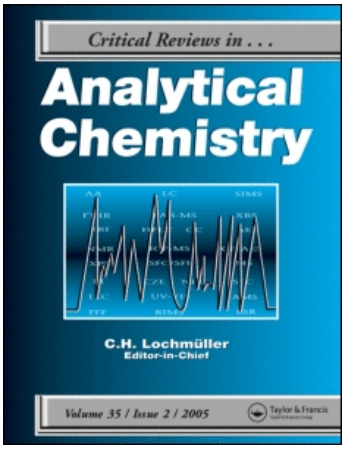

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Chemiluminescent Methods in Agrochemical Analysis

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Chemiluminescent Methods in Agrochemical Analysis

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ABSTRACT: The present review is devoted to applications of chemiluminescence for agrochemical analysis. The scope of chemiluminescent techniques to the determination of pesticides and fertilizers are presented, including methodology and practical considerations. Specific applications to organophosphorus, carbamates, nitrogen compounds, sulfur compounds, as well as the incorporation of chemiluminescent immunoassay are included for this purpose. The literature reviewed covers the papers of analytical interest that have appeared in approximately the last 5 years, but relevant earlier references are also included.

KEY WORDS: chemiluminescence, pesticide analysis, herbicide analysis, fertilizer analysis, chemiluminescent immunoassay.

I. INTRODUCTION

Pesticide is a term used in a broad sense for chemicals, synthetic or natural, that are used for the control of insects, fungi, bacteria, weeds, nematodes, rodents, and other pests.[1] The use of pesticides must be regulated in such a manner that the intake of a pesticide residue does not exceed the acceptable daily intake. The monitoring of pesticides in food is one of the most important aspects of minimizing potential hazards to human health. The accuracy and precision of the methods to be followed for the analysis of pesticide residues are important considerations to ensure that the data generated are meaningful. During recent years, the number of publications on pesticide residue analysis has increased significantly, and important advances have been made in the development of methods for a wide variety of sample types. Most pesticide analysis has been performed using methods involving solvent extraction of analyte from the sample matrix by blending, cleanup to remove interfering coextractans, qualitative and quantitative determination with element-selective detectors.[2] Although such methods continue to predominate, new and improved methods and technologies for the analysis of pesticide have continued to evolve rapidly over the last few years. In this review we expose chemiluminescent methods as employees in agrochemical product analysis of the last few years.

We can define chemiluminescence (CL) as the emission of electromagnetic radiation (ultraviolet, visible, or infrared) produced in a chemical reaction from the decay of an excited species to the electronic ground state.[3] If the reaction occurs in a living system or is derived from one, the process is called bioluminescence (BL). The term chemiluminescence was first coined in 1888 by Eilhardt Weidemann, as part of his classification of "cold light" (luminescence).[4] However, the first

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report of artificial chemiluminescence occurred more than 200 years earlier in 1669. The German physician Henning Brand, hoping to make his fortune from alchemy, isolated from urine a substance that glowed continuously in the dark. He called the substance "phosphorus mirabilis", but it is better known today as white phosphorus. This product was extensively studied by Sir Robert Boyle, who published the results in two pamphlets: *The Aerial Noctiluca* (London, 1680) and *The Icy Noctiluca* (London, 1681). In the latter part of the 19th century it was found that rather simple organic compounds could also give rise to chemiluminescence.[5] Thus, Radziszewski found that lophine (2,4,5-triphenylimidazole) emitted green light when it reacted with oxygen in alkaline solution. This discovery was published in the year 1877. Today many chemiluminescent systems are known, both biological and non-biological. A lot of inorganic and organic chemical reaction produce light because one of the reaction products is formed in an electronic excited state, and emits the radiation on falling to the ground state. A general description of the reactions is:[6]

$$A + B \rightarrow C^* + D$$

$$C^* \rightarrow C + \text{Light}$$

where (*) indicates an electronically excited state. This would be a rather simple case and would be classified as direct chemiluminescence. Sometimes, the excited product (C*) is an ineffective emitter, but by transfer of the excitation energy to an efficient fluorophore (F) added to the system, a considerable increase of luminescent intensity is obtained:

$$C^* + F \rightarrow C + F^*$$

$$F^* \rightarrow F + \text{light}$$

The emission is now identical with the fluorescence of F and this would be classified as indirect, sensitized, or energy transfer chemiluminescence. Hence, the fluorophore in sensitized CL is selected to provide the optimum compromise between excitation and emission wavelengths, chemical stability, and quantum yield. This is in contrast to direct CL, wherein reactivity and fluorescent properties reside in a single molecule.[7] Chemiluminescent emission can be characterized by the four parameters of color, intensity, rate of production, and decay of intensity and the reaction conditions have a significant effect on the progress of the chemiluminescent reaction. From the analyst's point of view, several factors must be considered in developing a chemiluminescent method.[8] Two of the most obvious are the efficiency of the CL reaction, which ultimately affects sensitivity and detection limits, and the reaction kinetics, which determine the precision and the sample throughput. In practice, these parameters are affected by reaction conditions, including solvents, concentrations, pH, and purity of reagents. Chemiluminescence in analytical chemistry has numerous advantages such as superior sensitivity, safety, rapid, and simple assays and controllable emission rate,[9] but it has some disadvantages, such as poor reproducibility and long observation times, although not all the reactions are slow. At the moment, there are numerous reports published on the importance of chemiluminescent methods in analytical chemistry and their applications in the determination of a great variety of compounds, for example, in the drug,[10] food,[11] environmental,[12,13] or clinical,[14] fields.

II. DETERMINATION OF ORGANOPHOSPHORUS AND CARBAMATES

Detection of environmental pollutants at the levels specified by regulatory agencies such as the Environmental Protection Agency (EPA) has been a challenging problem. Our focus in this section is on the detection and quantification of organophosphorus and carbamate pesticides. The persistence of organochlorine insecticides for long periods of time and their adverse effects on human health led to the development of laws for the control of their use. Progressively, they are being replaced by less toxic molecules such as organophos-

TABLE 1
Determination of Organophosphorus and Carbamate Pesticides

Method	Analyte	Ref.
Based on inhibition of acetylcholinesterase	Paraoxon and Aldicarb	6
	Carbofuran, dimethoate, bromophos, ethylparathion, chlorofenvinphos, and pyrimicarb	17
Based on alkaline phosphatase-catalized chemiluminescence	Paraoxon	18
	Paraoxon and methylparathion	20
Based on reaction with peroxide and indole	Diethoxyphosphorylcyanide	23

phorus and pyrethroid insecticides. The need for simple, rapid, and specific determination of organophosphorus and carbamate pesticides with a low detection limit has led to the development of a variety of analytical methods for their identification and quantification.[1,15] We now describe some chemiluminescent techniques based on several CL reactions that are summarized in Table 1.

A. Determination of Organophosphorus and Carbamates Based on Inhibition of Acetylcholinesterase

Organophosphorus compounds are powerful inhibitors of the enzyme involved in nerve function.[16] These compounds can form stable complexes with acetylcholinesterase (AChE), thus preventing, by phosphorylation, its function. AChE and acetylcholine are found in the nervous system. The neurotransmitter acetylcholine binds to the synapse receptors in order to transfer the electrical signal from one neuron to the other, or to the muscular cell.[17] The hydrolysis of acetylcholine by AChE allows its release from the receptor, which is then free and ready for a further stimulation. Organophosphorus and carbamate pesticides have the property to inhibit this esterase activity and in this way to break the nervous influx, leading to the death of the insects.

Roda et al.[16] describe a simple flow-injection determination of Paraoxon, as a representative organophosphorus pesticide, by three coupled enzyme reactions (where two or three enzymes are immobilized) with a chemiluminescent end-point detection according to the following scheme:

$$\text{Acetylcholine} \xrightarrow{\text{acetylcholinesterase}} \text{acetate} + \text{choline}$$

$$\text{choline} \xrightarrow{\text{choline oxidase}} \text{betaine} + H_2O_2$$

$$\text{luminol} + 2\,H_2O_2 + OH^- \xrightarrow{\text{peroxidase}} \text{aminophthaleine anion} + N_2 + 3\,H_2O + \text{light}$$

Because also carbamates inhibit, but reversibly, acetylcholinesterase, the authors have determined Aldicarb as a representative compound of this pesticide group.

Acetylcholinesterase in solution or immobilized on methacrylate beads (Eupergit C) was coupled to choline oxidase and peroxidase immobilized on Eupergit C. In this system choline formed by acetylcholinesterase was oxidized by choline oxidase and H_2O_2 produced was determined via the luminol/peroxidase luminescent reaction. The detection limits (3 σ) for Paraoxon and Aldicarb were 0.75 $\mu g\ l^{-1}$ and 4 $\mu g\ l^{-1}$, respectively, when soluble acetylcholinesterase was used under the following optimized experimental conditions: 56 μM luminol in working solution, sample volume 60 µl, flow-rate 0.3 ml min^{-1}, and 60 min incubation time. The results obtained by the developed method were in more in alignment with those obtained by a commonly used colorimetric test. The method is, in comparison with other systems described recently, similarly simple and fast, but with a greater sensitivity and good reproducibility.

A highly sensitive chemiluminescent assay was developed and optimized by Moris et al.[17] for acetylcholinesterase activity using the two consecutive reactions of choline oxidase and peroxidase that produce photons when luminol is used as a substrate. The assay has been used for the detection of carbofuran, a carbamate pesticide that is used often. The same assay can be adapted to all pesticides inhibiting the nervous AChE activity. Organophosphorus and carbamate molecules such as dimethoate, bromophos, ethylparathion, chlororfenvinphos and pyrimicarb were tested in this work. One advantage of this pesticide detection system is the possibility to adapt assay to a simple portable system since light emission is the end signal. The test is carried out using a multidish plate, which is disposed on a 20,000 iso ultrahigh speed black and white instant film (Polaroid type 612). The picture was then processed for 45 s and then coated for protection against fading. Four concentrations of pyrimicarb were tested. The results clearly show the lowering of the light emission with increasing pyrimicarb concentrations. The detection limits of the most of the developed methods are in the ngl^{-1} level. The described method is less sensitive, but it is cheaper and faster. The possibility of using a Polaroid camera mounted with ultra-sensitive films also affords valuable possibilities for such assays.

B. Determination of Organophosphorus Compounds Based on Alkaline Phosphatase Catalized Chemiluminescence

Recently, a chemiluminescence technique has been investigated in the University of Massachusetts, Lowell,[18-22] to detect organophosphorous based pesticides with high sensitivity. The same detection scheme can be applied in developing a fiber optic chemiluminescence-based biosensor. Ayyagari et al.[18,21,22] describe experiments involving detection of chemiluminescent signals generated by free and immobilized (on polymer matrix/glass surface) enzyme alkaline phosphatase. Alkaline phosphatase acts on a wide range of monoesters of orthophosphoric acid, both aliphatic, such as glycerol 1-phosphate, and aromatic, such as 4-nitrophenylphosphate. The enzyme catalyzes the dephosphorylation of chloro 3-(4-methoxy spiro [1,2-dioxetane-3-2′-tricyclo-{3.3.1.1}-decan(-4-yl) phenyl phosphate (CSPD). One of the products of this reaction is generated in a highly excited state, which, after subsequent de-excitation, emits blue light as a chemiluminescent signal that decays with time. In the presence of organophosphorous-based pesticides, the enzyme activity is inhibited, which leads to the generation of a weaker chemiluminescence signal. Thus, the signal intensity, which is inversely proportional to the inhibitor concentration, is related to the amount of the pesticide in solution. Detection of paraoxon at a concentration of about 50 ppb is achieved with this system. The detection methodology forms an integral part of a biosensor under development and is adaptable to incorporating optical fibers for remote detection of pesticides. The enzymes immobilized on the fiber tip catalyze the reaction and the chemiluminescent signal can be

collected and transmitted by the same fiber. Gao et al.[19] have investigated the effect of different tapered fiber tip configurations on coupling efficiency of chemiluminescence light. In their fiber optic biosensor, alkaline phosphatase was immobilized on the surface of the fiber tip by a sol-gel layer. The chemiluminescent signal was collected at the tapered fiber tip, with the shortest length, and tranmitted by the same fiber to a CCD array detector cooled to 0°C. The experiments demonstrated that weak CL signals can be detected by choosing the tip geometry of the optical fiber that has the highest coupling efficiency. Pande et al.[20] have described a methodology for the creation of a molecular assembly consisting of the enzyme alkaline phosphatase immobilized onto a glass surface using a biotinylated conjugated copolymer, poly(3-undecylthiophene-co-3-thiophenecarboxaldehyde) 6-biotinamido hexano hydrazone. The biotinylated polymer is attached to the inside walls of a silanized glass capillary via hydrophobic interactions and a streptavidin conjugated alkaline phosphatase is interfaced with the polymer through the classical biotin-streptavidin interaction. The authors have utilized this molecular assembly for the detection of paraoxon and methyl parathion at a concentration down to 500 to 700 ppb.

C. Determination of Organophosphorus Compounds by Reaction with Peroxide and Indole

Free oxalic acid is oxidized by peroxide in presence of a dehydrating agent to CO_2 and H_2O. In all probability monoperoxalate and dioxetane dione originate intermediately. The latter desintegrates thermically into two molecules of CO_2, one of them getting into an electronically excited single state and partial emitting light when returning to its ground state. In the presence of a suitable fluorescer it is possible to sensitize this chemiluminescence.

Albrecht et al.[23] propound a method to determine the organophosphorous compounds with the general formula: (RO)R'P(O)X (R = alkyl, aryl, alkoxy, dialkylamino, X = CN, F, etc.) by the generation of a highly fluorescent indoxyl and indigo white by the reaction of the phosphorus compound with peroxide and indole. An ethanolic solution of diethoxyphosphorylcyanide was incubated for 60 s with sodium perborate and indole. Dosed by the injector, the sodium oxalate solution and a solution of *bis*(ciclohexyl)carbodiimine in absolute ethanol were added to the sample adjusted to pH = 1 by HCl. This technique can be used as a suitable alternative for the determination of organophosphorus and it can obtain a detection sensitivity up to values < 1 μmol/l.

III. DETERMINATION OF NITROGEN COMPOUND

A wide variety of methods and techniques are available for the determination of N-compound in the agricultural field but only a few chemiluminometric methods have been established. In Table 2 we have summarized the most common Cl techniques that have appeared in the last year.

A simple continuous-flow chemiluminometric method for the determination of ammonium ion is described by Halvatzis et al.[24] The proposed automated method is the first direct chemiluminometric method developed for ammonium ions. The work involves the development and optimization of a method for the analysis of ammonium ion in fertilizers, based on a new chemiluminescent (CL) reaction between *N*-bromosuccinimide (NBS) and ammonium ion in alkaline medium. A number of fluorescent compounds enhance de CL emission intensity if present in the reaction medium. The reaction that occurs is:

$$NH_4^+ + OH^- \rightarrow NH_3 + H_2O$$

$$3NBS + 2NH_3 + 3OH^- \rightarrow 3NHS + N_2 + 3Br^- + 3H_2O$$

(NHS: succinimide)

TABLE 2
Determination of Nitrogen Compounds

Method	Analyte	Ref.
Based on reaction with *N*-bromosuccinimide	NH_4^+ in fertilizer	24
Oxidation to NO and CL reaction with O_3	Harmful nitrogen from nitrogen fertilizer	26
	Glyphosate	27
	Propazine, atrazine, simazine	28

The reaction belongs to the group of CL redox reactions that generate nitrogen. Nitrogen is probably produced in an excited state and has the ability to chemiexcite coexisting fluorophores, such as dichlorofluorescein. Dilution is the only sample pre-treatment required. The detection limit (blank + three times its standard deviation) was 0.032 μg/ml of ammonium ion and the relative standard deviations for 10 measurements were 1.6 and 0.4% for 0.540 and 3.60 μg/ml, respectively. The detection limit, sensitivity, and selectivity compare very well with existing analytical methods for ammonium ion. The results are repeatable and show that the method can be applied to the determination of ammonium ion in fertilizers.

Using a recently developed pyro-chemiluminescence (PCLM) method,[25] Burba[26] has determined harmful nitrogen as a criterion of beet quality. Harmful nitrogen represents the soluble nitrogen of sugar beet which cannot be eliminated during traditional juice purification. For this reason harmful nitrogen is an important criterion of beet quality. The nitrogen can be determined in beet extracts clarified with aluminum sulfate as aluminum sulfate soluble nitrogen (AIN). The harmful nitrogen content of sugar beet from a variety and nitrogen fertilizer trial is presented. The PCLM is able to meet the requirements of large-scale analyses in beet laboratories and can be used for harmful nitrogen determinations in beet brei extracts clarified with aluminum sulfate. The total soluble nitrogen content of these extracts is closely correlated to the total nitrogen of corresponding thick juices that represents the harmful nitrogen of sugar beet by definition. The reaction mechanism as principle of the pyro-chemiluminescence method is:

$$\text{N-compound} + O_2 \rightarrow NO + \text{other products}$$

$$NO + O_3 \rightarrow NO_2^* + O_2 \rightarrow NO_2 + O_2 + \text{light}$$

Organic and inorganic nitrogen (including nitrate) are oxidized in air at 1000°C to form nitric oxide, which is converted to an excited nitrogen dioxide by ozone. As this excited molecule decays, light is emitted and sensed by a photomultiplier. The resulting, amplified signal is specific for nitric oxide and thus proportional to the nitrogen content in the original sample. Total soluble nitrogen of clarified beet brei extracts and total nitrogen in thicl juices (≈ harmful nitrogen), are closely correlated. As expected, harmful nitrogen of sugar beet increases with N-fertilization parallel to the α-amino-N content.

The glyphosate (GP) is a nonselective herbicide widely used for weed and vegetative control. Sen and Baddoo[27] describe a method for the determination of this compound as N-nitrous derivative based on: (1) nitrosation of glyphosate to N-nitrous-GP, (2) HPLC separation on an anion-exchange column, (3) post-column denitrosation of N-nitrous-GP to nitric oxide, and (4) chemiluminescence determination of the liberated nitric oxide. The method was applied to the determination of GP in water, beer, and ale, lentils and beans, and a few cereals.

A novel interface of a chemiluminescent nitrogen detector with a packed column chromatographic system, utilizing supercritical methanol modified carbon dioxide, has been developed by Shi et al.[28] Packed-column SFC using 10% methanol modified CO_2 and pressure programming can readily elute triazine herbicides. Using this approach with simultaneous chemiluminescent nitrogen detection at 219 nm the authors have determined propazine, atrazine, and simazine. The detector provides equimolecular nitrogen response to nitrogen-containing compounds with high sensitivity, selectivity, and wide linear dynamic range. The procedure provides another alternative for chromatographic separation, and at the same time can unleash the power of nitrogen detection by simplifying analysis of complex samples.

IV. DETERMINATION OF SULFUR COMPOUNDS

Thiocarbamate insecticides are one of the new generation of biodegradable foliar spray insecticides developed to replace aromatic-halogenated pesticides, because they are less toxic to mammals and less persistent in the environment.[29] Analysis of sulfur compounds in the complex matrices typical of environmental or biological samples presents two distinct problems: separation and identification. The recent trend has been to couple selective detectors with separation processes for complex analyses.[30] A selective detector can alleviate both problems, separation and identification. Sulfur-selective chemiluminescence detection can be achieved by converting sulfur-containing compounds to sulfur monoxide and subsequently allowing the SO to react with ozone to produce electronically excited SO_2^*:

$$SO_{(g)} + O_{3(g)} \rightarrow SO^*_{2(g)} + O_{2(g)}$$

$$SO^*_{2(g)} \rightarrow SO_{2(g)} + \text{light}$$

The first reaction is highly exothermic, and the energy produced is large enough to excite SO_2 at the same time. The light emitted[31] from the reaction ranges from 260 to 480 nm with a maximum intensity around 350 nm. There are several means of producing SO from sulfur containing compounds, of these methods, the combustion has been shown to be of practical use in chromatographic S-compound detection. A termed the flame sulfur chemiluminescence detector (SCD)[32] has been shown to exhibit nearly equimolar response to sulfur compounds regardless of oxidation stated. The combination of high sensitivity and selectivity, wide linear range, and lack of quenching and interferences from non-sulfur species has made flame SCD an effective choice for chromatographic detection of sulfur compounds. Table 3 summarizes the SCD applications for agrochemical products.

A sulfur-selective chemiluminescence detector (SCD) has been interfaced with packed-capillary-column high-performance liquid chromatography (HPLC) by Karen Chang et al.[31] The detection chemistry for the SCD is based on the chemiluminescent reaction between ozone and sulfur monoxide, which is produced from the decomposition of sulfur-containing analytes in a H_2/air reducing flame. The detection limit was determined to be 4 pg/s of sulfur, while 50/50 methanol/water was employed as the mobile phase. Detector linearity was 3 orders of magnitude, and a selectivity of at least 10^6 was obtained. Analysis of sulfur-containing pesticides (dimethoate, triallate, aldicarb, fenitrothion, methiocarb, fenthion, and methomyl) are reported and the results have demonstrated that the SCD can be an alternative method for detection of these pesticides.

Howard and Taylor[33] have evaluated the use of the ozone-based sulfur chemiluminescence coupled with packed column supercritical fluid chromatography (SFC). Optimization studies include the quenching effect of the CO_2 mobile phase and organic species on the chemiluminescent species on the (SO_2^*) and the necessary air and hydrogen flow rates to gain maximum SCD sensitivity. The results obtained have indicated that the increased

TABLE 3
Determination of Sulfur Compounds

Method	Analyte	Ref.
Packed-capillary-column HPLC/SCD	Dimethoate, triallate, aldicarb, fenitrothion, methiocarb, fenthion, methomyl	31
SFC/SCD	Sulfonylurea herbicides	33
Micro HPLC/SCD	Thiocarbamate	34
LPSA	Aldicarb, methiocarb aldicarbsulfone, aldicarbsulfoxe, oxamyl, methomyl	30
SFE/HPLC/SCD	Methomyl, mathrocarb, eptam	29

flow of CO_2 does not further quench the chemiluminecent species from that occurring in capillary SFC. Methanol-modified CO_2 was also demonstrated to be compatible with the SCD even with the high mobile phase flow rate. The authors have applied the procedure to sulfonylurea herbicides. The separation of seven thermally labile sulfonylurea herbicides was achieved in under 8 min with a normal-phase silica HPLC column and a 5% methanol-modified mobile phase under isobaric conditions. The sensitivity of the SCD under the methanol-modified conditions was less than that of the HPLC/UV case, similar SFC/UV sensitivity was obtained. Unfortunately, SFC/SCD is not applicable to the analysis of many polar sulfur-containing compounds due to the nonpolar nature of the SF CO_2 mobile phase. An extension of this method is SCD interfaced to reversed-phase-mode HPLC, it can also accommodate analytes of higher molecular weight and polarity than SFC. Howard et al.[34] have optimized the process for micro-HPLC/SCD under various reversed-phase (methanol/water) elution conditions. Linear dynamic range, correlation coefficient, response factor, and limit of detection were found to vary with mobile-phase composition. The lowest sensitivity achieved was a detectivity of 666 fg of S/s at a mobile-phase composition of 40% methanol/60% water. The selectivity of the system was demonstrated by the analysis of thiocarbamate pesticides.

Ryerson et al.[30] report the development of a sensitive sulfur-selective detector that can be used at liquid flow rates characteristic of analytical-scale HPLC, ion chromatography (IC), and flow injection analysis (FIA). The new liquid-phase sulfur analyzer (LPSA), employing gas-phase chemiluminescence for selective detection of liquid phase sulfur compounds, operates by converting sulfur-containing compounds in the liquid phase, under pressure and elevated temperatures, into sulfur monoxide. The SO is allowed to permeate across a membrane, into a helium stream, and is swept into a reaction cell. Ozone is added to the cell, and photoemision resulting from $SO + O_3$ reaction is monitored by a photomultiplier tube. The method has been shown to operate effectively at 0.4 to 9.9 ml/min flow rates, typical of analytical HPLC, ion chromatography, and flow-injection analysis. The authors have applied the detector to analysis of standards of

sulfur-containing pesticides, with detection limits on the order of 10 ppb S.

Howard et al.[29] have studied the presence of tiocarbamate insecticides (methomyl, mathiocarb, and eptam) in apple samples. These authors use the supercritical fluid extraction (SFE) for sample preparation, which offers many attractive benefits when compared with traditional methods. The combination of SFE for sample preparation and HPLC-SCD for extract analysis would provide a powerful analytical scheme for the determination of these difficult analytes and complex matrices, and also provide valuable qualitative information about the nature of the apple coextracts.

V. DETERMINATION OF AGROCHEMICAL PRODUCTS BY CHEMILUMINESCENT IMMUNOASSAY

Although chromatographic techniques, gas chromatography or high-performance liquid chromatography have been applied extensively to monitor occupational and environmental exposure to toxic organic chemistry, in recent years immunoassay methods have proven its applicability on this topic. To sum up, for pesticide determination there are immunoassays for individual chemicals which are specific for each one of them, or in some cases, for a class of group, but not for different groups of pesticides,[35] and further research is necessary. Nevertheless, literature studied indicates that there are an important number of recent reports on the determination of agricultural product by these assays, as well as cited by reviews related with determination of pesticides.[36–39] As an alternative to chromatographic techniques, immunoassays have many attributes, they combine specificity and sensitivity with ease of use, large sample throughput, minimum sample clean-up, and cost-effectiveness among others.[40] Immunoassay can be defined as an analytical method based on an antigen-antibody reaction. Analyte (the antigen) is detected and generally quantified by the use of specific antibody. Antibodies, or immunoglobulins, are a diverse group of glycoproteins that make up part of the body's immune system, and each has the ability to selectively bind a particular foreign agent or antigen.[41] The typical immunoassay is characterized by the fact that it is a limited reagent assay. This means that there is less antibody present than there is antigen, and to quantitative the system a labeled form of the antigen is necessary.[42] In some cases it is a labeled antibody instead of the antigen and the technique is then called immunometric assay.[43] Several labels have been used for this purpose. In this way, radioisotopes were first employed as marked in immunoassay, called this technique radioimmunoassay (RIA). Recently, labeling can be carried out by different markers like enzymes or luminescent products, among others. The scheme of a classic imunoassay could be:

$$\underset{\text{(analyte)}}{\text{Antigen}^{*} + \text{Antigen} + \text{Antibody}} \rightarrow \begin{array}{ll} \text{Antigen-Antibody} & \text{Antigen} \\ + & \\ \text{Antigen}^{*}\text{-Antibody} & \text{Antigen}^{*} \\ \text{(bound fraction)} & \text{(free fraction)} \end{array}$$

where (*) means labeled.

The proportion of labeled antigen bound is inversely proportional to the number of unlabeled antigen. Derivation of standard curve and a suitable analytical measurement allows the determination of the analyte in the sample.

When the label used for detection is a chemiluminescent one, we are talking about chemiluminescent immunoassay (CLIA). In this way, marriage of chemiluminescent labels and immunoassay methods is now well established for the quantification of low concentration of analytes and reviews and books reflect the actuality of this theme.[44,45] Moreover, the extreme sensitivity of CL and BL assays (detection limits in the attomole-zeptomole range: 10^{-18} to 10^{-21} mol) has made this ideal for immunoassay and other applications,[46] and in recent years the scope of this type of analytical procedures has been extended out of its classic field of application, clinical testing, to other areas such as agriculture, food, or environmental chemistry, among others. Detection in CLIA may be based on either direct monitoring of conjugated labels or enzyme-mediated formation of luminescent products.[47] The last system is essentially enzyme immunoassay with chemiluminescent end-point and as such possess similar problems to conventional enzyme immunoassay.[48] Among luminescent compounds that have been used as labels include luminol, isoluminol, ABEI [*N*-(4-aminobutyl)-*N*-ethyl isoluminol] and the best established, acridinium esters. Due to the light signal produced by both luminol and acridinium esters being short-lived, enhanced luminescence has been used as a real alternative. Horseradish peroxidase (HRPO) has been used extensively as a stable enzyme label. The characteristics of enhanced luminescence, that is, an increased, prolonged, and relatively stable light output, provide a means of increasing assay sensitivity and of simplifying the end-point measurement of luminescent immunoassays.[49] In addition, these chemiluminescent labels are suitable for use in immunoassays as they are stable. In these cases the technique is called enhanced chemiluminescent immunoassay (ECLIA).

Alternatively, the light emission in chemiluminescent assays is usually measured with a photomultiplier tube or a silicon photodiode, but recently, as we point out later, a detection system based on emitted light impacting on photographic film results in an inexpensive and easy manner to detect analytes, allowing its adaptation to a portable luminometer the development of an "in field" technique and making the photographic end-point assay an interesting tool to achieve semiquantitative and qualitative measurements.

This section aims to compile the maximum information about the topics previously mentioned in order to demonstrate by the reviewed reports that immunological methods in combination with chemiluminescence, constitutes a cheap, practical, and easy way to carry out agrochemical product determination. Table 4 summarizes the CLIA applications for agrochemical products.

Navas Diaz et al.[50] carried out the determination of dichlorprop methyl ester (DME) by an enhanced chemiluminescent kinetic competitive ELISA, employing a secondary antibody labeled with horseradish peroxidase. The method is applied to extracts of wheat grains. The chemiluminescent reaction is developed with a luminol-H_2O_2-horseradish peroxidase-*p*-iodophenol (as enhancer) mixture by using a fiber optic to collect the light emission. The minimum detectable concentration was 0.11 ng ml^{-1}, and the relative standard deviation was 20.6% at a concentration level of 0.5 ng ml^{-1} and of 7.23% at 50 ng ml^{-1} of DME (n = 10). The authors carried out cross-reactivity studies (estimated by using the 50 and 75% displacement methods) of DME and related compounds, confirming those that ELISA procedure was selective for the analyte before structurally related compounds usually found in formulations of pesticides. The authors concluded that enhanced chemiluminescent detection in ELISA methods provide good detection limits and precision for this purpose.

A highly sensitive chemiluminescent immunoassay for the determination of the herbi-

TABLE 4
Determination of Agrochemical Products by CLIA

Analite	Immunoassay technique	Chemilunminescent system	Detection limit	Ref.
Dichlorprop methyl ester	ELISA	Luminol-H_2O_2-HRPO *p*-iodophenol	0.11 ng ml^{-1}	50
2-4 Dichlorophenoxy acetic acid	ELISA	CSPC Emerald II	0.006 ng ml^{-1}	51
Methyl phosphonic acid, *p*-aminophenol 1,2,2,-trimethyl-propyl diester	Direct competitive labeled-hapten EIA Competitive EIA with indirectly labeled antibody	Luminol Coumaric acid	10^{-6} mol l^{-1}	52
Chlortoluron	ELISA	HRPO Amerlite	—	40
Atrazine	Direct competitive assay	ECLIA	10–20 ng l^{-1}	53

cide 2,4-dichlorophenoxyacetic acid (2,4-D) is reported by Dzgoev et al.[51] employing a microformat thick-film technology to pattern a hydrophobic layer 100 μm thick onto glass microscope slides to form an array of 2×2 mm^2 squares. The detection system is based on chemiluminescent reaction using a slow-scan cooled CCD camera. For practical purposes, an exposure time of 90 s is chosen. The substrate solution for the enzyme employed by these investigators contains an enhancer (Emerald II), disodium3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1] decan]-4-yl) phenyl phosphate, (CSPD) and diethanolamine buffer (pH 10.0). The detection limit for single-sample microformat assay of 2.7×10^{-11} *M*, or 6 pg of 2,4-D is reported and compared with the detection limits of a tube-based chemiluminescent assay using standard luminometer and of a colorimetric ELISA, resulting these last 20 times and 4000 times lower than the microformat imaging ELISA proposed in this paper.

Erhard et al.[52] report about a sensitive, specific, and easy test to perform detection system for an organophosphate that can be used under field conditions. They carry out the semiquantitative determination of the organophosphorus compound methyl phosphonic acid, *p*-aminophenyl 1,2,2,trimethyl-propyl diester (MATP), by development of two assays (direct and indirect competitive immunoassay), both with chemiluminescence determination of peroxidase. Luminol (5-amino-2,3-dihydro-1,4-phalazinedione), coumaric acid (as an enhancer) and H_2O_2 are preferred as substrate and the emitted light is determined with a camera luminometer selecting a 5-min interval as exposure time. In the direct assay a MAb F71D7 (IgG1 antibody) is use to coat the solid phase. For the indirect assay a second antibody (anti-mouse IgG) labeled with peroxidase as conjugate is used. Authors report detection limits for both assays at a concentration of 10^{-6} mol/L MATP using a polaroid film type 612, 20,000 ASA to ensure maximum sensitivity. For validation of the direct competitive assay a microtiter luminometer was used reducing the detection limit to 5×10^{-8} mol/l MATP. The authors conclude that the test developed does not require electrically and can be used as a field test and they inform of its

feasibility to determine MATP at low concentrations. Moreover, they propose the chemiluminescent test system described as a true alternative to ELISA systems.

Fawaz et al.[40] describe the determination of Chlortoluron [*N*-(3-chloro-4-methylphenyl)-*N'*-dimethylurea], a phenylurea herbicide widely used, making use of an enhanced chemiluminescent immunoassay (ECLIA) method with photographic detection of the light emitted using a Dynatech camera luminometer, loaded with Polaroid Type 612 film. The enhancement reagent added in this assay is Amerlite. A peroxidase chlortoluron label is prepared by the authors in a nominal molar ratio of 1:20 (horseradish peroxidase:hapten). Film exposed for 40 s using different concentrations of chlortoluron standards result in a grade response, which allows visual discrimination between the chlortoluron standards. Moreover, a significant correlation between the ELISA and ECLIA results is reported. Authors conclude that proposed assay is able to identify samples containing the mentioned herbicide at or above the European limit for individual pesticide in drinking water (The EU Maximum Admissible Concentration (MAC) = 0.1 $\mu g\ l^{-1}$).

Lowe et al.[53] report about a novel chemiluminescent immunoassay method, that is now being used for regulatory testing of pesticide by the UK water authorities. The system employed is based on the competition between pesticide in the sample (water: natural surface and natural bore, both raw and treated) and labeled pesticide for a limited number of antibody binding sites. The method has been applied to the detection of atrazine, although the authors expose its applicability to any small molecule which is possible to produce an antibody. No significant interferences (NaCl, HNO_3, cadmium, nickel, copper....) are found and, moreover, cross-reactivity studies carried out show that propazine presented a large one, but it is not a handicap because of its prohibition of use. On the basis of the results obtained by these authors and compared with gas chromatography-mass spectrometry led to the conclusion that the reported method supplies analytical advantages relative to sensitivity and improved sample recoveries.

Determination of triasulfuron, the active ingredient of logran, in soil is carried out.[54] The authors have developed a fully automated immunoassay with high sample throughput. The competitive assay made use of hapten-bound paramagnetic particles and acridinium ester-labeled monoclonal antibody. Purified Mab 4147-19-4 is labeled by reacting for 20 min at room temperature with 20 μg of *N*-hydroxisuccinimide-activated dimethyl acridinium ester dissolved in 20 ml of DMSO. The authors compare four different solid-phase antigens, one of then corresponding to the chloroethoxy phenylsulfonylurea moiety of triasulfuron with and extended aminopentyl spacer obtaining limits of detection for triasulfuron of 0.02 μg/l in aqueous media and 0.05 μg/kg in soil. They conclude that comparison between HPLC measurements and CLIA was favorable (with a correlation coefficient of 0.96) and that the method proposed offers the possibility of performing automated multiresidue analysis by immunoassays.

Some of the methods previously described are now well established as regulatory tests by the corresponding authorities and we can conclude that although there are not many reports, the results obtained by the authors endorse the fact that CLIA is a valuable alternative to conventional methods for pesticide determination.

VI. CONCLUSIONS

This paper has examined several chemiluminescent techniques used for agrochemical product measurements that have shown an increasing interest in recent years. CL reactions have considerable analytical potential because they have numerous advantages, such as low detection limits, due to the absence of source noise, wide linear range, and

fast response. In addition, the posibility of robust and inexpensive instrumentation make CL an attractive analytical tool. Alhough some difficulties associated mainly with selectivity must be pointed, these can be avoided in several ways, so a combination of CL detection with chromatographic techniques has enabled the selectivity of measurement to improve considerably, solving in addition, the limiting factor (stray light) of fluorescent detection.

The literature reviewed shows that determination of pesticides making use of chemiluminescence present, in general, the topics previously mentioned for chemiluminescent techniques. Chromatographic systems are described for organophosphorus, carbamates, nitrogen compounds and sulfur compounds become highly selective. However, these methods often involve extensive sample preparation to result in an increase of assay cost and time. Reports about immunochemical coupled with chemiluminescence allow us to point out that both disadvantages are minimized and alternatively, the use of a photographic endpoint has great potential as an extra-laboratory monitoring technique, although it results in a semiquantitative determination, less sensitive but cheaper and faster.

We suggest that the current literature when reviewed allows us to conclude that chemiluminescent methods, including CLIA, constitute a real alternative to the traditional detection system and can be used for the determination of agrochemical products not only in many laboratories but as "in field" techniques, although further investigation is necessary.

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