proteins on a 5 μm Atoll MP (50 mm × 2 mm I.D.) column (Interchim, Montluçon, France). Mobile phases consisted of ACN with 0.05% (v/v) trifluoroacetic acid or 0.1% (v/v) formic acid or 0.3% (v/v) acetic acid (mobile phase A) and water with the same concentration of the same acid (mobile phase B). The separations were carried out with single-step gradients described in Table 1, at a flow rate of 0.4 ml/min followed by a plate at 60/40 (v/v) ACN/water during a minute and reversed gradients to equilibrate columns between runs to the initial conditions.

2.4. Detector design

A scheme of the new laser-induced native fluorescence (LINF) detector is presented in Fig. 1. A compact deep-UV He–Ag laser provided by PhotonSystems (Covina, CA, USA) was used here as excitation source for fluorescence detection of biomolecules This new laser has been already described in another publication [21]. It emits pulsed radiation at 224.3 nm with a power of few mW. Pulse duration is set up at 100 μs and pulse frequency at 3 Hz.

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Table 1
Details of gradients used for peptides and proteins separation

| Additive | Peptides separation | | | Proteins separation | | |
| | Mobile phase A/B (v/v) | | | Mobile phase A/B (v/v) | | |
	From	To	Time (min)	From	To	Time (min)
TFA 0.05% (v/v)	8/92	30/70	20	26/74	80/20	10
Formic acid 0.1% (v/v)	5/95	25/75	20	15/85	70/30	10
Acetic acid 0.3% (v/v)	3/97	23/77	20	15/85	55/45	10

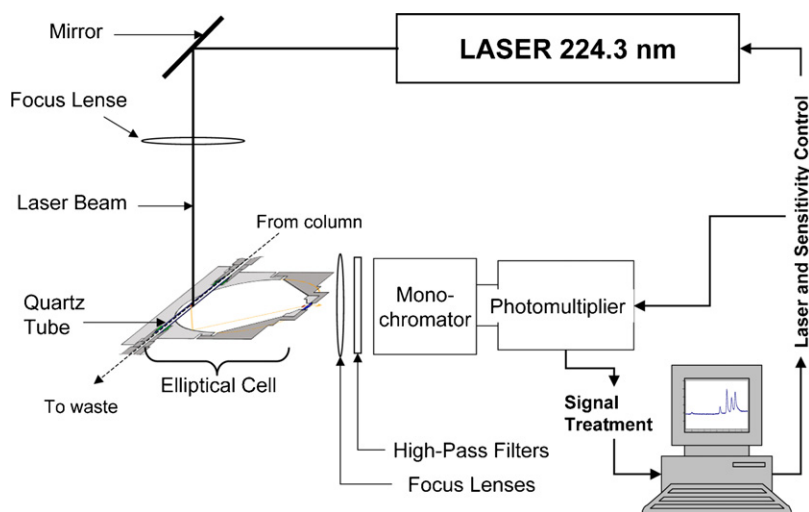Mobile phase A: acetonitrile + additive, mobile phase B: ultra pure water + additive.

Fig. 1. Scheme of LINF detection system.

Flowgene (St. Beauzire, France) provided its detection technology as an innovating elliptical detection cell [22] based on an interesting property of the ellipse: each ray emitted at one focus pass through the second one. Chromatographic flow is excited in a quartz tube (2 mm ED, 1 mm I.D.) placed on one focus. The 2 mm diameter laser beam is focused on this focal point. The elliptical cell behaves like a concentrating mirror so that 60% of the fluorescence emitted can be collected at the second focus. It is important to note that the fluorescence collection efficiency is independent of the wavelength. This is important for the linearity of the response of the cell, when most of the optical components have non-linear response in the UV (between 200 and 300 nm).

Lenses were used to focus the fluorescence from the detection cell onto a H1034 monochromator (Jobin Yvon, Longjumeau, France) equipped with UV gratting and with a bandwidth of 20 nm. Two filters block reflected laser radiation and all wavelengths below 250 nm before the monochromator and a 6780 UV photomultiplier tube module (Hamamatsu Photonics, Massy, France) collects the fluorescence emission at the wavelength selected.

2.5. Determination of LOD and LOQ

The limit of detection (LOD) is the minimum quantity or concentration that can be distinguished from zero. The limit of quantification (LOQ) is the minimum quantity or concentration that can be evaluated with a certain precision. In this study, 3σ and 10σ criteria were applied for the calculation of LOD and LOQ values according to guidelines for validation of analytical methods [23], using the formula (1):

$$L = \frac{kh_{max}}{S} \qquad (1)$$

where L stands for LOD or LOQ; k is a factor depending on the limit wanted, whose value is 3 for LOD and 10 for LOQ; h_{max} is the mean of maximal signal amplitude of a blank measured during 20 times the peak width at half-height, on at least 5 blanks; S is the slope of a linear regression equation of calibration using 3 different concentrations.

3. Results and discussion

3.1. LINF detector advantages

The He–Ag laser used in this study has the size, weight and consumption of a He–Ne laser but with output in the deep-UV. Therefore, it is preferred to large laser units usually used for analysis by LIF in deep-UV. Moreover, He–Ag laser is far more affordable (about $8000). Based on manufacturer's specifications, the laser lifetime should be over 10,000 h when used with an emission frequency of 1 Hz and between 1500 and 2000 h with an emission frequency of 10 Hz. A frequency of 3 Hz is generally enough for a correct peak description and allows a long lifetime. The efficiency of fluorescence emission depends

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on the laser power and the duration of excitation. Here, the laser emits at a low nominal power (10 mW typical) but with a sufficient excitation time (100 μs typical) that allows an interaction between light and matter as if it was a quasi-cw operation, which avoids biomolecules thermal destruction.

As the laser does not emit continuously, the fluorescence collection must be synchronized with the laser emission. The delay between the beginning of laser pulse and the beginning of fluorescence collection can be adjusted, so can the duration of collection, also called as gate time. This allows a perfect optimization of the detector depending on the compound analyzed. Here, it has been optimized for ibuprofen's detection, which is used as reference for comparison with a fluorimeter, with a delay time of 3 μs and a gate time of 200 μs.

The fluorescence collecting system is also a great improvement in regards to conventional fluorimeter. Whereas conventional systems collect about one-fourth of the fluorescence emission perpendicularly to the excitation direction, the elliptical detection cell permits a collection of 60% of the fluorescence emission.

3.2. LINF versus conventional fluorimeter

Performances of this new LINF detector have been evaluated with ibuprofen, a chemically defined molecule widely available with a known purity. Ibuprofen's maximum excitation was measured at 224 nm, which is the wavelength of the laser used in this detector. Ibuprofen's fluorescence quantum yield has been evaluated according to the comparative method of Williams et al. [24] using tryptophan ($\Phi_F = 0.14$ [25]) as reference. The measured value for ibuprofen is $\Phi_F = 0.062$ at pH 7 in ultra pure water as solvent.

Chromatograms of ibuprofen with the LINF detector and with a Shimadzu RF-551 fluorimeter are shown in Fig. 2a and b. LOD and LOQ for ibuprofen determined for an emission at 300 nm with the fluorimeter are 0.8 ± 0.2 nmol (0.04 ± 0.01 mM) and 2.7 ± 0.7 nmol (0.13 ± 0.03 mM), respectively. With the LINF detector, LOD and LOQ of 1.5 ± 0.1 pmol (74 ± 6 nM) and 4.5 ± 0.3 pmol (0.22 ± 0.02 μM) respectively have been reached, which is an improvement of about a factor 500. Detection limit obtained with the LINF detector is also lower than the 2.5 nmol LOD obtained with UV-absorption detector at 220 nm by Rustum [26]. Although it can be argued that the recently marketed fluorescence spectrometers could offer a better sensitivity or better signal to noise ratio than the RF-551, the important improvement we observed makes us confident that this first generation of LINF detector allows better LOD and LOQ performances than conventional spectrofluorimeters.

3.3. Analysis of aromatic amino acids

Phenylalanine, tyrosine and tryptophan are the three amino acids responsible for proteins high absorption in the 250–280 UV region. Their maximal wavelengths of excitation are 250 nm for Phe and 270 nm for Tyr and Trp, which does not correspond with the laser wavelength. On the other hand, the laser wave-
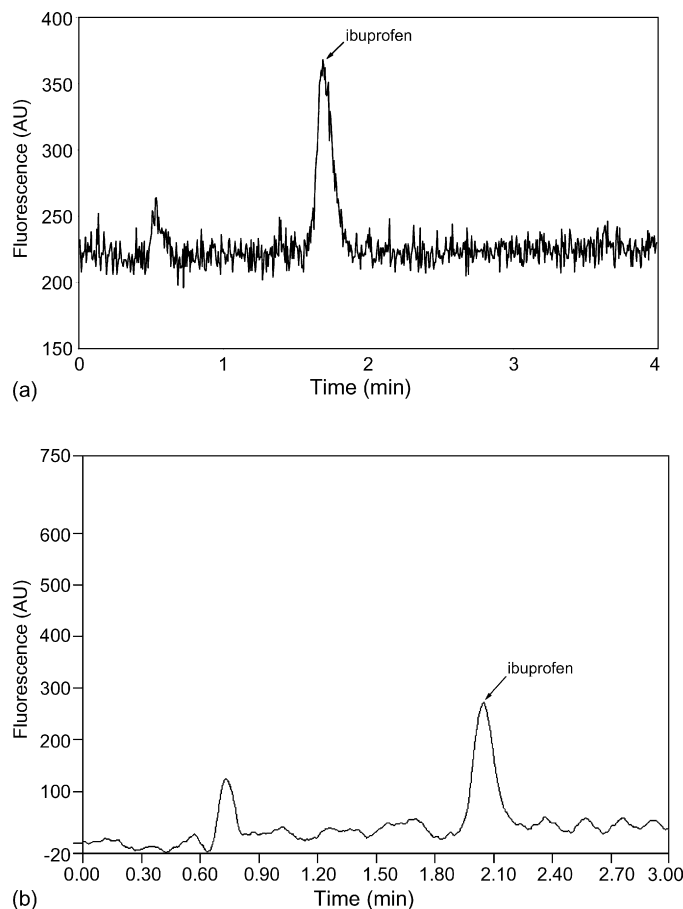

(a)

(b)

Fig. 2. Chromatograms of ibuprofen (experimental conditions: injection volume 20 μl; column Hypersil BDS C18 5 μm × 50 mm × 4.6 mm; mobile phase ACN—phosphate buffer pH 7 30/70 (v/v) 1 ml/min; excitation wavelength 224 nm, emission wavelength 300 nm). (a) Obtain with the LINF detector for a 76.5 nM solution (1.5 pmol) with a signal to noise ratio of 3.7. (b) Obtain with the RF-551 fluorimeter for a 51.5 μM solution (1.0 nmol) with a signal to noise ratio of 4.2.

length is closer to a second maximum of excitation measured at 210 nm for Phe and 220 nm for Tyr and Trp.

LODs and LOQs were determined for an emission at 290 nm for Phe, 310 nm for Tyr and 360 nm for Trp. They were measured using both the LINF detector and the RF-551 spectrofluorimeter, first with excitation at 224 nm, and second with excitation at the maximum excitation wavelength of each amino acid. Data are summarized in Table 2. As expected, detection and quantification limits with the conventional fluorimetric detector are lower when the molecules are excited at their maximum excitation wavelength. But they are still much lower with the LINF detector, even if the laser does not excite amino acids at their maximum. Improvements of factors 25–170 are observed over the RF-551 spectrofluorimeter exciting amino acids at their maximum. An improvement of at least a factor 2 over detection limits reported in literature for tyrosine and tryptophan analysis by HPLC fluorimetric detector is also achieved: Wood and Hall only reach a LOD for tyrosine of 2 pmol [27] and Bailey and Ayling show a linearity of detection for tyrosine and tryptophan from 2.5 to 25 pmol [28]. The LINF detector is, therefore, particularly suitable for aromatic amino acids detection.

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Table 2
LODs and LOQs ± standard deviation for aromatic amino acids determined with the LINF detector and the RF-551 fluorimeter (quantities in mol, concentrations (in brackets) in mol/l)

Detector	Excitation		Phe	Tyr	Trp
LINF	224 nm	LOD	$3.6 \pm 0.2 \times 10^{-10}$ ($1.82 \pm 0.08 \times 10^{-5}$)	$8 \pm 2 \times 10^{-13}$ ($3.9 \pm 0.9 \times 10^{-8}$)	$7.1 \pm 0.8 \times 10^{-13}$ ($3.5 \pm 0.4 \times 10^{-8}$)
		LOQ	$1.1 \pm 0.05 \times 10^{-9}$ ($5.5 \pm 0.3 \times 10^{-5}$)	$2.4 \pm 0.5 \times 10^{-12}$ ($1.2 \pm 0.3 \times 10^{-7}$)	$2.1 \pm 0.3 \times 10^{-12}$ ($1.1 \pm 0.1 \times 10^{-7}$)
RF 551	224 nm	LOD	$3 \pm 2 \times 10^{-8}$ ($1.4 \pm 0.8 \times 10^{-3}$)	$2.2 \pm 0.6 \times 10^{-10}$ ($1.1 \pm 0.3 \times 10^{-5}$)	$6 \pm 1 \times 10^{-11}$ ($3.1 \pm 0.5 \times 10^{-6}$)
		LOQ	$9 \pm 5 \times 10^{-8}$ ($4 \pm 2 \times 10^{-3}$)	$6 \pm 2 \times 10^{-10}$ ($3.3 \pm 0.9 \times 10^{-5}$)	$1.9 \pm 0.3 \times 10^{-10}$ ($9 \pm 2 \times 10^{-6}$)
	Maximum	LOD	$9 \pm 1 \times 10^{-9}$ ($4.5 \pm 0.7 \times 10^{-4}$)	$1.3 \pm 0.1 \times 10^{-10}$ ($6.7 \pm 0.6 \times 10^{-6}$)	$2.6 \pm 0.6 \times 10^{-12}$ ($1.3 \pm 0.3 \times 10^{-6}$)
		LOQ	$2.8 \pm 0.4 \times 10^{-8}$ ($1.4 \pm 0.2 \times 10^{-3}$)	$4.0 \pm 0.4 \times 10^{-10}$ ($2.0 \pm 0.2 \times 10^{-5}$)	$8 \pm 2 \times 10^{-11}$ ($3.9 \pm 0.9 \times 10^{-6}$)

Table 3
LOD ± standard deviation for some peptides determined with the LINF detector, the UV-absorbance detector and the ESI-ion trap spectrometer (quantities in mol, concentrations (in brackets) in mol/l)

Detector	Additive	Peptides				
		Gly-Tyr	Val-Tyr-Val	Met-enképhaline	Leu-enképhaline	Angiotensine II
LINF	TFA 0.05%	$5 \pm 1 \times 10^{-12}$ ($2.6 \pm 0.6 \times 10^{-7}$)	$4.7 \pm 0.5 \times 10^{-12}$ ($2.4 \pm 0.3 \times 10^{-7}$)	$4.9 \pm 0.4 \times 10^{-12}$ ($2.4 \pm 0.2 \times 10^{-7}$)	$4.7 \pm 0.9 \times 10^{-12}$ ($2.3 \pm 0.4 \times 10^{-7}$)	$3.2 \pm 0.7 \times 10^{-12}$ ($1.7 \pm 0.4 \times 10^{-7}$)
	Formic acid 0.1%	$3.5 \pm 0.3 \times 10^{-12}$ ($1.8 \pm 0.1 \times 10^{-7}$)	$5.5 \pm 0.3 \times 10^{-12}$ ($2.7 \pm 0.2 \times 10^{-7}$)	$5.2 \pm 0.8 \times 10^{-12}$ ($2.7 \pm 0.4 \times 10^{-7}$)	$5.8 \pm 0.7 \times 10^{-12}$ ($2.9 \pm 0.4 \times 10^{-7}$)	$4.6 \pm 0.8 \times 10^{-12}$ ($2.3 \pm 0.4 \times 10^{-7}$)
	Acetic acid 0.3%	$3.5 \pm 0.6 \times 10^{-12}$ ($1.7 \pm 0.3 \times 10^{-7}$)	$6.3 \pm 0.5 \times 10^{-12}$ ($3.3 \pm 0.3 \times 10^{-7}$)	$8 \pm 2 \times 10^{-12}$ ($3.8 \pm 0.8 \times 10^{-7}$)	$7.9 \pm 0.7 \times 10^{-12}$ ($3.9 \pm 0.4 \times 10^{-7}$)	$6 \pm 1 \times 10^{-12}$ ($3.2 \pm 0.5 \times 10^{-7}$)
UV	TFA 0.05%	$3 \pm 0.5 \times 10^{-11}$ ($1.5 \pm 0.3 \times 10^{-6}$)	$6 \pm 1 \times 10^{-11}$ ($3.2 \pm 0.5 \times 10^{-6}$)	$5.7 \pm 0.2 \times 10^{-11}$ ($2.9 \pm 0.8 \times 10^{-6}$)	$7 \pm 1 \times 10^{-11}$ ($3.6 \pm 0.7 \times 10^{-6}$)	$2.5 \pm 0.6 \times 10^{-11}$ ($1.3 \pm 0.3 \times 10^{-6}$)
	Formic acid 0.1%	$1.5 \pm 0.1 \times 10^{-10}$ ($7.7 \pm 0.7 \times 10^{-6}$)	$2.7 \pm 0.3 \times 10^{-10}$ ($1.3 \pm 0.1 \times 10^{-5}$)	$2.1 \pm 0.2 \times 10^{-10}$ ($1.1 \pm 0.1 \times 10^{-5}$)	$2.4 \pm 0.2 \times 10^{-10}$ ($1.2 \pm 0.1 \times 10^{-5}$)	$1.1 \pm 0.1 \times 10^{-10}$ ($5.3 \pm 0.4 \times 10^{-6}$)
	Acetic acid 0.3%	$8 \pm 2 \times 10^{-10}$ ($4 \pm 1 \times 10^{-5}$)	$5 \pm 2 \times 10^{-10}$ ($2.3 \pm 0.8 \times 10^{-5}$)	$3.5 \pm 0.4 \times 10^{-10}$ ($1.7 \pm 0.2 \times 10^{-5}$)	$4.0 \pm 0.5 \times 10^{-10}$ ($2.1 \pm 0.3 \times 10^{-5}$)	$1.7 \pm 0.2 \times 10^{-10}$ ($9 \pm 1 \times 10^{-6}$)
MS TIC	TFA 0.05%	$1.0 \pm 0.7 \times 10^{-9}$ ($5 \pm 4 \times 10^{-5}$)	$7 \pm 5 \times 10^{-10}$ ($4 \pm 2 \times 10^{-5}$)	$2.0 \pm 0.8 \times 10^{-10}$ ($1.0 \pm 0.4 \times 10^{-5}$)	$1.6 \pm 0.9 \times 10^{-10}$ ($8 \pm 4 \times 10^{-6}$)	$1.0 \pm 0.6 \times 10^{-10}$ ($5 \pm 3 \times 10^{-6}$)
	Formic acid 0.1%	$3 \pm 1 \times 10^{-10}$ ($1.3 \pm 0.7 \times 10^{-5}$)	$1.4 \pm 0.7 \times 10^{-10}$ ($7 \pm 3 \times 10^{-6}$)	$7 \pm 2 \times 10^{-11}$ ($3 \pm 1 \times 10^{-6}$)	$7 \pm 2 \times 10^{-11}$ ($3 \pm 1 \times 10^{-6}$)	$3 \pm 1 \times 10^{-11}$ ($1.5 \pm 0.6 \times 10^{-6}$)
	Acetic acid 0.3%	$8 \pm 3 \times 10^{-11}$ ($4 \pm 2 \times 10^{-6}$)	$1.8 \pm 0.4 \times 10^{-11}$ ($9 \pm 2 \times 10^{-7}$)	$1.3 \pm 0.5 \times 10^{-11}$ ($6 \pm 2 \times 10^{-7}$)	$1.2 \pm 0.5 \times 10^{-11}$ ($6 \pm 2 \times 10^{-7}$)	$6 \pm 2 \times 10^{-12}$ ($3 \pm 1 \times 10^{-7}$)
MS extracted ion	TFA 0.05%	$1.5 \pm 0.6 \times 10^{-11}$ ($8 \pm 3 \times 10^{-7}$)	$2 \pm 1 \times 10^{-12}$ ($1.1 \pm 0.6 \times 10^{-7}$)	$1.4 \pm 0.4 \times 10^{-12}$ ($7 \pm 2 \times 10^{-8}$)	$2.2 \pm 0.8 \times 10^{-12}$ ($1.1 \pm 0.4 \times 10^{-7}$)	$3.8 \pm 0.6 \times 10^{-13}$ ($1.9 \pm 0.3 \times 10^{-8}$)
	Formic acid 0.1%	$3 \pm 2 \times 10^{-12}$ ($1.5 \pm 0.8 \times 10^{-7}$)	$3.2 \pm 0.3 \times 10^{-13}$ ($1.6 \pm 0.1 \times 10^{-8}$)	$4.5 \pm 0.4 \times 10^{-13}$ ($2.3 \pm 0.2 \times 10^{-8}$)	$2.9 \pm 0.8 \times 10^{-13}$ ($1.4 \pm 0.4 \times 10^{-8}$)	$1.3 \pm 0.6 \times 10^{-13}$ ($7 \pm 3 \times 10^{-9}$)
	Acetic acid 0.3%	$1.6 \pm 0.6 \times 10^{-12}$ ($8 \pm 3 \times 10^{-8}$)	$3 \pm 1 \times 10^{-13}$ ($1.2 \pm 0.6 \times 10^{-8}$)	$1.4 \pm 0.7 \times 10^{-13}$ ($6 \pm 3 \times 10^{-9}$)	$2 \pm 1 \times 10^{-13}$ ($8 \pm 5 \times 10^{-9}$)	$2 \pm 1 \times 10^{-13}$ ($1.2 \pm 0.5 \times 10^{-8}$)

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3.4. Analysis of peptides and proteins

As noteworthy limits of detection and quantification for aromatic amino acids are reached by the LINF detector, detection of peptides and proteins containing one or several of these amino acids should also be attractive. Five peptides (Gly-Tyr, Val-Tyr-Val, Met- and Leu-enkephalins and angiotensin II) and four proteins (ribonuclease A, cytochrome *c*, holotransferrin and apomyoglobin) were studied. These peptides contain one tyrosine but no tryptophan; therefore, they are detected at 310 nm. Proteins are detected at 360 nm because they contain one or more tryptophan, except ribonuclease A.

Peptides and proteins are generally detected by UV absorption at 214 or 254 nm or by electrospray (ESI) mass spectrometry so the LINF detector was compared to these modes of detection. The UV detection was here performed at 214 nm because the response was higher for peptides than at 254 nm (data not shown). The ESI-ion trap spectrometer was used in scan mode and optimized successively for each peptide or protein. Three different additives in mobile phase were used: trifluoroacetic acid which is the best acid for proteins separation by liquid chromatography but does not suit for mass spectrometry, formic acid which is better for MS detection but not for UV detection, and acetic acid which is intermediate between these two acids [5,6]. LODs and LOQs for peptides and proteins were measured with the LINF detector, the UV detector, and the mass spectrometer on total ionic current and on each extracted ion. Some peptides LODs are shown in Table 3.

First, these results show that LINF detection is not influenced by the additive used in mobile phase. UV detection (10-factor variation for detection limits) and especially MS detection (100-factor variation) provided variable LOD/LOQ depending on the additive. The LINF detector reaches detection limits in the picomole range for peptides and proteins, which is 10–100 times lower than detection at 214 nm. Limits with LINF are also intermediate between those with the ESI-ion trap spectrometer on the TIC and on the extracted ions. Thus, LINF detector can be used to analyze peptides and proteins alone or together with a mass spectrometer, thanks to its stability faced with mobile phases composition and to the detection and quantification limits reached.

4. Concluding remarks

This new laser-induced native fluorescence detector is a compact and highly sensitive apparatus. Its performances have been proven by the limits of detection and quantification reached for ibuprofen, which are at least 500 times lower than those reached by a conventional fluorimeter. It has been successfully applied to the detection of aromatic amino acids and peptides or proteins containing them, with LODs in the nanomole range for phenylalanine and in the picomole range for tyrosine, tryptophan, peptides and proteins. As it is able to detect the same levels of peptides as an ESI-ion trap spectrometer and its response does not depend on the mobile phase used, the LINF detector is clearly an excellent choice for peptides and proteins detection.

LIF detection is more often used with CE than with HPLC. It is therefore uneasy to compare the LINF detector used in HPLC, where the mass injected is exactly known, with the other LIF detectors used in CE, where injection volumes are more difficult to evaluate. For tyrosine, LODs of 20 nM with a laser operating at 284 nm [13] and from 2.1 to 2.3 μM with an excitation at 248.6 nm have been reported [10,11]. For tryptophan, LODs of 0.2 nM with lasers operating at 266 or 284 nm [12,13] and from 4 to 63 nM with a laser at 248.6 nm have been reached [10,11,14]. LODs for several proteins are in the 5–50 nM range with excitation by a laser operating at 248.6 nm or by a LED at 280 nm [14,15]. With this first LINF detector at 224.3 nm, the smallest concentrations detected were approximately in the same range, for a 20 μl injection: 40 nM for tyrosine, 36 nM for tryptophan and from 22 to 550 nM for peptides and proteins. As this detector can also be used in CE, a comparison with these types of LIF detectors will be soon possible.

Although limits of detection reached by the LINF detector are very low, they can be improved even further. They are now limited by background noise due to fluorescence of compounds when they are excited at 224 nm, including impurities in materials. There is few data available on the fluorescence emitted by a compound when excited by a 224 nm laser. Some unexpected compounds may emit fluorescence at this wavelength like impurities in the quartz tube or in water. The study of special materials for a use at 224 nm is an important part of the next investigations for the improvement of the signal/noise ratio.

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