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Short communication

Micellar electrokinetic capillary chromatography with laser-induced fluorimetric detection of amines in beer

LeAnn M. Preston, Michael L. Weber, George M. Murray*

Department of Chemistry and Biochemistry, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250, USA

Abstract

Capillary electrophoresis with simultaneous ultraviolet absorbance and laser-induced fluorescence detection is applied to identify and quantify selected amines in beer following derivatization with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. Quantitation was performed using the method of standard addition in order to avoid pH-dependent variations in the reactivity of the derivatizing agent with the added benefit of verifying peak identity. An inexpensive and "easy-to-use" on-column fiber optic fluorescence detection cell is described and implemented in the analysis.

Keywords: Amines

1. Introduction

Interest in the determination of amines in foodstuffs and beverages arises not only from their effect on palatability, but also because they may serve as precursors to the in vivo production of carcinogenic nitrosamines [1,2]. Certain amines in beer have been shown to have pharmacological effects [3]. Biogenic amines are commonly used as markers for the determination of food spoilage or tissue degeneration [4]. Also, many methods for the analysis of amino acids, peptides and proteins are based on the amine functionality [5].

Laser-induced fluorescence detection has been shown to be more selective and sensitive than direct photometric detection methods. Commercial electropherographs that provide for laser fluorimetric detection are costly. A novel fluorescence detector scheme is proposed for the detection of amines in foodstuffs and beverages that is inexpensive and may be built simply and without the need for micropositioners. In this detector, two optical fibers, a 125 μm excitation and a 400 μm collection fiber, are positioned against the bare capillary wall using commercial HPLC fittings. The alignment and positioning provided a detection limit of 1 pmol for derivatized dibutylamine using a modest laser power of 5 mW.

2. Experimental

2.1. Reagents and materials

All materials were obtained from commercial suppliers and used without further purification. Analytical reagent grade chemicals were used along with deionized water to prepare solutions. Standard amines were purchased from Aldrich (Milwaukee, WI, USA). 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was supplied by Pfaltz and Bauer (Stanford, CT, USA). Buffer solutions were prepared

*Corresponding author.

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using sodium dodecyl sulfate (SDS) from Sigma Chemical Co. (St. Louis, MO, USA) and boric acid from J.T. Baker (Phillipsburg, NJ, USA). The pH was adjusted using 1.0 *M* sodium hydroxide. A National Bohemian beer was used for all analyses.

2.2. Instrumentation

CE experiments were performed using an Isco Capillary Electropherograph Model 3850 (Lincoln, NE), equipped with a UV absorbance detector and a vacuum/electro-migration injection accessory. The fluorescence detector was built in-house as shown in Fig. 1. The 488 nm line from a 20 mW American Model 60x argon-ion laser (MWK Industries, Corona, CA, USA) operating at approximately 5 mW was chopped at 240 Hz with a locally fabricated chopper and focused into a 125 μm optical fiber for fluorescence excitation. The 125 μm excitation fiber and the 400 μm collection fiber were positioned flush to the separation capillary at a 90° angle. The 400 μm collection fiber terminated into a 540 nm interference filter (Esco Products, Oak Ridge, NJ, USA) in line with a Model HNF-488-1.0 holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI, USA) and finally, a Model R-928 photo-multiplier tube (Hamamatsu Corporation, Bridgewater, NJ, USA). No focusing lenses were used for collection of fluorescence, rather the collection fiber was placed flush against the first filter. The signal from the PMT

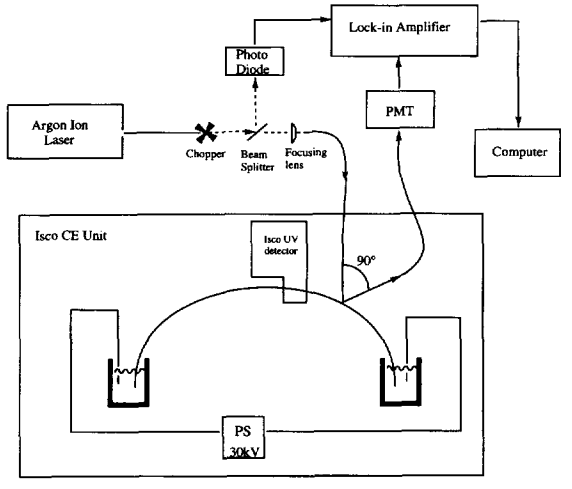

Fig. 1. Schematic diagram of the fluorescence detector.

was monitored using a Model 5101 lock-in amplifier (Princeton Applied Research, Princeton, NJ, USA) and processed using a National Instruments Lab-NB computer board and Lab View software (Austin, TX, USA) installed on a Macintosh II computer. The use of a chopper and a lock-in amplifier helped reduce the baseline drift due to variations in the laser power. Data were analyzed using an Igor Pro software package from WaveMetrics (Lake Oswego, OR, USA).

The fluorescence cell was built in-house using an HPLC cross and a tee, both purchased from Upchurch Scientific (Oak Harbor, WA, USA). The tee was attached, using epoxy, to the cross that had a central opening. The polished ends of the collection and excitation fibers were fitted into sleeves with the appropriate inner diameter and inserted into the cross or tee and held in place by Micro Fingertight fittings (also purchased from Upchurch Scientific). The capillary, with a 2 cm window, passed through the cross and was also held in place by sleeves and Micro Fingertight fittings on both sides of the cross.

Fluorescence emission and excitation spectra were acquired using an SLM 8000C spectrophosphorimeter equipped with SLM 480005 software (SLM Aminco Instruments, Urbana, IL, USA).

2.3. Separation

An 81 cm long bare fused silica capillary with an inner diameter of 75 μm and an outer diameter of 360 μm was used for all separations. The outer polyimide coating was burned off for the UV absorbance and fluorescence cells positioned in-line 23.5 and 6.5 cm from the outlet, respectively. Samples were vacuum-injected at a pressure of −3.4 Pa for 2 s. The separation buffer was 50 m*M* SDS and 10 m*M* borate adjusted to pH 9.3 with sodium hydroxide. The capillary was washed between runs with a 0.1 *M* NaOH solution, rinsed with water and then equilibrated with the separation buffer for 5 min. A separation voltage of 24 kV was applied resulting in a current of 68 μA.

2.4. Sample preparation

The pH of the beer was adjusted to 9.0 with 1.0 *M* NaOH prior to derivatization. For standard addition

and peak identification, beer samples were spiked using concentrated amine solutions (8 to 24 *M*).

Samples were derivatized by adding equal volumes of the beer sample, methanol saturated with sodium acetate, and a solution of 10 mg/ml NBD-Cl in methanol. The mixture was then heated at 60°C for 2 h in a sealed container. The derivatized material was diluted 1:4 prior to injection. The sample preparation procedure was designed to be easily automated. A standard amine solution consisting of 33 ppm of methylamine, dimethylamine, ethylamine pyrrolidine, isobutylamine and isoamylamine in water was derivatized. The derivatized material was diluted 1:100 with deionized water or with 50 m*M* SDS solution and used to obtain fluorescence spectra.

3. Results and discussion

Direct UV absorbance detection of amines in beer at 195 nm is impractical due to the complexity of the sample matrix. Derivatization of the amines with a visible chromophore leads to improved selectivity for absorbance detection and an added ability to measure fluorescence using a 488 nm excitation wavelength. A variety of derivatizing agents are available for use with amines. Following Sepaniak and co-workers [6,7], we chose 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) as a fluorescent tag for amine determinations. This reagent forms a highly fluorescent adduct with primary and secondary amines in good yield and in a reasonable time (1 h). The 7-fluoro compound reacts more rapidly but is much more expensive [8]. NBD-Cl has strong absorbances at both 488 and 337 nm allowing for excitation by either an argon-ion or nitrogen laser. Amines are pre-column derivatized with NBD-Cl and examined with simultaneous UV absorbance and fluorescent detection. It was determined that the use of a holographic notch filter combined with an interference filter gave better signal-to-noise ratio than the use of a 1/8 m monochromator. The use of a pulsed nitrogen source could allow for time-resolved measurements that might result in better detectivity.

The fluorescence excitation spectrum, Fig. 2a, of NBD-derivatized amines shows that the absorbance is well matched to the 488 nm line of the argon-ion laser. Fig. 2b shows the fluorescence emission spectra of the NBD-derivatized amines when dissolved in de-ionized water and in a 100 m*M* SDS solution. An increase in fluorescence intensity is observed in the SDS solution, indicating that the amine derivatives reside within the micelles and thus are insulated from quenching by water. Hence the micelles are useful for enhancing detection limits as well as allowing for separation by MECC.

3.1. Qualitative analysis

The amines of interest were separated in less than 11 min with separation efficiencies ranging from 60 000 to 85 000 theoretical plates. A sensitivity enhancement of the fluorescence detection can easily be seen when compared to the UV absorbance detection as shown in Fig. 3. The unreacted NBD-Cl appears as the largest peak in the UV absorbance electropherogram at 5.0 min but gives a rather weak peak in the fluorescence electropherogram at 5.6 min. Unreacted NBD-Cl exhibits weak fluorescence, but is a strong absorber. The difference in the retention times between the two electropherograms is due to the in-line arrangement of the two detectors. To better show the correlation between the two electropherograms, the absorbance electropherogram was offset slightly in Fig. 3.

Identification of the amines in beer was based on spiking the beer sample with standard amines. Of the amines tested, ammonia, ethylamine, isoamylamine and dibutylamine were found in the beer sample. The peaks were identified as shown in Fig. 3 and the retention times from the fluorescence electropherogram are presented in Table 1. The amines identified correlate with amines known to be found in beer [6].

Since NBD-Cl reacts readily with primary and secondary amino groups, the additional peaks in the fluorescence electropherogram are likely to be NBD-derivatives of other amines yet to be determined, and may possibly be due to amino acids [9].

3.2. Quantitative analysis

The quantitative results from the fluorescence data of the amine analyses are also presented in Table 1. Quantitation was based on linear calibration plots using the peak heights form the fluorescence elec-

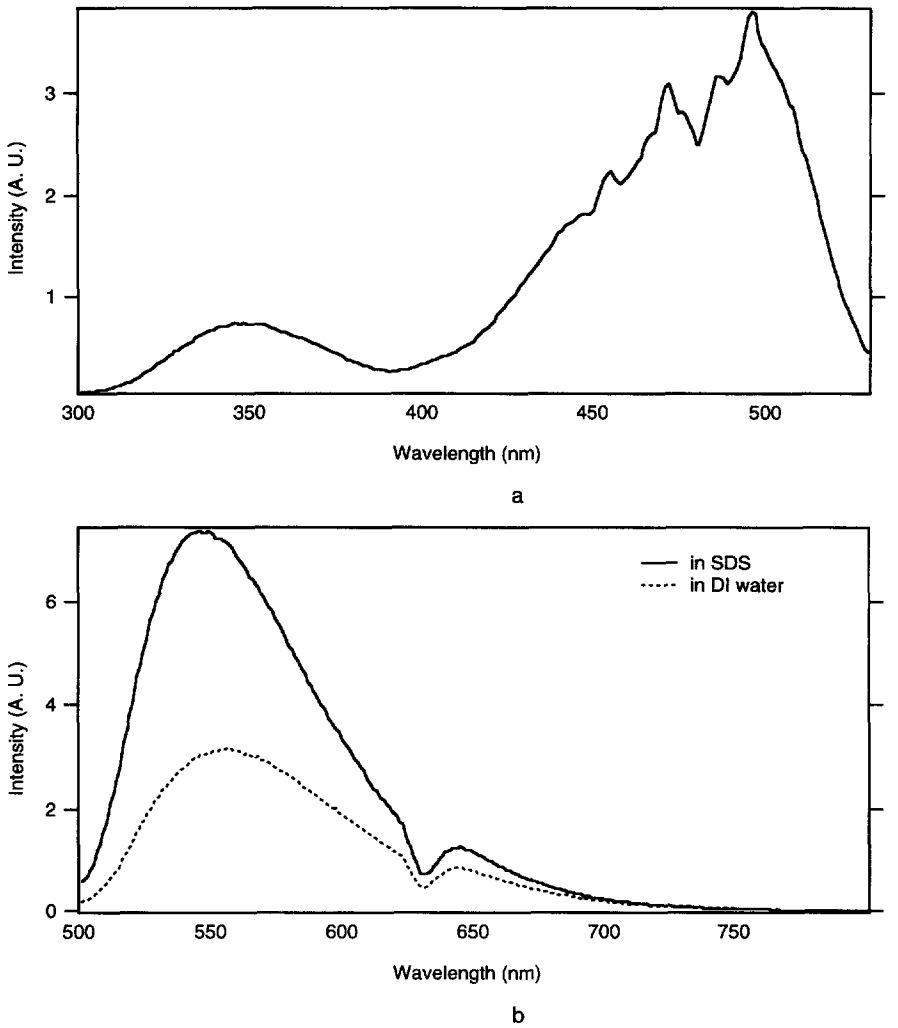

Fig. 2. (a) Excitation (A) of six derivatized amines in a standard mixture monitored at 556 nm. The mixture consisted of 33 ppm of methylamine, dimethylamine ethylamine pyrrolidine, isobutylamine and isoamylamine derivatized with NBD-Cl. (b) Emission spectrum of the standard mixture in DI water (– – –) and in 100 m*M* SDS (——) monitored at 488 nm excitation wavelength.

tropherograms. Detection limits are estimated to be in the lower m*M* range at 3*S*/*N*. Variation in reactivity of the NBD-Cl with the amines is suspected to contribute to the relatively high R.S.D. values. Isoamylamine was also found to be in the beer, but its presence was below the limit of quantitation. The detection limit for NBD-isoamylamine was found to be 0.01 m*M*.

While easily observed using fluorimetric detection, many heavier amines are generally not observable with absorbance detection. This is a result of the increased fluorescence yield of the heavier adducts.

3.3. Applications

The ultimate aim of this research is to develop a method of analysis applicable to the study of in vivo nitration. An important step in the nitrosamine determination is the reduction of the nitrosamine to an amine that could be derivatized. This aspect of

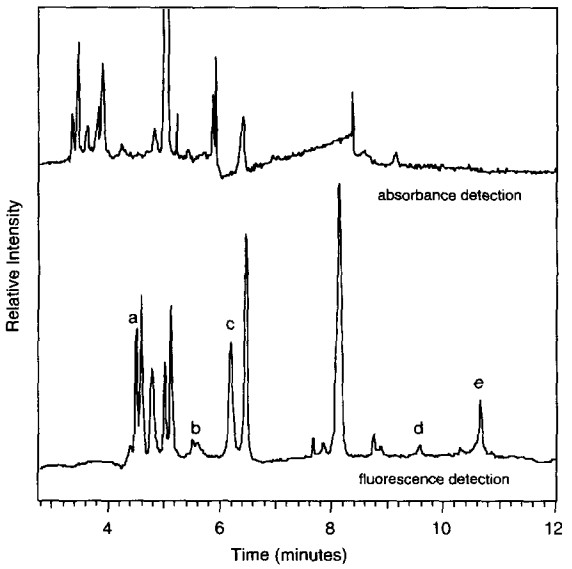

Fig. 3. Absorbance and fluorescence detection of derivatized beer showing differing selectivity and enhanced sensitivity of fluorescence detection. Retention times are shown in Table 1. Peak identification: a=NBD-ammonia, b=excess derivatizing agent, c=NBD-ethylamine, d=NBD-isoamylamine, and e=NBD-dibutylamine.

research is of great significance to the project, since this would allow for the bulk of the experimental work to be performed with innocuous amines rather than the carcinogenic nitrosamines. On the outset we were not sure whether the reduction would stop at the hydrazine compound (which is also derivatizable), or if it would proceed to the amine compound with cleavage of the N=O group. The use of cyclic voltammetry allowed us to ascertain the appropriate reduction potential and the proper conditions for efficient reduction of nitroso-pyrrolidine. Tests were performed using N-nitroso-pyrrolidine in a micro-vial cell with micro-electrodes using a total cell volume of 500 μl. These small devices and small volumes were used to ascertain the practicality of handling and examining very small volume biological samples. The results of the reduction were verified and the recovery calculated (70% in a first trial lasting 10 min at −1.0 V vs. SCE) by analysis of the reduced solution against a pyrrolidine standard using MECC with UV–Vis absorbance detection.

4. Conclusions

It is demonstrated that the selectivity and sensitivity of laser fluorimetric detection can be applied to capillary electrophoresis by the use of a few common fittings, sleeves and optical fibers. The fluorescence detection method is found to be more selective and sensitive for the determination of aliphatic amines in beer than the UV–Vis absorbance detection. The method lends itself to easy automation, if more sophisticated equipment is available. The amine method can be extended to the analysis of carcinogenic N-nitrosamines upon their electrochemical reduction. Nitrosamines may be reduced to their corresponding amines electrochemically and analyzed by the proposed method for amine determination.

Acknowledgments

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Table 1
Summary of the quantitative analysis from fluorescence data for determination of amines in beer

Amine	t_m (min)	Concentration (m*M*)	Concentration (ppm)	R.S.D. (%)	Injected (pmol)	LOD (m*M*)
Ammonia	4.2	1.23	18.5	17	4.2	0.0090
Ethylamine	6.1	4.86	219.1	19	16.6	0.042
Isoamylamine	9.6	na	na	na	na	0.13
Dibutylamine	10.8	0.32	40.8	10	1.1	0.038

na=not analysed.

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