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**ARTICLE** *in* ANALYTICAL CHEMISTRY · DECEMBER 1993

Impact Factor: 5.64 · DOI: 10.1021/ac00070a010 · Source: PubMed



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# Comparison of a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay and Gas Chromatography for the Determination of Nicotine in Cigarette Smoke Condensates

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**A competitive enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies was developed to measure nicotine in smoke condensates of cigarettes. The ELISA standard curve displays a detection limit of 70 ng/mL and an effective working range of 0.3–4.0 μg of nicotine/mL. Using standard samples, ELISA results compare well with theoretical values ($r$ = 0.997). The suitability of the method for industrial routine application was evaluated by measuring the nicotine concentration in 245 cigarette smoke condensates. When ELISA results were compared with those obtained by gas chromatography, a correlation coefficient $r$ = 0.886 was obtained. The assay allows the specific, precise, and accurate determination of nicotine, without any significant interference from other alkaloids also present in tobacco smoke. Preliminary data suggest that the immunoassay could also be applied to other matrices, e.g., tobacco extracts. ELISA equipment requirements are minimum, simple, and low-cost, and an unskilled person could perform 150 analysis in a working day. On the basis of these features, ELISA is proposed as a promising alternative to instrumental methods for some industrial applications.**

## INTRODUCTION

The analysis of nicotine, the major alkaloid present in tobacco leaves and smoke, has gained importance due to increasing public concern for the potential harmful effects of chemicals on human health. Tobacco manufacturing industries are also aware of this problem, and therefore the analysis of nicotine is included in the routine quality control of tobacco products. Industrial determination of nicotine is normally carried out by instrumental techniques, gas chromatography (GC) being the most broadly applied.[1-3] Apart from its high analytical performance, some disadvantages associated with GC—complex equipment, laborious cleanup procedures, expensive analysis—make it advisable to search for complementary or alternative methods. Since their introduction three decades ago, immunological methods have been extensively and successfully used in clinical analysis and biomedical applications, where they offer simplicity, sensitivity, and specificity.[4] More recently, immunoassays have been applied to the analysis of pesticides and other organic chemicals in fields such as environmental control, the food industry, etc.[5-8] In this respect, radioimmunoassays[9] and enzyme immunoassays[10,11] have been developed for the determination of nicotine and its metabolites in biological fluids, but no direct application to the analysis of tobacco products has been reported. In the present paper, a new competitive enzyme-linked immunosorbent assay (ELISA), specifically for the measurement of nicotine in smoke condensates of cigarettes, is described. Monoclonal antibodies (MAb) to nicotine are used in this assay as immunological tools. The main analytical features of the developed ELISA are compared with GC, and its suitability to measure real industrial nicotine-containing samples is also discussed as an example of the potential applications of immunochemical methods in industrial analysis.

## EXPERIMENTAL SECTION

**Reagents and Cell Lines.** All chemical and biological products were of the highest purity grade commercially available and they were purchased from Sigma-Aldrich Química (Madrid, Spain) and Merck (Darmstadt, Germany), unless otherwise indicated. Culture plastic ware was from Bibby Sterilin Ltd. (Stone, UK). P3-X63-Ag8.653 mouse plasmacytoma line was from American Type Tissue Culture Collection (Rockville, MD). All cell lines were cultured in high-glucose Dulbecco modified Eagle's medium (Gibco BRL, Paisley, Scotland) supplemented with 4 mM L-glutamine, 1 mM nonessential amino acids, and 15% fetal calf serum (Gibco).

**Production and Characterization of MAbs to Nicotine.** In order to elicit an immune response against nicotine, 3′-(hydroxymethyl)nicotine hemisuccinate was synthesized as described by Langone and Van Vunakis.[9] This hapten was conjugated to bovine serum albumin (BSA) using the modified active ester method[9] and the mixed-anhydride method.[12] From mice immunized with these conjugates, the MAb production was carried out essentially as described by Nowinski et al.[13] Two cell fusions were performed, and 10 hybridomas secreting MAbs that recognized nicotine were cloned and stabilized. These MAbs were subsequently characterized with regard to their affinity and specificity to nicotine. MAb affinity was estimated as the

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concentration of nicotine that inhibits 50% of the maximum response ($IC_{50}$) in competitive assays, and specificity was evaluated from the cross-reactivity with other alkaloids, using the Abraham 50% displacement method.[14] The MAb with the lowest $IC_{50}$ for nicotine and the lowest cross-reactivity with nicotine analogs also present in tobacco smoke was chosen for further ELISA development.

**Preparation of Nicotine Standards and Samples.** *Nicotine Standards for ELISA.* A stock solution (3.5 mg of nicotine/mL) was used, from which standards in the range 10 ng/mL to 2 mg/mL were prepared daily by serial dilution in 10 mM phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS).

*Nicotine Standards for GC.* Covering the range 120–960 μg of nicotine/mL, GC standards were prepared independently in 2-propanol containing *n*-heptadecane (0.5 mg/mL) and ethanol (5 mL/L) as internal standards.

*Nicotine Standards for Spectrophotometry.* They were prepared from a stock solution (1.5 mg of nicotine/mL) by independent dilutions in 5% acetic acid to cover the range 15–225 μg/mL.

*Smoke Condensate Samples.* Cigarettes were conditioned following the ISO norm 3402, and smoke condensates were then prepared according to ISO norm 4387 (revision 1991) using a smoking machine (Filtrona, Model SM 350) that observes the ISO norm 3308. Smoke alkaloids from five cigarettes were retained in a 44-mm glass fiber filter and extracted with 20 mL of 2-propanol containing the internal standards. These alkaloid extracts were used for performing both GC and ELISA nicotine determinations: samples for GC were injected directly onto the column, while samples for ELISA were diluted in PBS until they entered the immunoassay working range.

*Tobacco Extract Samples.* Tobacco samples (leaves or reconstituted) were milled (0.5-mm particle size) in a Tecator Cyclotec 1093 mill and dried at 40 °C for 24 h. Alkaloids were then extracted with 5% acetic acid (5 mg of tobacco/mL), using an Ika-Werk, HS 500 mechanical shaker, for 15 min at 150 rpm. Finally, extracts were filtered on Whatman filter paper No. 1, and clear filtrates were used for the spectrophotometric determination of alkaloids and ELISA determination of nicotine.

**Nicotine Immunoassay.** A competitive indirect ELISA format was chosen. Immunoassays were performed in 96-well polystyrene microtiter plates (Bioreba), by the following procedure:

*First.* Plates were coated with a nicotine–ovalbumin conjugate obtained by covalently attaching 3′-(hydroxymethyl)nicotine hemisuccinate to the protein using the mixed anhydride method. A 100-μL aliquot of a conjugate solution (0.3 μg/mL in 50 mM sodium carbonate buffer, pH 9.6) was added to each well and incubated overnight at 4 °C. Plates were washed three times with 0.9% NaCl containing 0.05% Tween-20, in a microplate automatic washer (Wellwash 4 from Denley Ltd., Sussex, UK).

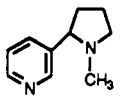
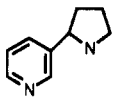
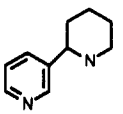
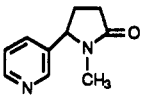
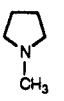
*Second.* Nicotine standards or samples (50 μL) in PBS were added to the wells, followed by 50 μL/well of LIB-N4 MAb [0.1 μg/mL in PBS containing 0.05% Tween-20 (PBST)]. Each sample was assayed at two different dilutions (1/150 and 1/200 from 2-propanol extracts of smoke condensates, 1/20 and 1/40 from acetic acid extracts of tobacco), and an aliquot of each dilution was placed in triplicate wells. The competitive immune reaction was incubated for 1 h at room temperature, and plates were washed as previously described.

*Third.* Peroxidase-labeled rabbit anti-mouse immunoglobulin (100 μL/well) (Dako, Glostrup, Denmark), diluted 1/2000 in PBST, was added and incubated for 30 min at 37 °C. Following this incubation, plates were washed four times.

*Fourth.* In order to determine the peroxidase activity bound to the wells, 100 μL/well of *o*-phenylenediamine (2 mg/mL in 62 mM phosphate, 25 mM citrate buffer, pH 5.4, containing 0.012% $H_2O_2$) was added. After being incubated for 10 min at room temperature, the colorimetric reaction was stopped by adding 100 μL/well of 2.5 M sulfuric acid, and the absorbance was read at 490 nm and recorded with a Dynatech MR 700 microplate reader (Sussex, UK).

Nicotine standard curves were obtained by plotting absorbance vs the logarithm of nicotine concentration and were mathematically fitted to a four-parameter logistic equation as described by Rodbard.[15] A standard curve was run on each plate along with the samples.

**Table I. Immunological Characterization of LIB-N4 Monoclonal Antibody with Respect to Nicotine and Related Alkaloids**

| compound | structure | $IC_{50}$ (μM) | CR[a] (%) |
|---|---|---|---|
| nicotine |  | 4.9 | 100.0 |
| nornicotine |  | 702.1 | 0.7 |
| anabasine |  | 247.6 | 2.0 |
| cotinine |  | 1623.5 | 0.3 |
| pyridine |  | $>10^5$ | $<5 \times 10^{-3}$ |
| *N*-methylpyrrolidine |  | 187.0 | 2.6 |

[a] CR, cross-reactivity.

**GC Determination of Nicotine.** Nicotine determinations by GC were performed on cigarette smoke condensates according to ISO norm 10315 (revision 1991), on a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector, a HP 7673 autosampler, and a packed column (2 mm × 2 m Chromatosorb Q, 80–100 mesh). The temperatures used for analysis were 170 (column), 250 (injection) and 300 °C (detector). The injected volume was 2 μL, and each determination was performed in duplicate.

**Spectrophotometric Determination of Alkaloids.** Tobacco extracts were analyzed by the cyanogen chloride-based method described by Harvey and Handy.[16]

## RESULTS AND DISCUSSION

**Immunochemical Properties of LIB-N4 Monoclonal Antibody.** As mentioned before, several MAbs to nicotine were obtained following standard fusion procedures. MAbs were tested by competitive ELISA for their ability to recognize nicotine, and the one which best suited the assay requirements in terms of affinity and specificity was chosen. The immunochemical features of the chosen MAb (LIB-N4) are summarized in Table I.

Although the calculated affinity of this MAb to nicotine ($IC_{50}$ = 0.8 μg/mL = 4.9 μM) is not as high as some previously reported data,[11] it is suitable for the application described here. In fact, the nicotine concentration in smoke condensates of cigarettes normally ranges from 25 to 750 μg of nicotine/mL, which is clearly more than that required to produce significant inhibition.

The nicotine extraction procedure from cigarettes (see Experimental Section) also extracts other alkaloids. Therefore, the choice of the MAb was based mainly on its cross-reactivity with compounds structurally very similar to nicotine and/or usually found in significant amounts in tobacco smoke.

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(16) Harvey, W. R.; Handy, B. M. *Tob. Sci.* **1981**, *25*, 131–133.

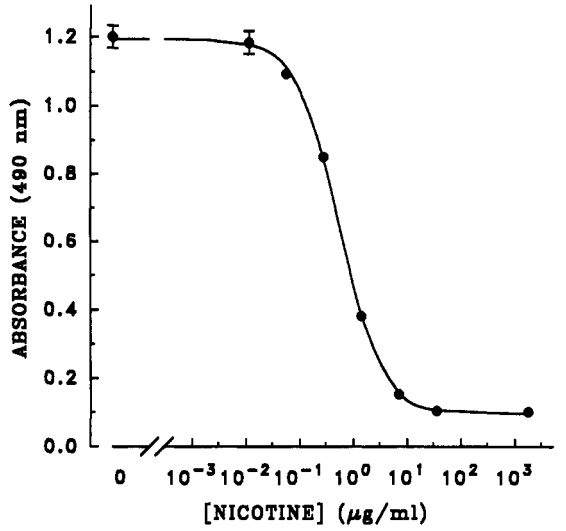


**Figure 1.** Representative standard curve for determining nicotine by indirect competitive ELISA. Each point represents the mean of triplicate wells ± standard deviation.

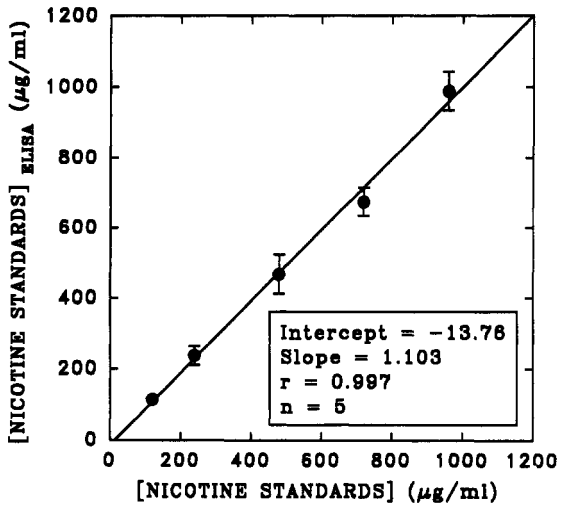


**Figure 2.** Linear regression analysis between the theoretical nicotine concentration in standard samples and nicotine concentration determined by ELISA. Points represent the mean of six determinations ± standard deviation.

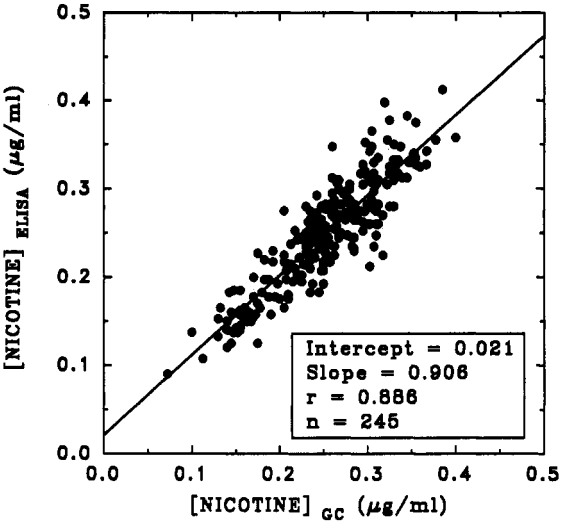


**Figure 3.** Linear regression analysis between nicotine concentration in cigarette smoke condensates determined by ELISA and by GC.

LIB-N4 MAb did not significantly recognize nicotine-related compounds, since much higher concentrations (at least 100 times) were required to produce the same inhibition as seen for nicotine. The highest cross-reactivity observed was 2.6% for *N*-methylpyrrolidine, which is not present in smoke condensates. Any minimal change in the chemical structure of nicotine (e.g., the presence of a carbonyl group in continine, the lack of a methyl group in nornicotine, or the change of the pyrrolidine ring by a piperidine ring in anabasine) drastically reduced the MAb affinity.

**Standard Curve for the Nicotine ELISA.** A typical standard curve for the nicotine competitive assay using the LIB-N4 MAb is shown in Figure 1. The sigmoidal curve, obtained by mathematical fitting of experimental points, showed the characteristic features of competitive inhibition assays. The ELISA detection limit, calculated as the nicotine concentration corresponding to the absorbance of the zero dose minus three standard deviations, was 70 ng/mL. The working range of the assay was calculated from the imprecision profile, in such a way that the coefficient of variation (CV) did not exceed 10%. According to this condition, the working range was 0.3–4 μg of nicotine/mL. To ensure that the samples are adequately buffered, the cigarette smoke condensates must be diluted at least 100 times. The ELISA working range effectively allowed such a dilution and still provided the required sensitivity.

**Comparison of ELISA and GC Nicotine Determinations.** According to guidelines in developed countries, the nicotine content of cigarettes should be precisely determined and properly indicated on packets. Nicotine analysis in tobacco manufacturing industries is normally carried out by GC on cigarette smoke condensates. Thus, in order to validate the immunochemical assay of nicotine, it was compared with GC as a reference method. First, precision and accuracy were determined for both analytical methods by measuring the nicotine concentration in standard samples. Second, with the aim of assessing the suitability of ELISA for routine analysis, the concentration of nicotine in industrial real samples of cigarette smoke condensates was measured by ELISA and GC, and the results were compared to each other.

*Analysis of Standard Samples.* Five standard samples ranging from 120 to 960 μg of nicotine/mL were prepared in 2-propanol and measured six times independently by ELISA and by GC. As shown in Figure 2, a good linear correlation between ELISA and theoretical values was obtained (correlation coefficient $r = 0.997$). The ELISA intraassay precision, represented by the mean CV, was 8.7% (ranging from 5.6 to 11.9%). The mean accuracy, obtained from the percent difference between the calculated nicotine concentrations and the theoretical values, was ±3.4% (ranging from −6.3 to 3.0%). Under these conditions GC was 12 times more accurate and ~60 times more precise than ELISA.

These results suggest that ELISA cannot replace GC for this particular application. However, as discussed below, other properties of ELISA may justify its use in routine applications.

*Analysis of Real Samples.* More than 200 blind real samples of cigarette smoke condensates were analyzed by the immunochemical method, and the same samples were determined by GC at the R&D Center of the Spanish tobacco industry (Tabacalera S.A.). The values obtained by both methods were compared by linear regression analysis after analyzing 77, 113, 124, and 245 cigarette smoke condensates. The statistical results did not significantly change as a function of the number of samples.

The intermethods linear regression analysis corresponding to 245 samples (Figure 3) showed a correlation coefficient $r = 0.886$, which is in the same range as other reported values for hapten immunoassays.[17-21] The applied statistical tests indicated a high significance for the correlation coefficient

**Table II. Comparative Analysis of Nicotine in Original and Spiked Samples by Gas Chromatography and ELISA**

| nonspiked | | | spiked | | |
|---|---|---|---|---|---|
| | nicotine (mg/mL) | | | nicotine (mg/mL) | |
| sample | GC | ELISA | sample | GC | ELISA |
| 1 | 0 | 0.02 | 1S1 | 0.28 | 0.27 |
| | | | 1S2 | 0.37 | 0.36 |
| 2 | 0.04 | 0.04 | 2S1 | 0.26 | 0.26 |
| | | | 2S2 | 0.41 | 0.46 |
| 3 | 0.11 | 0.12 | 3S1 | 0.25 | 0.24 |
| | | | 3S2 | 0.41 | 0.41 |
| 4 | 0.19 | 0.18 | 4S1 | 0.26 | 0.26 |
| | | | 4S2 | 0.42 | 0.40 |
| 5 | 0.27 | 0.25 | 5S | 0.41 | 0.40 |
| 6 | 0.28 | 0.20 | 6S | 0.41 | 0.36 |
| 7 | 0.32 | 0.26 | 7S | 0.42 | 0.37 |
| 8 | 0.29 | 0.31 | 8S | 0.44 | 0.42 |
| | $r = 0.960$ | | | $r = 0.941$ | |

(Student's $t$ test, $p < 0.001$) and a very good linear fitting between ELISA and GC results (variance analysis, $p < 0.005$). Deviations from linearity between ELISA and reference methods are often explained either in terms of interferences caused by compounds that cross-react with the antibody or by matrix effects.[8] Since nicotine analogs in cigarette smoke condensates are present in very low concentrations as compared with nicotine and, as previously discussed, LIB-N4 proved to be a very specific antibody, matrix effects are more likely the responsible for the difference observed.

In order to assess whether there are true matrix interferences, the analysis of several spiked samples was carried out. Because all smoke condensate samples contained nicotine, spiked samples were obtained by adding nicotine to real samples. Table II shows the results obtained by GC and ELISA when original (nonspiked) and spiked samples were analyzed. The linear correlation coefficient between GC and ELISA was essentially the same in the two sets of samples, which tends to support the conclusion that true matrix effects are involved in the nicotine assay, more than immunological interferences due to cross-reactivity of unknown compounds.

A question that could arise in relation to the practical application of ELISA is the necessity for performing a standard curve on each plate. To evaluate this question, a mathematical approach was chosen consisting of the interpolation of a significant number of samples both in their own-plate original standard curve and in an average curve. The comparison was carried out using 38 samples and 26 curves performed in the course of the routine determination of nicotine. In order to be properly averaged, individual curves were normalized by expressing the absorbances as a percentage of the maximum response. When the results obtained by interpolation in the original curves and in the average curve were compared, similar overall precision was found. On the other hand, the estimated nicotine concentration was similar in 60% of the samples, but in the remaining 40% it changed significantly. Therefore, we feel the inclusion of a standard curve into each ELISA plate is advisable in order to ensure the accuracy of quantitative measurements, although the use of an average curve could be applicable for semiquantitative purposes.

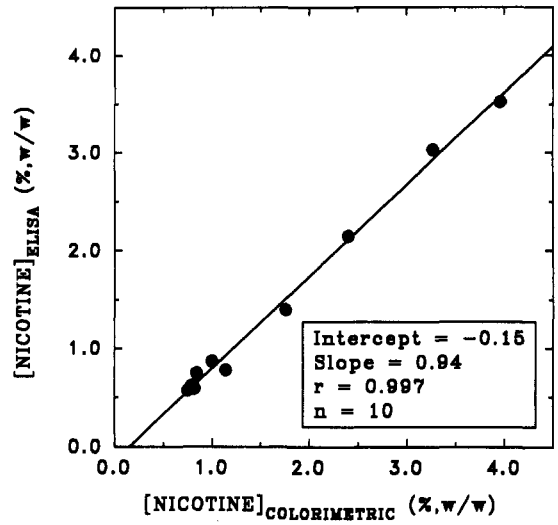


**Figure 4.** Linear regression analysis between nicotine concentration in tobacco samples determined by ELISA and spectrophotometrically.

A significant point to be considered when the industrial suitability of a method is being assessed is the *sample throughput*. In the conditions described above, with a standard curve included in each ELISA plate, ~150 samples can be easily analyzed in a working day (7–8 h). This analysis capability seems quite reasonable and should be enough to fulfill most industrial requirements.

Another important aspect in the evaluation of a new method is the *cost effectiveness* of the analysis. In this respect, a benefit generally attributed to immunochemical methods is the low cost of routine analysis when compared with instrumental analysis.[7] Besides its analytical aspects, the ELISA discussed here was also compared with GC in economical terms. ELISA was effectively 4–5 times cheaper than GC when applied to the analysis of real samples. This cost ratio, although far from some reported cases in which immunoassays are argued to be highly advantageous, could become a weighty reason in itself to adopt the ELISA for industrial applications, especially where profits are of great importance and a less demanding analytical performance is required.

An example of these potential applications could be the nicotine analysis in some matrices other than cigarette smoke condensates, such as raw material. In fact, ELISA was tentatively applied to the determination of the nicotine content in several samples of tobacco leaves and reconstituted tobacco. The reference method for comparison was in this case the automated spectrophotometric technique normally applied in tobacco manufacturing industries to determine total alkaloids in those matrices. An excellent correlation between both methods was found ($r = 0.997$, Figure 4), which supports the expectation that this ELISA, although originally developed for cigarette smoke condensates, can also be a promising analytical alternative for other applications.

## CONCLUSIONS

The ELISA presented here constitutes, to our knowledge, the first immunoassay especially devoted to nicotine analysis in tobacco-related samples. In order to validate the proposed immunoassay, a high number of real industrial samples were analyzed and the results submitted to a rigorous statistical study as compared to GC, the current method of choice. The immunochemical method uses minimum equipment, is easy to perform by unskilled people, and effectively allows the specific determination of nicotine in cigarette smoke condensates with acceptable precision and accuracy. In addition, preliminary data suggest that this ELISA could be also

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(18) Thurman, E. M.; Meyer, M.; Pomes, M.; Perry, C. A.; Schwab, A. P. *Anal. Chem.* **1990**, *62*, 2043–2048.
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(21) Skerrit, J. H.; Hill, A. S.; Beasley, H. L.; Edward, S. L.; McAdam, D. P. *J. AOAC Int.* **1992**, *75*, 519–528.

applicable to matrices other than smoke condensates of cigarettes.

Most industrial requirements in the analysis of low molecular weight organic compounds are properly satisfied by using instrumental methods such as GC and HPLC. These methods normally offer excellent analytical performance but require expensive equipment and experienced personnel. In practice this may impede many industries in installing these techniques and performing their own analysis. Even in industries with a high economical potential, instrumental methods are often available only in a centralized laboratory to which samples from peripheral production plants have to be sent, which subsequently produces delays and cost increases. In this respect, the actual need for using complex instrumental techniques for any routine analysis could be questioned, especially if rapid in situ determinations are required and a simple, inexpensive, and efficient method is available. Apart from its interest for the analysis of nicotine in itself, this immunoenzymatic approach could serve as a model system to evaluate the suitability of immunochemical methods for industrial analytical applications. As illustrated in this case, some applications exist in which a well-designed ELISA is able to overcome the limitations associated with classic instrumental techniques, reaching satisfactory analytical properties. With the only limitation the availability of specific antibodies, ELISA should therefore be considered as a viable and valuable alternative for many routine analysis in industrial laboratories.

## ACKNOWLEDGMENT

Part of this work was presented as a communication at the 1992 Coresta Congress (Jerez de la Frontera, Spain). We express our sincere gratitude to Dr. Jaime Primo for his scientific advice in organic synthesis and to Dr. Shirley J. Gee for reading the manuscript. Our thanks to the R&D Center of Tabacalera S.A. for supplying industrial samples and performing GC measurements and to Ms. M. J. Lianes for her technical assistance. The financial support of Tabacalera S.A. is also acknowledged.

RECEIVED for review March 12, 1993. Accepted August 22, 1993.®

• Abstract published in *Advance ACS Abstracts*, October 1, 1993.