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Journal of Chromatography A, 717 (1995) 335–343

JOURNAL OF CHROMATOGRAPHY A

Ball-lens laser-induced fluorescence detector as an easy-to-use highly sensitive detector for capillary electrophoresis Application to the identification of biogenic amines in dairy products

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Abstract

A ball-lens laser-induced fluorescence detector based on the conventional collinear arrangement is described for use with capillary electrophoresis. It allows better mechanical tolerances for capillary adjustment in front of the laser beam. Its sensitivity is equal to that of the conventional collinear arrangement. Micellar electrokinetic chromatographic determination of biogenic amines in dairy products is described as an application of this detector.

1. Introduction

Since Gassman et al. [1] introduced laser-induced fluorescence detection (LIFD) for capillary electrophoresis (CE) instead of the xenon lamp fluorescence proposed by Jorgensson and Lukacs [2], LIFD has gradually become the most sensitive technique for analyte detection in narrow-bore capillaries. The large number of papers that have been published testify to the usefulness of this kind of detection [1,3–8].

The two most popular on-column LIF systems are based on two different optical arrangements: (1) an orthogonal arrangement detection as described by Jorgensson and co-workers [2,9] and Zare and co-workers [1,10] and (2) a collinear arrangement as described by Hernandez and co-workers [5,11,12]. A third on-column arrangement, with axial beam illumination [13], has been described, but its use today is limited.

Wu and Dovichi [14] used a sheath flow cuvette detector as an "end-column" detector. The sheath flow cuvette and an on-column collinear arrangement seems to allow the same sensitivity limits of around ten fluorescent molecules detected [3,15,16]. In most work on LIF an on-column detector is used because of its ease of use.

The sensitivity level of orthogonal and collinear on-column arrangements has been discussed earlier [11] and is slightly better for the collinear arrangement. Hernandez and co-workers [5,11,12] have summarized the advantages of using the coaxial arrangement, viz., capillary adjustment easy to realize with an *XYZ* displacement device, minimum Raman noise level and the possibility of using objectives with a very high numerical aperture.

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0021-9673/95/$09.50
SSDI 0021-9673(95)00464-5

The principal difficulty with collinear CE–LIFD is adjusting the capillary window in front of the light beam. For instance, the *XYZ* mechanical tolerances of adjustment of the capillary in front of the objective may be less that 2.5 μm (*XYZ* axes are defined in Fig. 1).

We report here a new optical device, containing a ball-lens, which allows one to make this adjustment with a higher mechanical tolerance of ca. 40 μm. The sensitivity results are almost identical with those obtained with Hernandez and co-workers' optical arrangement, but no further adjustment is necessary after removing the capillary. The optimum laser power and the photomultiplier tube (PMT) voltage were studied. A linear range from 10^{-8} to 10^{-12} *M* was observed with two standard fluorescent dyes [fluorescein isothiocyanate (FITC) and Rhodamine 123]. This new optical device detector was used for the determination of biogenic amines in dairy products.

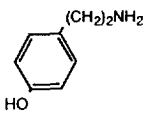
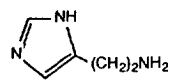
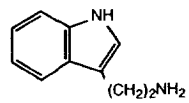
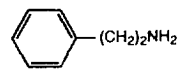
During the ripening of cheese, casein is slowly degraded by proteolitic enzymes. This mostly leads to a steady increase in the content of free amino acids, some of which can be subjected to subsequent breakdown reactions. Decarboxylation is such a reaction. It is catalysed by specific bacterial decarboxylases and gives rise to the formation of carbon dioxide and amines. These amines are designated as biogenic because they are formed by the action of living organisms. The most important biogenic amines that can be found in cheese are listed in Table 1 together with their precursors [17].

Whereas in most hard paste cheeses the content of biogenic amines is low [17], some soft cheeses have fairly high levels [18]. The consumption of large amounts of these amines can bring about symptoms of intoxication such as headache, nausea, hypo- or hypertension, cardiac palpitation and possibly shock [19]. As a result, biogenic amines are indicators of food quality. Therefore, it is important to determine certain biogenic amines, in the presence of amino acids in different food matrices.

Different methods have been used to separate and detect these amines, but the separations may be time consuming, might give poor chromatographic or electrophoretic resolutions and have poor sensitivity levels. Thin-layer chromatography [20–22] and thin-layer electrophoresis [21–23] with different detection reactions have been used. Gas chromatography of volatile biogenic derivatives has also been described [24,25]. More recently, ion-exchange chromatography [21,26,27], isotachophoresis [28,29] and reversed-phase-high-performance liquid chromatography (HPLC) [30–34] have given the most sensitive results. Most amines or non-aromatic amino acids show neither natural UV absorption nor fluorescence, and different chemical precolumn derivatization reagents have been tested for the analysis of amines, e.g., ninhydrin in amino acid analysers with postcolumn derivatization [35], 5-dimethylaminonaphthalene-1-sulfonylchloride, *o*-phthaldeialdehyde and 9-fluorenylmethyl chloroformate [32,33,36]. The best sensitivity is estimated as ca. $0.5 \cdot 10^{-6}$ mol of biogenic amine [36].

In this work, we evaluated the sensitivity performance of our "easy-to-use" LIF detection

Table 1
Biogenic amines and their precursor

Biogenic amine	Formula	Amino acid precursor
Tyramine		Tyrosine
Putrescine	$H_2N(CH_2)_4NH_2$	Ornithine
Histamine		Histidine
Cadaverine	$H_2N(CH_2)_5NH_2$	Lysine
Tryptamine		Tryptophan
Phenylethylamine		Phenylalanine

system for the determination of fluorescein thiocarbamyl (FTC) biogenic amines and FTC-amino acids in fresh and 1-month-old French soft cheese (Camembert).

2. Experimental

2.1. Instrumentation

For the ball-lens experiments, a capillary electrophoresis manual sampler and a high voltage power supply from a Spectra-Phoresis 100 (TSP, Freemont, CA. USA) were used. The instrumental design of the LIF detector is illustrated in Fig. 1. Laser radiation of 488 nm from a low-power argon ion laser (7 mW) (ILT, Salt Lake City, UT, USA) (1) passes through a laboratory-made optical fibre device (2), then the laser light is reflected by a 45°, 488 nm dichroic mirror (3) (Andover, Salem, NH, USA) and focused by an achromatic 5 × 0.2 lens (focal length 11 mm) (Zeiss, Le Pecq, France) (4) and a capillary ball-lens cell from a TSP capillary electrophoresis detector (5), where the sapphire ball-lens is placed in direct contact with the detection window of a fused-silica capillary of dimensions 50–75 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) (6). The capillary holder is mounted on an *XYZ* micropositioner table (Melles Griot, Irvine, CA, USA). The detection window (4 mm length) is formed by burning off the polyimide coating with an electrical coiled resistance. Light emitted from the capillary is collected by the ball-lens and the achromatic lens and passed through the dichroic mirror, a laboratory-made spatial filter (7), a notch filter centred on wavelength 488 nm (Andover) (8) and a high-pass filter (Oriel, Straford, CT, USA) (9). The fluorescence signal is then detected by a PMT (Type 928, Hamamatsu, Bridgewater, NJ, USA), the high voltage supply (10) of which may be adjusted. The signal is collected at a 10-Hz sampling rate with a Boreal data acquisition system (JMBS-Developpements, Grenoble, France) running on an IBM PC.

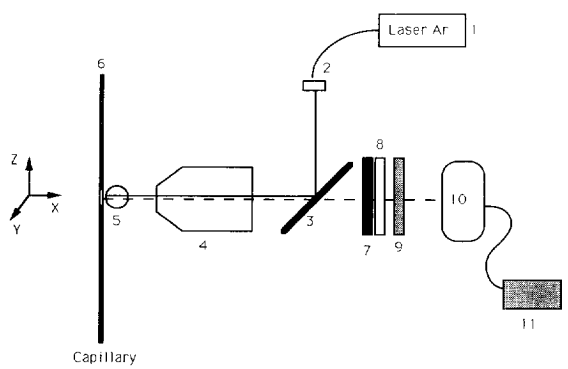


Fig. 1. Schematic diagram of ball-lens laser-induced fluorescence detector.

For classical collinear arrangement experiments, an IRIS 2000 [Europhor Instruments (now Zeta Technology), Toulouse, France] was used.

2.2. Optimization of optical arrangement

Studies on optical optimizations were carried out by flushing a 10^{-8} *M* rhodamine 123 solution in the capillary and recording the fluorescence intensity for each position of the capillary ball-lens cell.

For pinhole diameter experiments, the optical arrangement was adjusted to its optimum fluorescence intensity *XYZ* positions. The fluorescence of the 10^{-8} *M* rhodamine 123 solution and water flushed in the capillary was recorded for each diameter. Between rhodamine and water measurements, the capillary was washed consecutively for 3 min with 0.1 *M* NaOH solution and for 3 min with water. The signal-to-water ratio is defined as the ratio of the fluorescence intensity of the 10^{-8} *M* rhodamine 123 solution to that of water.

2.3. Chemicals and reagents

All chemicals and reagents were purchased from Aldrich (St. Quentin Fallavier, France), and used without purification.

2.4. Procedure for FITC derivatization

Rhodamine 123 and FITC were diluted with water (HPLC grade) to give concentrations from

10^{-8} to 10^{-13} *M* were obtained. These dyes have a maximum absorption near 488 nm.

Biogenic amines and amino acids were diluted with water–methanol (90:10, v/v) to give concentrations from $5 \cdot 10^{-7}$ to $5 \cdot 10^{-11}$ *M*. Derivatization with FITC was performed as described in Ref. [11].

2.5. Preparation of cheese sample

Fresh and 1-month-old Camembert (French soft cheese), pasteurized and unpasteurized, were chopped up and homogenized with 0.1 *M* HCl in the proportions: 0.1 *M* HCl = 4:3 (w/w). A 0.23-g amount of the paste was suspended in 5 ml of 0.1 *M* HCl. After centrifugation, the supernatant solution was filtered. The residue was extracted twice with 5 ml of 0.1 *M* HCl and filtered. After neutralization of the resulting combined extract with 0.2 *M* sodium carbonate solution, derivatization of amino acids and biogenic amines with FITC was carried out as described elsewhere [11]. The derivatized samples were diluted 100 000-fold and analysed by CE–LIF.

2.6. Separation conditions

All separations were carried out using an 80-cm long fused-silica capillary (50 cm effective length). For Rhodamine 123 and FITC studies, a 75 mm I.D. capillary and 50 m*M* sodium tetraborate borate buffer (pH 8.3) were used with 15 s, 16 kV electrokinetic injection and a separation potential of 16 kV. The separation buffer for FTC-amino acids and biogenic amines consisted of 100 m*M* SDS–100 m*M* boric acid. The pH was adjusted to 9.2 by addition of sodium hydroxidy solution. A 50 mm I.D. capillary was used with 2 s hydrodynamic injection (17.5 nl) and a separation potential of 24 kV. In both cases the capillary was rinsed for 3 min with 0.1 *M* NaOH, 3 min with distilled water and 3 min with buffer.

2.7. Analysis

Peaks were identified by spiking diluted cheese samples with standard solutions of amino acids and biogenic amines. Biogenic amines were determined using linear calibration graphs based on peak height. Each calibration graph contained data points at a minimum of six different concentrations, and each graph spanned the range of concentrations found in diluted cheese samples. Linearity was assessed using standard least-squares analysis of the logarithm of peak height vs. logarithm of concentration plots. Detection limits were estimated at twice the peak-to-peak noise by extrapolation from plots of peak height vs. concentration.

3. Results and discussion

3.1. Detector optimization

Difficulties met with the classical collinear arrangement for adjusting with extreme precision the capillary in front of the capillary window led ut to seek a new procedure to eliminate such small mechanical tolerances. Hlousek [37] proposed for spectrophotometric analysis in fused-silica capillaries the use of a small ball-lens, to obviate the importance of mechanical tolerances and to allow easier mounting and replacement of the capillary.

Fig. 2A and B show the intensity of the fluorescence signal vs. *XY* position of the ball-lens in front of the laser beam for the two optical arrangements. We can see (Fig. 2A) that the *Y* position is critical for the two systems, but the mechanical tolerance is much greater for the new system (ca. 180 mm compared with 8 mm for a signal reduced by 50%). The ball-lens mounted on the capillary allows the mechanical tolerances to be increased by a factor of 22. The ball-lens acts as a very short focal length lens to convert the slowly converging light [low numerical aperture (*NA*) of microscope objective; here *NA* = 0.2] to a rapidly converging cone of light (high numerical aperture, here *NA* = 0.88) that will impage the source into the area of interest in the sample cell. Fig. 2B shows the *X* positioning, provided that *X* is lower than the microscope objective focal length and that the laser spot illuminating the ball lens has a smaller area than the circular area of the ball-lens. Studies on the

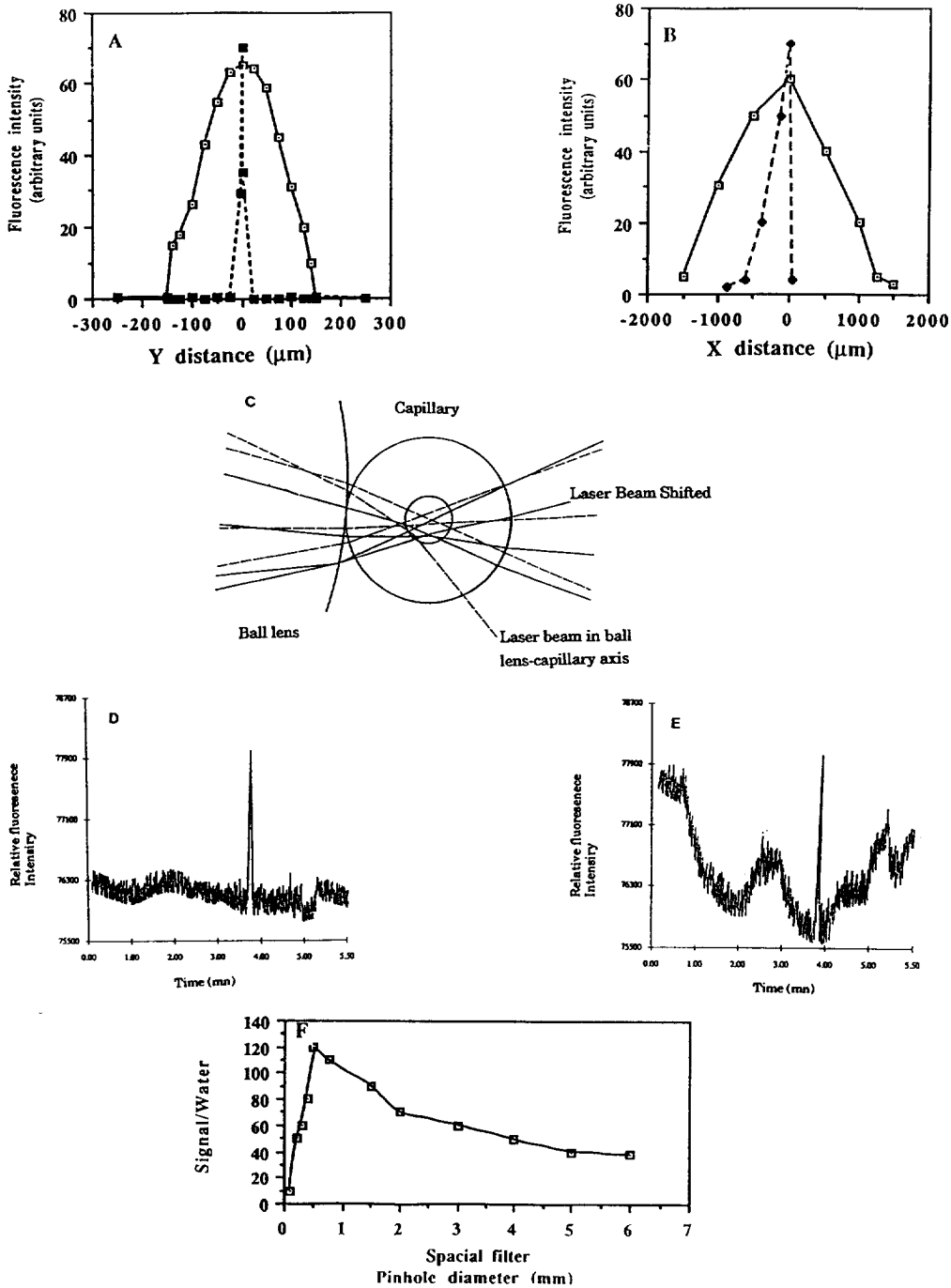


Fig. 2. Influence of X and Y positions of ball-lens–capillary cell relative to the laser beam. The zero point is defined as the optimum for relative fluorescence intensity of a 10^{-8} M Rhodamine 123 solution. Solid line, ball-lens detector; dashed line, conventional collinear detector. (A) Influence of Y position; (B) influence of X position, point $0 = 10\,200$ μm for ball-lens detector, 160 μm for classical collinear arrangement detector; (C) schematic ray diagram of a ball-lens showing ray traces through the ball-lens and the capillary cell; (D) analysis of $1.5 \cdot 10^{-12}$ M Rhodamine 123 solution after optimization of LIF detector; (E) analysis $1.5 \cdot 10^{-12}$ M Rhodamine 123 solution after removing the capillary ball-lens cell from the detector and putting it in the detector again without any new adjustment; (F) influence of pinhole diameter.

Z distance indicated that the laser spot has to be collimated on the ball-lens circular area. These results confirm that a high NA allow small variations of the laser beam position with regard to the ball-lens and capillary to be compensated (Fig. 2C) [37].

Because the adjustment of the capillary is critical for the conventional collinear arrangement, removing the capillary implies adjusting its position in front of the microscope objective. Using the ball-lens optical arrangement we show that this post-removal adjustment is not so critical. The sensitivity level is the same and the laser beam is collimated on the ball-lens and capillary as when the ball-lens cell and capillary are removed and replaced in front of the laser beam. A $1.5 \cdot 10^{-12}$ M solution of Rhodamine 123 gave the same signal-to-noise ratio in both cases (S/N = 21) (Fig. 2D and E).

The influence of the spatial filter pinhole diameter was also studied. Fig. 2F shows that the optimum diameter for such a filter is 500 μm. This means that when the diameter is becoming smaller, reflections of the laser beam on the microscope objective, the ball-lens and capillary are less important: the signal-to-water ratio increases. When the hole has a diameter of less than 500 μm, the fluorescence signal is much too reduced and it becomes of the same order as dark noise; the signal-to-water ratio decreases.

3.2. Detector performance

Scott [38] stated that if a response, R, arises as a function of concentration, C, then the function $R = AC^B$ where A and B are constants, will describe the response curve. Therefore, the equation $\log R = \log A + B \log C$ should describe a straight line with a slope B. Scott arbitrarily proposed that for a "linear" dependence of response on concentration, B should lie in the range 0.98–1.02. Values outside this range imply degrees of non-linearity. Standard curves for "non-linear" responses can be used provided that B is uniform over the analytical concentration range and that R can be accurately determined.

For the classical collinear arrangement [39], we found a linear dynamic range close to four orders of magnitude for Rhodamine 123 between 10^{-8} and 10^{-12} M and a slope B of 1.01. For the ball-lens detector a slope of 0.96 ($r^2 = 0.998$) for FITC and 1.05 for Rhodamine 123 ($r^2 = 0.999$) was obtained. As reported elsewhere, the LIF detector might be non-linear with Scott's definition for some fluorophores [40] but give a linear dynamic range of at least 5 (10^{-8}–$3.5 \cdot 10^{-13}$ M) and 4 (10^{-8}–$5 \cdot 10^{-12}$ M) orders of magnitude for Rhodamine 123 and FITC, respectively.

3.3. Fluorescein thiocarbamyl (FTC)-amino acids and FTC-biogenic amines in soft cheese samples

Amino acids are commonly studied with LIF–CE [41–43]. Because most amines show neither natural UV nor fluorescence, chemical derivatization is necessary for detecting derivatives of amines prior to CE separation. As reported by Nouadje et al. [44], contrary to UV detection, LIF allows the selective detection of labelled compounds, independently of micellanous non-fluorescent compounds.

Standard mixture of histamine, tyramine, putrescine, β-phenylethylamine cadaverine and tryptamine mixtures were analysed. All six biogenic amines are separated with baseline resolution in less than 15 mn. Separation efficiencies obtained range from 60.000 theorical plates for tryptamine (tr = 13,48 mn) up to 200.000 theorical plates for histamine (tr = 9.65 mn). Detection limits for the six biogenic amines range from $0.5\ 10^{-10}$ M for B phenylamine up to $1{,}5 \cdot 10^{-10}$ M for tryptamine.

3.4. Analysis of cheese samples

Analyses of dansylated samples with CE–UV detection give very complex electropherograms (data not shown), which cannot be used to identify amino acids and to quantify biogenic amines. Cheeses are very complex mixtures which contain many compounds that absorb in the UV wavelength range.

The selectivity of fluorescent primary amine labelling with FITC allows one to detect only

ammonia, primary amines, amino acids and proteins. The use of FTC-amino acids and FTC-amines reduces the interference with fluorescence of other natural compounds which are generally excited in the UV range [44]. Moreover, high dilution prevents the detection of such naturally fluorescent molecules, which have low concentrations, and which have very low fluorescent quantum yields at 488 nm.

The two electropherograms of fresh and matured pasteurized 1-month-old cheese in Fig. 3A and B demonstrate the use of LIF detection. For cadaverine and putrescine we did not take into account the two labelling site due to the presence of diamino group. The two labelled molecules are eluted with a longer elution time. We did not use an internal standard because the extraction procedure is achieved without multiple steps, in a very short time, and moreover the reproducibility of 2-s hydrodynamic injections of a mixture of 10^{-9} *M* FITC and Rhodamine 123 showed a relative standard deviation of 8.9% ($n = 6$).

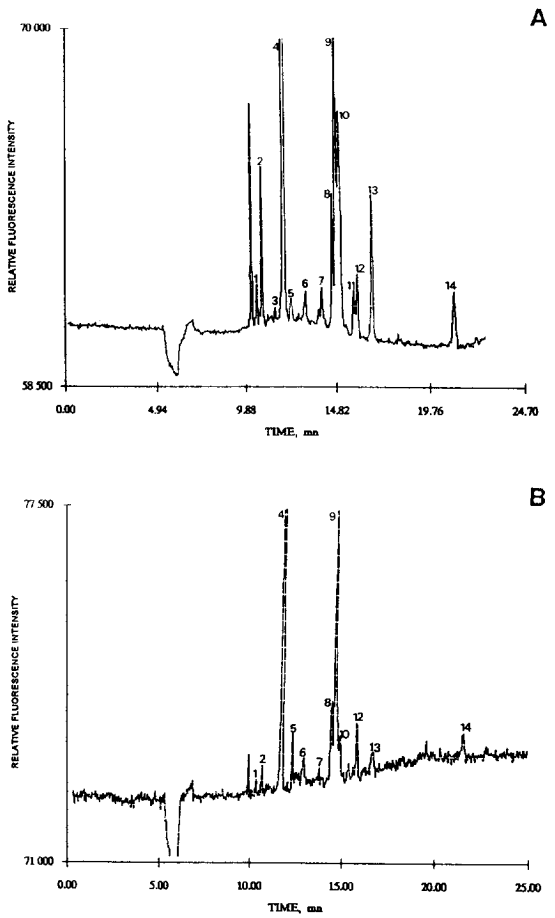


Fig. 3. LIF electropherograms of 100 000-fold diluted samples of (A) fresh and (B) 1-month-old pasteurized cheese. Hydrodynamic injection time 2 s, 80 cm length capillary, 100 m*M* SDS, 100 m*M* boric acid buffer (pH 9.2), 24 kV separation. Peaks: 1 = Lys; 2 = Arg; 3 = ornithine; 4 = ammonia; 5 = putrescine; 6 = blank; 7 = β-phenylethylamine; 8 = cadaverine; 9 = Tyr; 10 = Phe; 11 = Ser; 12 = Ala; 13 = Gly; 14 = Asp.

Table 2 shows the amounts of biogenic amines detected in 100 000-fold diluted samples. Ammonium ions, arginine, lysine, phenylalanine, alanine, aspartic acid, glycine and tyrosine were identified in fresh and matured cheese, but the concentrations are about ten times higher in matured than in fresh cheese. Amino acids come from β-casein degradation during ripening [45,46]. We found relatively high cadaverine and putrescine concentrations in matured and fresh cheese, as described Ten Brink et al. [18] in fermented foods.

Histamine, histidine and tyramine were not detected, but we identify a large amount of tyrosine. We may explain the absence of histamine and histidine by the rapid transformation of histidine into histamine and degradation of the latter during prolonged ripening [47]. No explanation was found for the absence of tyramine, which is normally found in soft cheese [22]. No significant differences were found between pasteurized and unpasteurized cheese.

4. Conclusion

Our "easy-to-use" LIF detector offers very good sensitivity and can be used many times without adjusting the capillary position in front of the laser beam. The ball-lens cell leads to very high sensitivity and decreases the importance of mechanical tolerances. Moreover, compared with other optical collinear arrangements which use a poor UV transmission, high-magnification objective, our optical device includes a small magnification objective and a ball-lens, both of which allow either visible or UV wavelengths to

Table 2
Determination of biogenic amines in fresh and 1-month-old pasteurized or unpasteurized cheese

Sample	Concentration (10^{-9} M)					
	Histamine	Tyramine	Tryptamine	Putresceine	Cadaverine	β-Phenylethylamine
Unpasteurized fresh cheese	UD[a]	UD	UD	0.80 ±0.05	3.10 ±0.12	0.50 ±0.02
Unpasteruised 1-month-old cheese	UD	UD	UD	1.80 ±0.08	34.95 ±1.37	1.40 ±0.05
Pasteurized fresh cheese	UD	UD	UD	3.10 ±0.12	5.62 ±0.21	0.44 ±0.02
Pasteurised 1-month-old cheese	UD	UD	UD	<0.08	28.05 ±0.95	2.65 ±0.05

[a] Undectected.

be transmitted very well. UV-transmitting, high-magnification objectives are very expensive, prices varying between US $5500 and 20 000.

The linear range of external standard calibrations of close to five orders of magnitude allows easy quantification and we have determined the concentrations of certain biogenic amines in soft cheese at the sub-attomole level.

Acknowledgements

We thank Professor Gilles Couderc and B. de Vandière de Vitrac de Bellussière for their comments on the manuscript.

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