High-Performance Liquid Chromatography Coupled with Chemiluminescence Nitrogen Detection for the Study of Ethoxyquin Antioxidant and Related Organic Bases

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Abstract

The Chemiluminescent Nitrogen Detector (CLND) for use with high-performance liquid chromatography (HPLC) allows for the low-level detection of nitrogen-containing compounds with simple quantitation. The nitrogen selective detector's equimolar response (i.e., equal response for nitrogen independent of its chemical environment) allows for any nitrogen-containing compound to be quantitated as long as the number of nitrogens are known. The HPLC–CLND provides a new detection method for analytes that are not available in large quantities or have unknown chemical or physical characteristics such as oxidation products, metabolites, or impurities. Ethoxyquin is a primary antioxidant that is used to preserve many food products and animal feeds. HPLC–CLND is used in the study of the oxidation products of ethoxyquin because limited quantities of these compounds are available and subsequent calibration curves are difficult to maintain. HPLC–CLND as a new method of detection has been evaluated for its equimolarity of response, linear range, limit of detection, and limit of quantitation.

Introduction

Nitrogen analysis through the use of a chemiluminescence method offers many advantages over the older Kjeldahl analysis method (1). Chemiluminescence nitrogen detection (CLND) after chromatography has been demonstrated and is of considerable current interest. Typically, chemiluminescence detection after chromatography takes place in the gas phase and employs a post-column chemiluminescence reaction that involves nitrogen monoxide and ozone as follows:

$$NO + O_3 \rightarrow NO_2 + O_2 \qquad \text{Eq. 1}$$

$$NO_2 \rightarrow NO + h\nu \qquad \text{Eq. 2}$$

where hν is the electromagnetic radiation.

The use of this chemistry as a chromatographic detector is based on the early work of Fontijn et al. (2). The first reaction step involves the combustion of nitrogen-containing molecules in a high-temperature furnace (approximately 1000°C) with an excess of oxygen. The products from this combustion include nitrogen monoxide, water, carbon dioxide, and other combustion products or oxides. Nitrogen dioxide is not formed because in a combustion process that takes place above 600°C, all nitrogen dioxide will dissociate fully (3). Nitrogen monoxide is then reacted with an excess of ozone, and the energy of the reaction produces nitrogen dioxide in an excited state, which provides the release of photons (i.e., chemiluminescence).

In order to provide total nitrogen determination, the reaction sequence mentioned must meet certain criteria. First, the reaction converting nitrogen-containing analytes to nitrogen monoxide must be quantitative. Secondly, all nitrogen (regardless of their chemical environment) must provide the same signal for quantitation. Finally, the chemiluminescence that takes place must be able to be monitored by a common detection process. These criteria were achieved and the detection process was patented by Parks and Marietta in 1977 (4).

The CLND was initially developed for gas chromatography (GC) (5–12). Although, it has also been found useful in supercritical fluid chromatography (SFC) with both 100% CO_2 and limited levels of a methanol modifier (13–15). The basic design of the postcolumn reaction does not make the same CLND that is used with GC and SFC directly amenable for use with high-performance liquid chromatography (HPLC). A liquid sample must be nebulized for introduction into the high-temperature furnace. The rate of introduction must be optimized in order to allow for proper combustion of the introduced liquid. Finally, water must be eliminated from the system prior to the reaction of nitrogen monoxide with ozone.

Prior to 1992, several HPLC–CLND methods had been reported (16), but they did not find wide application because of the problem of water elimination, which meant long drying times for reaction chambers and inlet lines of the detector. In 1992, a CLND for use with HPLC was first reported. Fujinari et al. (6) reported the determination of ammonium nitrogen in waste water with

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the CLND, which exhibited a nitrogen detection limit of 5 ng.

Fujinari et al. (8) continued to investigate the HPLC–CLND system wherein a reversed-phase separation of peptides was conducted. The research focused on the ability of the CLND to simplify the separation of complex mixtures by only giving a response to nitrogen-containing compounds. Fujinari et al. (17) also used the CLND with size-exclusion chromatography in order to estimate the molecular mass distribution of food-grade protein hydrolyzates. In this research, CLND provided an accurate peptide profile, but simultaneous UV detection could not. In recent years, Fitch et al. (18) have briefly discussed the strengths of CLND for use with HPLC in combinatorial chemistry. Taylor et al. (19) also showed the use of HPLC–CLND in combinatorial chemistry by first evaluating the detector and then showing its ability to simplify and quantitate chromatograms.

Elemental selective detection offers many advantages over other commonly used detection processes. The CLND allows for a selective, sensitive, and simple analysis to be performed as compared with other spectroscopic detection processes (20) that require a molecule to have a chromophore or the ability to fluoresce. Ultraviolet detectors, although the most common and useful in HPLC, can only detect molecules that undergo an absorption process when irradiated with a specific wavelength of light. Fluorescence detectors are also based on a quantum process of the absorption and release of energy. A nitrogen selective detector only requires nitrogen be present.

In addition, CLND has also been reported to have an equimolar response (19) regardless of the chemical state of nitrogen. This allows nitrogen-containing compounds to be easily quantitated. A known standard is required to accurately quantitate an analyte with UV, fluorescence, and mass spectrometry (MS), because analytes have different molar absorptivities, response factors, or ionization efficiencies. The fact that CLND does not require a standard makes the CLND an indispensable tool in combinatorial chemistry and any situation in which degradants, byproducts, oxidation products, or metabolites must be detected and quantitated in a timely manner. We wish to report in this study the HPLC–CLND of a selection of nitrogen-containing compounds of varying basicity with emphasis on ethoxyquin and its metabolites. The CLND employed has a greater capacity for larger liquid sample volumes and a membrane dryer that basically prevents water from reaching the detection zone. The oxidation products of ethoxyquin have never been monitored to our knowledge.

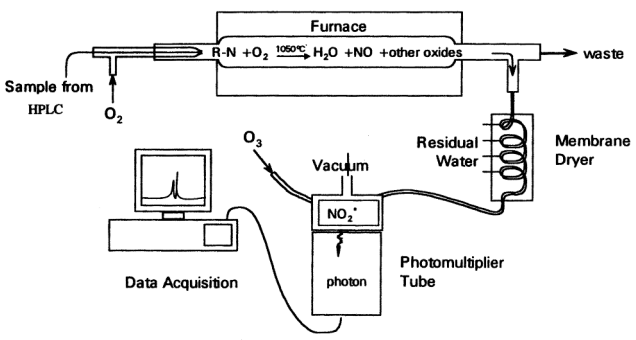

Figure 1. Schematic for a CLND.

Experimental

Instrumentation

A Model 8060 CLND nitrogen-specific HPLC detector from Antek Instruments (Houston, TX) was interfaced to a Hewlett-Packard (Little Falls, DE) Model 1050 HPLC. The HPLC system was plumbed with 0.005-inch PEEK tubing from ChromoTech (Apple Valley, MN). The CLND allowed patented pryo-chemiluminescence nitrogen detection with a sample introduction system and electronic mass flow controllers. Fused silica (50-µm i.d., 150-µm o.d.) from PolyMicro Technologies (Tucson, AZ) was used in the Antek CLND nebulizer as a replacement for the steel tubing that came with the CLND. Data were recorded by a Hewlett-Packard 3394 Integrator. A schematic of the HPLC–CLND system is shown in Figure 1.

A chromatographic separation of the mixture of nitrogen-containing compounds was achieved using 3-µm particle-size C18

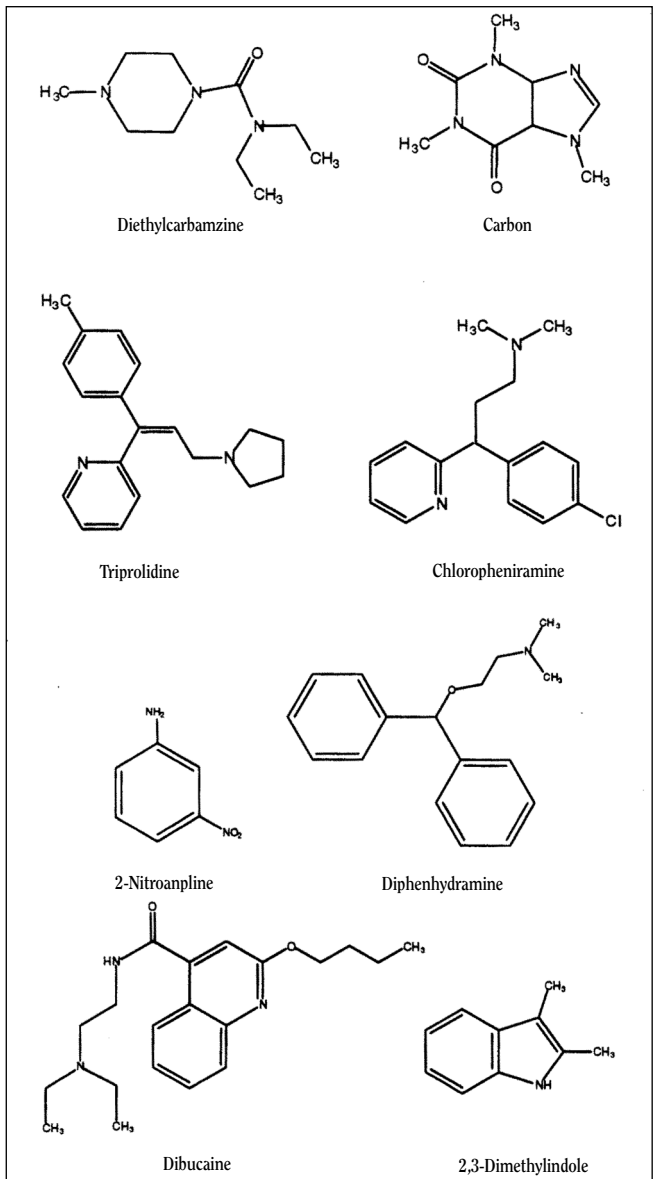

Figure 2. Chemical structures of the eight nitrogen-containing compounds used in the HPLC–CLND evaluation.

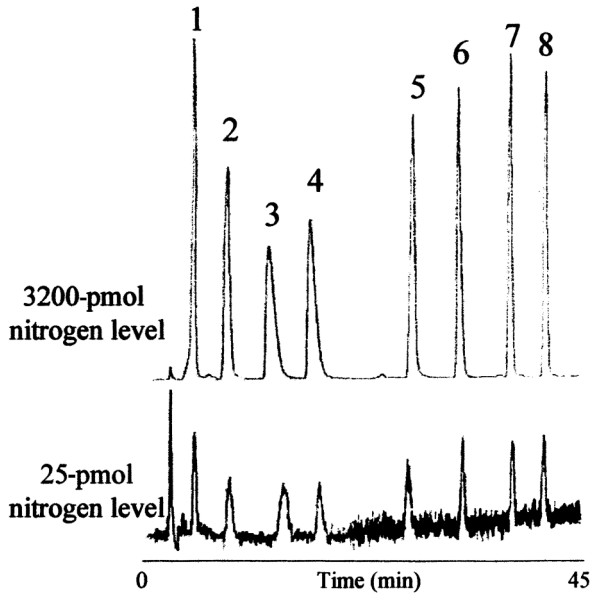

Figure 3. Reversed-phase HPLC–CLND separation of eight nitrogen-containing compounds: diethylcarbamazine, 1; caffeine, 2; triprolidine, 3; chlorpheniramine, 4; 2-nitroaniline, 5; diphenhydramine, 6; dibucaine, 7; and 2,3-dimethylindole, 8.

Table I. CLND Equimolarity Data

Compound	MW	N atoms	No. of (pmol N)	LOD* (pmol N)	LOQ† (CLND/pmol N)	Slope R^2
Diethylcarbamazine	391.4	3	25.6	51.2	10057	0.9996
Caffeine	194.2	4	24.2	48.5	10157	0.9999
Triprolidine	390.9	2	24.7	49.4	9513	0.9995
Chloropheniramine	314.9	2	24.8	49.5	10552	0.9997
2-Nitroaniline	138.1	2	26.0	52.0	10639	0.9997
Diphenhydramine	291.8	1	24.6	49.2	10613	0.9998
Dibucaine	343.5	3	24.7	49.4	10105	0.9999
2,3-Dimethylindole	145.2	1	24.6	49.2	9964	0.9999

* LOD, Limit of detection. Signal-to-noise ratio = 3:1.
† LOQ, Limit of quantitation. Signal-to-noise ratio = 5:1.

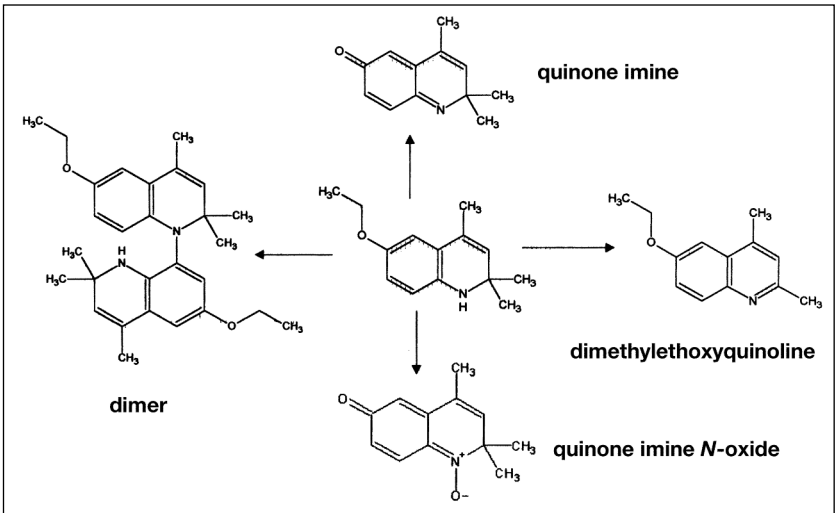

Figure 4. Possible ethoxyquin oxidation products and their origin.

LUNA, cyano LUNA, or silica LUNA columns (150 × 1 mm) from Phenomenex (Torrence, CA). For the evaluation of the CLND, the C18 column was eluted at 50 µL/min with a linear gradient (15% to 85%) of solvent B for 30 min and held until all analytes eluted. Solvent A was 0.15% trifluoroacetic acid (TFA) in water, and solvent B was 75% methanol, 25% isopropanol, and 0.15% TFA. Samples were injected onto the column using a 2.5-µL injection loop that was filled using a threefold overfilling technique.

For the normal-phase separation of the oxidation products of ethoxyquin, both a cyano and silica column were used. The column was eluted at a flow rate of 50 µL/min using an isocratic mobile phase made up of previously prepared mixtures of hexane and isopropanol. For the reversed-phase separation of the oxidation products of ethoxyquin, a C18 column was used with a gradient of 55% to 95% solvent A (water) for 20 min. The mobile phase component B was 75% methanol and 25% isopropanol.

Chemicals and reagents

HPLC-grade methanol, isopropanol, TFA, and hexane were purchased from EM Science (Gibbstown, NJ). HPLC-grade water was purchased from Mallinckrodt (Paris, KY). Dibucaine, ethoxyquin, chloropheniramine, triprolidine, caffeine, diphenhydramine, diethylcarbamazine, and 2,3-dimethylindole (Figure 2) were purchased from Sigma (St. Louis, MO). 2-Nitroaniline was purchased from Aldrich Chemical Co. (Milwaukee, WI). All chemicals were used without further purification. HPLC solvents were used in preparing solutions of the various nitrogen-containing compounds. The argon that was used for the CLND nebulizer and grade 4.3 oxygen for the nebulizer, pyrolysis furnace, and ozone generator were purchased from Airco (Murray Hill, NJ). Ethoxyquin oxidation products were prepared by previously reported procedures, taken to dryness, and then diluted with either isopropanol for normal-phase separations or methanol for reversed-phase separations.

Results and Discussion

The objective of this study was to evaluate the equimolarity, linearity, linear range, limit of quantitation, and limit of detection of an HPLC–CLND system. These evaluations were performed using a variety of nitrogen-containing compounds and gradient mobile phase elution. Once the detector's characteristics were confirmed, the HPLC–CLND system was used to study the separation and detection of ethoxyquin and selected oxidation products of the antioxidant.

Eight compounds containing nitrogen were separated via a reversed-phase separation using a C18 stationary phase. The mobile phase gradient used two buffer solutions made at 0.15% with TFA. The TFA was added to change the mobile phase acidity to pH 2 in order to insure that all

basic compounds were in the protonated form. A low pH was chosen instead of a high pH to inhibit mobile phase dissolution. The CLND nebulization process was optimized for proper combustion and to minimize sample loss on the side of the pyrotube. A mixture of 75% methanol/25% isopropanol and water allowed the viscosity of the mobile phase to remain constant during gradient delivery as compared with a gradient using water and only methanol, which would gradually decrease in viscosity as the percent methanol increased. Changes in the mobile phase viscosity were believed to affect the nebulization process and could deteriorate detector response, thus influencing equimolar response.

Reversed-phase separation with the CLND of the eight nitrogen-containing compounds, with the gradient conditions previously noted, yielded a baseline resolution of all compounds. The gradient was started at a high concentration of water (85%) in order to get a proper retention of diethylcarbamazine. Triprolidine and chloropheniramine provided poorer peak shapes when compared with other compounds.

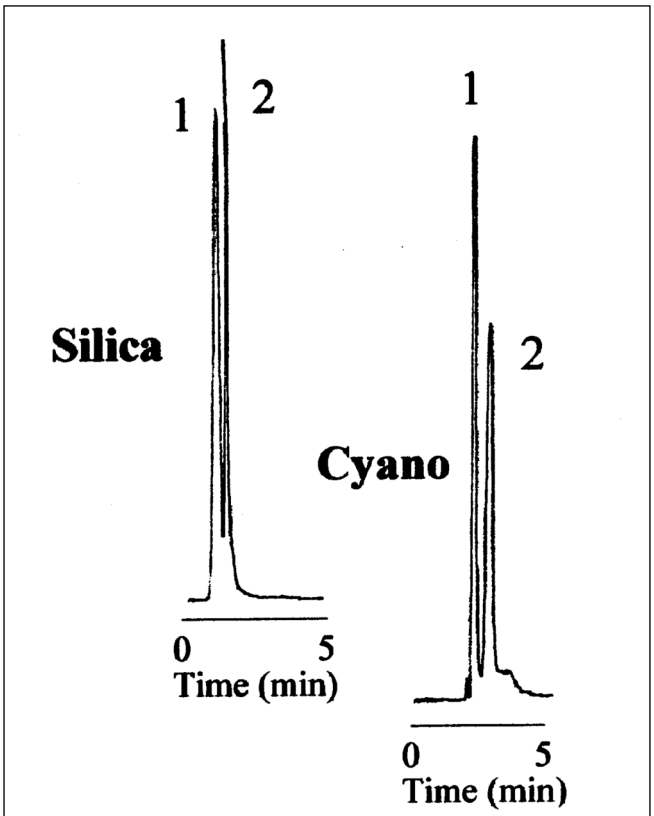

Figure 5. Normal-phase HPLC–CLND of selected nitrogen-containing compounds on two stationary phases: (1) ethoxyquin dimer and (2) ethoxyquin.

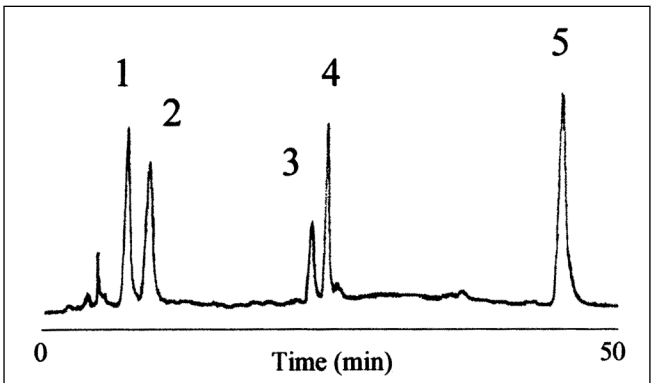

Figure 6. Reversed-phase HPLC–CLND of ethoxyquin oxidation products: quinone imine *N*-oxide, 1; quinone imine, 2; dimethylethoxyquinoline, 3; ethoxyquin, 4; and dimer, 5.

An analysis of the peak areas resulting from these compounds at levels of 25, 50, 100, 400, 800, 1600, 3200, and 6400 pmol of nitrogen provided a family of calibration curves and detector response data (Table I). Figure 3 illustrates the separation with nitrogen at the 3200-pmol and 25-pmol level. The calibration curves showed equal CLND response for all eight compounds investigated. Triprolidine was the only real outlier, as can be seen from the slope data in Table I. The slope data for the remaining seven components indicated that no other slopes on a per-mole nitrogen basis differed more than a few percent from the total average. Table I also shows the linearity, limit of detection, and limit of quantitation for all of the studied compounds. Although each compound had a different number of nitrogen atoms in various environments, dilution to a similar level of nitrogen content provided the same detection limit and response factor for all compounds. These data proved the equimolarity of the HPLC–CLND system and also provided the detector characteristics for a comparison with other HPLC detectors. In summary, the HPLC–CLND system showed a response independent of nitrogen type, linearity from 50 to 6400 pmol of nitrogen, and a sensitivity of 25 pmol nitrogen (i.e., 0.35 ng nitrogen).

It was of interest to determine the feasibility of CLND detection for normal-phase liquid chromatography. The only change made to the chemiluminescence detector for normal-phase conditions was an increase in the oxygen flow to 300 mL/min and a turning off of the argon flow. This was done because the normal mobile phase is 100% organic. Many require a more oxygen-rich environment to fully combust all of the mobile phase and analytes in the pyrotube. Also, the argon flow was turned off so that nebulization would not be affected by an increase in the oxygen flow. The normal-phase HPLC–CLND system was tested on an in-house prepared mixture of ethoxyquin and associated oxidation products. Structures of these compounds are shown in Figure 4. Their molecular formulation was established via GC–MS. After method development, the best chromatogram achieved is illustrated in Figure 5. Separations were achieved with 100% hexane on both cyanopropyl and silica stationary phases.

A reversed-phase separation of the antioxidant mixture with CLND detection was investigated next. Using a binary mobile phase of 75% methanol/25% isopropanol (A), 100% water (B), and a gradient of 55% A/45% B to 95% A/5% B for 20 min resulted in the separation shown in Figure 6. In this separation, the parent and its four metabolites are over 90% resolved. A total ethoxyquin determination could be made because the partially unresolved peaks each contained the same number of nitrogen atoms.

The HPLC separation of ethoxyquin and some of its oxidation products with CLND evaluation should provide the groundwork for future studies concerning the fate of antioxidants such as ethoxyquin. Primarily, the appearance and disappearance of nitrogen-containing oxidation products over time could provide insight into the mechanisms that describe ethoxyquin's antioxidant action for a variety of chemical systems.

Acknowledgments

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