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Abstract

Six 2,5- and 5,6- dihalonicotinic acids in a mixture were converted to their corresponding methyl esters and then analyzed by gas chromatography and gas chromatography-mass spectrometry. Four methods of conversion were compared for their GC sensitivity, efficiency and analysis time. In Method #1, using HCl and methanol as the reagents, the displacement of the halogens by chlorine (from HCl) at 2- and 6-positions was a common occurrence, rendering the method inefficient. In Method #2 (BF_3/methanol), the displacement of halogens by methoxide was evident. Method #3 (dicyclohexylcarbodiimide/methanol) produced a mixture of derivatives with a poor yield. Method #4 (diazomethane) gave a quantitative yield of the corresponding methyl esters without any side reactions and was suitable for analytical method development. The latest method provided short analysis time with all six methyl dihalonicotinates eluting within nineteen minutes. The resolution of the ester peaks was excellent and the detection limit was about 1 ng/μL for the dihalonicotinic acids.

Introduction

Nicotinic acid, also known as niacin, is an essential vitamin that contributes to the metabolism of carbohydrates and fats, the production of certain hormones, the functioning of the nervous and digestive systems, and the maintenance of healthy skin (Clayman, 1989). It is interesting, therefore, to study some of its derivatives to determine any beneficial properties. As many as twenty-six of the possible thirty-two 2,5- and 5,6-dihalonicotinic acids have been synthesized (Setliff, 1970; Setliff and Rankin, 1972; Setliff and Price, 1973; Setliff and Lane, 1976; Setliff and Greene, 1978; Setliff and Huie, 1981). The primary purpose of the synthesis of these compounds was to investigate their potential anti-arthritic, anti-viral, anti-psoraisis, or anti-bacterial properties. Other researchers have also studied these compounds as potential hypolipidemic agents (Gacek et al., 1972) Consequently, it has become quite important to develop sensitive analytical methods to determine the presence of these compounds in trace quantities, so that their potential application as pharmaceutical or agricultural agents can be evaluated in terms of their potency, toxicity, and metabolic transformation in living systems.

A survey of the literature reveals no analytical method available for the separation of the dihalonicotinic acids in a mixture. However, nicotinic acid and some of its other derivatives have been analyzed by high performance liquid chromatography (Iwaki et al., 1996; Manceau et al., 1992) and gas chromatography and gas chromatography-mass spectrometry (Zoellner, 1994). The HPLC method was employed to study the bioconversion of nicotinic acid to nornicotine in biological matrices such as blood plasma and urine (Manceau et al., 1992). The GC and GC-MS methods have been used to investigate the structural fragmentation patterns of the mono and diacylglycerol derivatives of nicotinic acid (Zoellner, 1994).

The purpose of the present research was to develop an acceptable analytical method for the separation and quantification of several dihalonicotinic acids in a mixture using GC and GC-MS techniques. Four methyl esterification methods, well-documented in the literature, were compared in terms of GC sensitivity, effficiency, and analysis time using six dihalonicotinic acids in a mixture as a test sample, as listed in Fig. 1. The methods used were (1) HC1/methanol, (2) BF_3/methanol, (3) Dicyclohexylcarbodiimide/methanol, and (4) diazomethane. These methods are commonly used for the methyl esterification of a variety of aliphatic and aromatic acids. Method #4 has been used earlier to synthesize the methyl esters of eight dihalonicotinic acids (Setliff and Huie, 1981). The conversion into the esters was about eighty percent in most cases. However, halogen replacement by chlorine at 2- and 6- positions from HC1 produced in-situ was observed for some esters when the preparation of the esters was attempted by reacting the dihalonicotinic acids with $SOC1_2$ and methanol.

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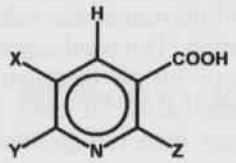
SIX DIHALONICOTINIC ACIDS USED FOR ANALYSIS



X	Y	Z	COMPOUND NAME
Cl	H	Cl	2,5-dichloronicotinic acid
Br	H	Cl	5-bromo-2-chloronicotinic acid
Br	H	Br	2,5-dibromonicotinic acid
Cl	Cl	H	5,6-dichloronicotinic acid
Br	Cl	H	5-bromo-6-chloronicotinic acid
Br	Br	H	5,6-dibromonicotinic acid

Table 1: The six dihalonicotinic acids used for the analysis.

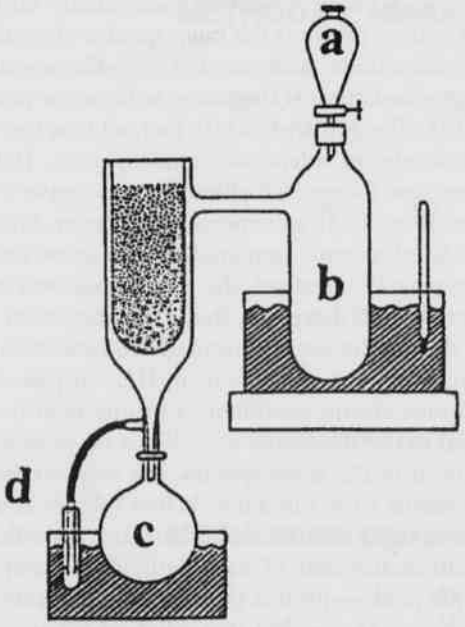


Fig. 1. Mini diazald apparatus and its set-up to produce diazomethane. The four components of the set-up are: a - a 100 mL separatory fumlel with a teflon stopcock and stopper; b - the mini diazald apparatus, consisting of a reaction vessel and condenser in one compact piece; c - a 50 mL round bottom flask; d - an ether trap at the side arm.

In general, esterification is necessary for the GC analysis of organic acids in order to increase the volatility of the test compounds so that they will be more amenable to separation in a GC column with little or no peak-tailing. The success of a method depends largely on the structures of the acids, and to some extent on their solubilities and other physical characteristics. It is imperative, therefore, to attempt various methods in order to develop the most suitable analytical method for a class of compounds.

Materials and Methods

A Varian Model 3400 gas chromatograph, coupled with a flame-ionization detector, was used to perform the GC analysis of the compounds. The GC-MS analyses were conducted with a Varian 3400 GC coupled with a Varian Saturn II Ion-Trap Detector, and also with a Hewlett-Packard Model 5890 Series II GC with a Hewlett-Packard Model 5970 Mass Selective (Quadrupole) Detector. Further details on the experimental conditions for data collection are provided later with the gas chromatograms and the mass spectra.

A mini-diazald kit (Aldrich Chemical) was used to prepare minute amounts of diazomethane from diazald (N-methyl-N-nitroso-p-toluenesulfonamide) for the esterification of the test compounds (Method #4). Standard laboratory glassware was used for all other esterification and sample preparation. All solvents and reagents were of analytical grade. Hydrochloric acid, benzene, dicyclohexylcarbodiimide (DCC), diethylether (99.9% pure, HPLC grade) and sulfuric acid were obtained from Sigma-Aldrich, whereas methanol, methylene chloride and pyridine were purchased from Fisher Scientific. The six test compounds were provided as analytical samples by F.L. Setliff of our department. The procedures for the esterification methods are given below:

(1) Method #1: HCl/Methanol Esterification (Knapp, 1979) - The samples (1.0 to 10.0 mg each) were dissolved in 4.0 mL of 0.50 M HCl in methanol to which 0.50 mL benzene was added. The solution was heated at 80°C for an hour with frequent shaking, cooled to room temperature, and then 10 mL of distilled water was added to it. The resultant esters were extracted three times with 3.0 mL of diethylether. The ether extract was dried over a 4:1 (w/w) mixture of anhydrous Na_2SO_4/$NaHCO_3$. Because of the interference from HCl, as will be described later, the aforementioned standard method was modified by replacing HCl with H_2SO_4. The methyl ester crystals obtained were then dissolved in 3.0 mL of methylene chloride. The solution was refrigerated until ready to analyze by GC and GC-MS.

(2) Method #2: BF_3/Methanol Esterification (Knapp, 1979) - The samples (10.0 mg each) were dissolved in 3.0 mL of methanol to which 3.0 mL of a 50% BF_3 in methanol solu-

tion (obtained from Sigma Chemicals) was added. The solution was refluxed at 100°C for ten minutes. Excess methanol was removed under vacuum at room temperature. After 20.0 mL of distilled water was added, the solution was shaken vigorously for five minutes. The layers were allowed to separate. The top layer contained most of the methyl esters. The acidic aqueous bottom layer was extracted three times with 10 mL of diethylether. The ether extracts were mixed and then were evaporated under vacuum at room temperature. The methyl esters were then dissolved in enough methylene chloride to bring the final volume up to 1.0 mL.

(3) Method #3: DCC/Methanol Esterification (Knapp, 1979) - The samples (10.0 mg each) were dissolved in a solution containing 4.0 mL of methanol and 1.0 mL of pyridine, the latter being the catalyst. The DCC was added in excess (10.52 mg), and the resulting solution was heated to about 80°C with gentle stirring for thirty minutes. The reaction produced N,N'-dicyclohexylurea as a byproduct, which was allowed to settle at the bottom and was decanted off. The excess DCC was decomposed at the end of esterification by adding acetic acid. The supernatant liquid, containing the methyl esters, was evaporated to dryness under vacuum, and the crystals then dissolved in enough methylene chloride to make a 1.0 mL solution for GC and GC-MS analysis.

(4) Method #4: Diazomethane Esterification (Aldrich Chemical, 1990)–The minidiazald kit was assembled according to the instruction provided by the manufacturer, as shown in Fig. 1. The condenser was filled with dry ice, then acetone was added slowly until the cold finger was about one-third full. Ethanol (5.0 mL) was added to a solution of KOH (2.5 g) in 4.0 mL of distilled water in the reaction vessel. A 50.0 mL receiving flask (with clear-seal joint) was attached to the condenser and then cooled in an ice bath. The trap, which had approximately 2 mL of diethylether, was cooled in an ice:salt (3:1) bath, which was also used for the receiver. A separatory funnel (with clear-seal joint) was placed over the reaction vessel, and the funnel was charged with a solution of diazald (2.5 g, 11.5 mmol) in 22.5 mL of diethylether. The reaction vessel was heated in a water bath at 65°C, and the diazald solution was added in small amounts periodically over a period of twenty minutes. The rate of addition of diazald solution was about the same as the rate of distillation. The cold finger was replenished with dry ice as necessary. When all of the diazald was consumed, 10.0 mL of diethylether were slowly added, and the distillation was continued until the distillate was colorless. The diethylether distillate should have contained about 350 mg (8.3 mmol) of diazomethane. The diazomethane was kept in a freezer at 0°C until its use for the esterication of the samples.

For the esterification, the samples (10.0 mg each) were dissolved in 1.0 mL of methylene chloride to which the diazomethane solution was added dropwise until a light yellow color persisted, thus insuring complete conversion of the test compounds into their corresponding methyl esters. In general, about 1.5 mL of diazomethane solution was necessary to complete the reaction. The resulting solution was allowed to stand overnight to insure complete reaction and the decomposition of the excess (if any) diazomethane. The sample solutions were then refrigerated until the GC and GC-MS analysis.

Results and Discussion

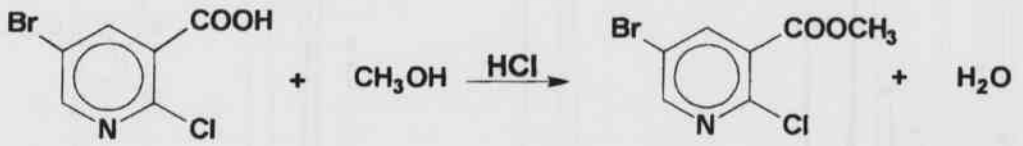
While a large number of methods for the esterification of organic acids is available in the literature, the four chosen are, in general, very successful in terms of high percentage of esterification, fast conversion rate, and the ease of carrying out the reactions. The chemistry involved in these methods are shown in Table 2, with 5-bromo-2-chloronicotinic acid as the test compound.

In Method #1, involving HCl (or H_2SO_4) and methanol, four of the six test compounds (5-bromo-6-chloro-; 2,5-dibromo-; 5,6-dibromo-; and 5-bromo-2-chloronicotinic acids) were esterified individually and also in a mixture. Figure 2 presents the mass spectra of methyl 5-bromo-6-chloronicotinate and methyl 2,5-dibromonicotinate. The spectra look almost the same, with major peaks at m/z: 76, 110, 170, 192, 220 and 251. In fact, all four test compounds showed almost identical mass spectra. Both 5-bromo-2-chloro- and 5-bromo-6-chloro-isomers gave a large parent ion peak at m/z 249 as expected. However, both 2,5-dibromo- and 5,6-dibromo- isomers failed to show the parent ion peak at m/z 293; instead, the major peak was at m/z 249. It was concluded, therefore, that a displacement reaction occurred during the esterification of the latter two compounds, during which the chlorine from HCl displaced one of the two bromine atoms, producing a bromo-chloro- compound identical to the first two esters. Since no peak at m/z 205 was observed in the mass spectra, the displacement of both bromine atoms were ruled out. When sulfuric acid was used instead of hydrochloric acid in the esterification process, the chromatogram of each individual compound showed multiple peaks with less than quantitative esterification. Therefore, it was decided that Method #1 would be unsuitable for a quantitative method development.

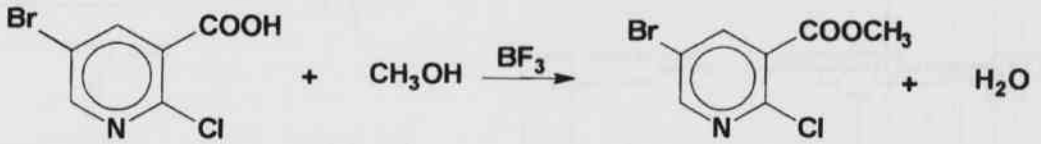
When BF_3/methanol was used for esterification (Method #2), incomplete esterification was evident. Figure 3 shows the chromatogram obtained when 5,6-dibromonicotinic acid was esterified by this method, indicating the presence of two compounds in the reaction product. GC-MS study revealed that the first peak was due to the unconverted acid, whereas the second one was an esterified product of the acid; only about 60% conversion was noted. When a

CHEMISTRY OF VARIOUS METHODS OF ESTERIFICATION

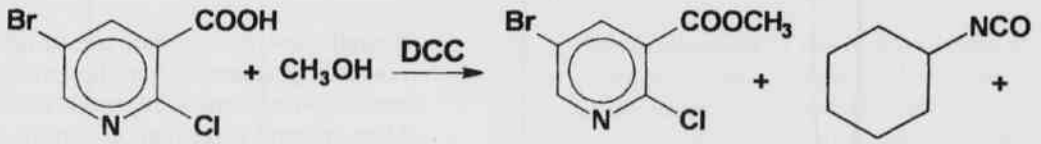
METHOD #1: Methanol and Hydrochloric Acid (or Sulfuric Acid).

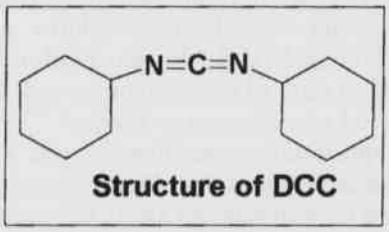


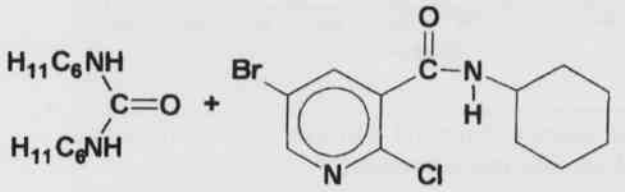
METHOD #2: Methanol and Boron Trifluoride.



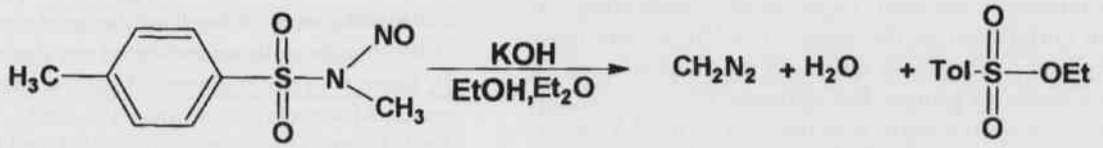
METHOD #3: Methanol and Dicyclohexylcarbodiimide.







METHOD #4: Diazomethane Produced form Diazald.



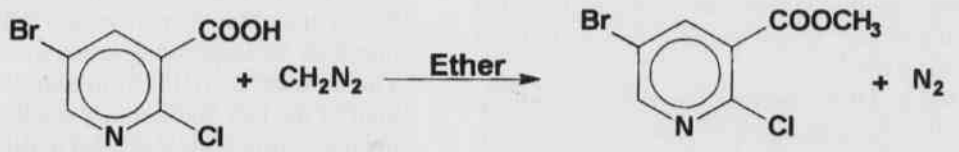


Table 2: Chemistry of the various methods of esterification.

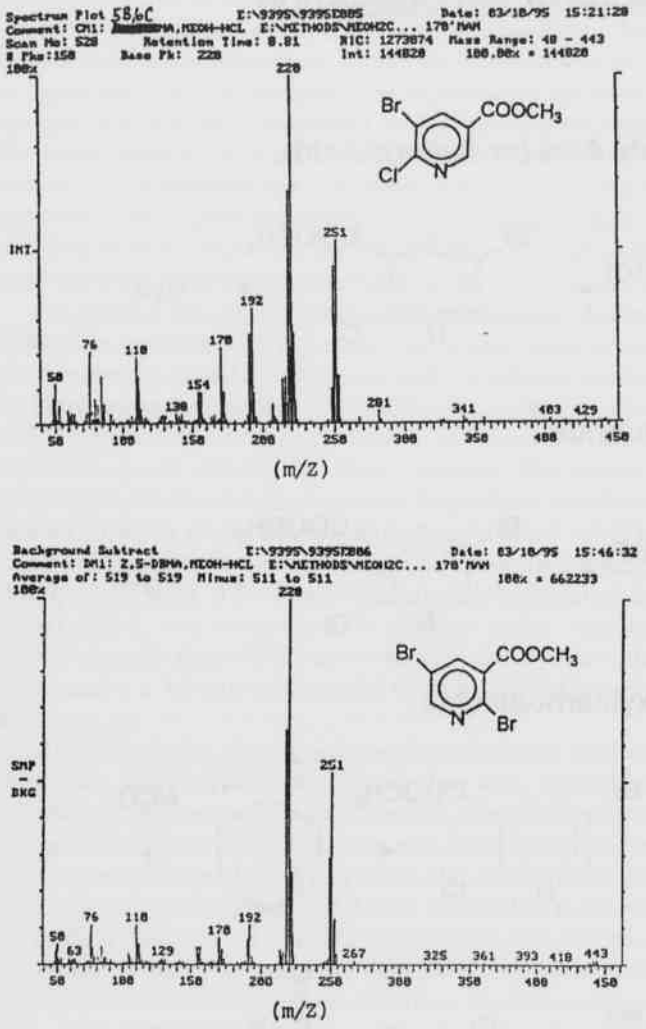


Fig. 2. Mass spectra of methyl 5-bromo-6-chloronicotinate and methyl 2,5-dibromo nicotinate.

mixture of all four test compounds was esterified, the total ion chromatogram showed eight peaks, indicating an incomplete conversion of the acids. In addition, the mass spectra of some of the esters indicated the displacement of halogens by methoxy groups. For example, the esterification of 5-bromo-6-chloronicotinic acid resulted in the formation of methyl 5-bromo-6-methoxynicotinate instead of methyl 5-bromo-6-chloronicotinate. Consequently, this method of esterification was discarded.

When subjected to DCC/methanol/pyridine esterification (Method #3), the chromatogram of 5-bromo-2-chloronicotinic acid produced multiple peaks (Fig. 4). As many as five peaks were obtained (excluding the solvent peak) from

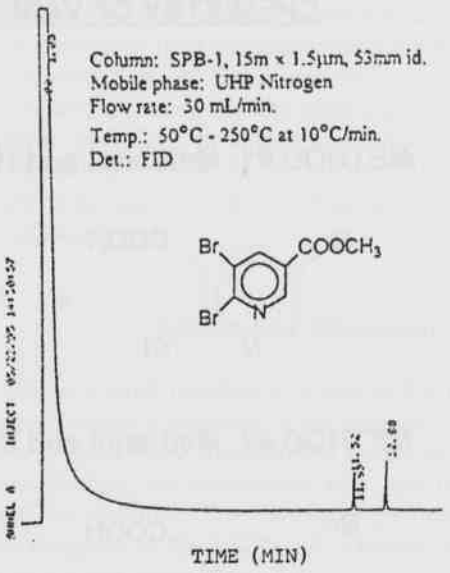


Fig. 3. Chromatogram of the reaction products following the esterification of 5,6-dibromonicotinic acid with BF_3/ methanol.

a single test compound, presumably due to a number of reaction products. After the product of this reaction was treated with acetic acid, there were still four peaks visible. Mass spectral investigation confirmed that a number of side reactions occurred, resulting in the formation of isocyanatocyclohexane, N,N'-dicyclohexylurea and its protonated form, N(cyclohexyl)-5-bromo-2-chloronicotinamide, and the methyl ester of the test compound. Obviously, this was not a good esterification technique.

A quantitative esterification was achieved using diazomethane (Method #4). Figure 5 shows a chromatogram obtained for a mixture of all six test compounds, along with 2-chlorobenzoic acid as the internal standard (at 12.26 min). One notices that only six peaks, well-resolved, were obtained with no indication of any side reaction. Mass spectral investigation of each of the peaks revealed the identity of the compounds as indicated on the chromatogram (Fig. 5); there was no evidence of mixed products in any of the peaks. Moreover, a comparative study, using purified standards from a previous study (Setliff and Huie, 1981) showed that a quantitative conversion ofthe dihalonicotinic acids into their corresponding esters was achieved by this method. The increase in yield (from about 80% to 100%) was attributed to the fact that a much smaller quantity (1.0 -10 mg) of the test compounds was used in the present study. The small peak preceding the chlorobenzoic acid peak was due to an

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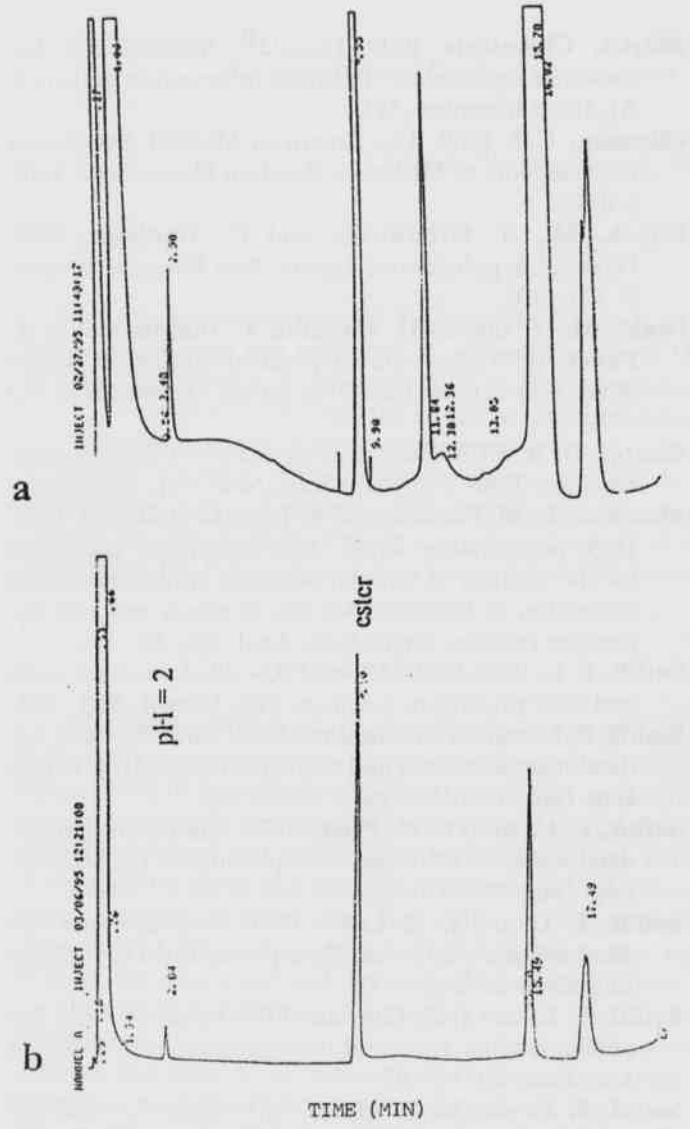

Fig. 4. Chromatograms of the reaction products following the esterification of 5-bromo-2-chloronicotinic acid using DCC/ methanol; a - chromatogram obtained at the reaction pH; b - chromatogram after the acidification. The chromatographic conditions are similar to those in Fig. 3.

impurity in the internal standard (used without purification) which was not identified. All six test compounds were also esterified individually to confirm their retention times and the order of elution from the GC column, which agreed with those obtained for the mixture. Both GC and GC-MS studies used identical column and separation parameters as described in Fig. 5.

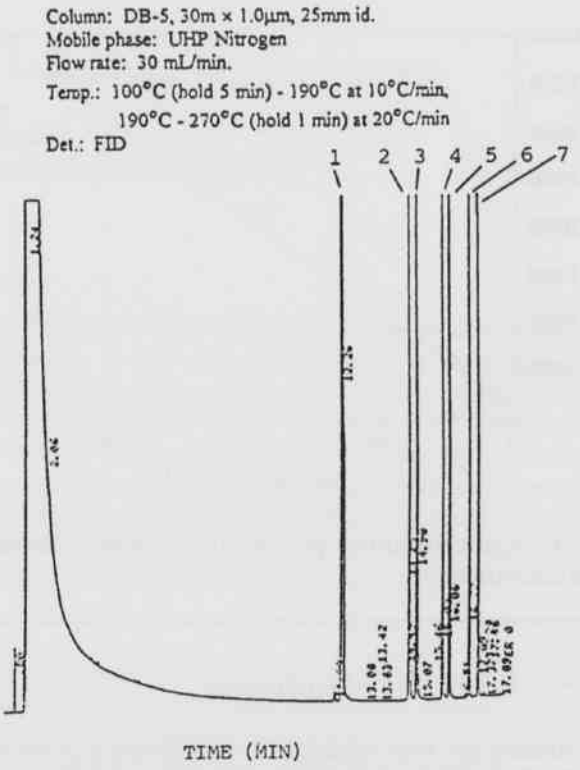

Fig. 5. Chromatograms of the methyl esters of the mixture of six test compounds by diazomethane esterification. The peaks are identified as follows: (1) methyl 2-chlorobenzoate (internal standard); (2) methyl 2,5dichloronicotinate; (3) methyl 5,6-dichloronicotinate; (4) methyl 5-bromo2-chloronicotinate; (5) methyl 5-bromo-6-chloronicotinate; (6) methyl 2,5dibromonicotinate; (7) methyl 5,6-dibromonicotinate.

It is obvious that Method #4 meets the criteria of a good analytical method for the separation and quantification of the dihalonicotinic acids. The methyl esters were produced quantitatively and the GC peaks were well-resolved. Since other dihalonicotinic acids were not readily available, the method development was continued using only those six test compounds. The concentration profile for each compound was studied both individually as well as in a mixture, ranging from 2 ng/μL to 1000 ng/μL. A typical plot of the data is shown in Fig. 6. Excellent linearity was observed up to about 500 ng/μL, above which the detector response was lower than expected. From these graphs the detection limit was estimated to be about 1 ng/μL. The reproducibility for the analysis in triplicate showed relative standard deviation between 5 and 15% for the compounds.

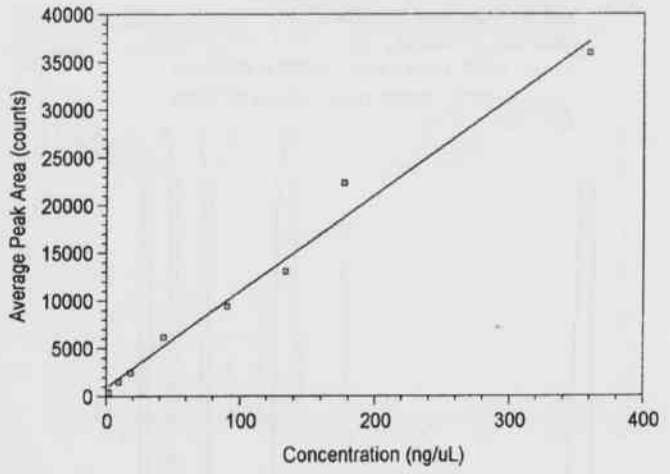

Fig. 6. Concentration profile of methyl 5-bromo-2-chloronicotinate.

Conclusions

Among the four methods of esterification studied, the one involving diazomethane was the only suitable technique for the separation and quantification of the dihalonicotinic acids. The method provided short analysis time with all six methyldihalonicotinates eluting within nineteen minutes. The methyl esters left no "memory effect" from the column, as compared to those observed with unesterified dihalonicotinic acids; in the latter cases, the column needed to be flushed repeatedly with methylene chloride to remove the traces of the compounds. The methyl ester peaks were also much sharper than the original acid peaks, with no tailing. We are confident that this method would yield separation and quantification of all twenty-six dihalonicotinic acids when they are present in a mixture. Lack of availability of all those test compounds prevented us from attempting such a separation at the present time. However, the observed pattern of elution of the methyl esters is that the 2,5- isomer is preceded by the 5,6- isomer of each methyldihalonicotinate. Also, among various dihalonicotinates, the dichloro esters elute before the bromo-chloro esters, which are followed by dibromo esters. Our future plan includes the use of all twenty-six dihalonicotinic acids in a sample. Also, an electron-capture detector will be used to improve the sensitivity and the detection limit of the method.

ACKNOWLEDGMENTS.—The authors wish to thank Mr. Tom Heinz of the National Center for Toxicological Research, Jefferson, AR for his help in obtaining some preliminary GC-MS data and their interpretation.

Literature Cited

Aldrich Chemicals. 1990. Diazald^{R}, MNNG and diazomethane generators. Technical Information Bulletin # AL-180, Milwaukee, WI.

Clayman, C.B. 1989. The American Medical Association Encyclopedia of Medicine; Random House, New York, p.1058.

Gacek, M., S. Gronowitz, and **C. Hedbom.** 1972. Potential hypolipidemic agents. Acta Pharma. Suecica. 9: 373 - 376.

Iwaki, M., T. Ogiso, H. Hayashi, T. Tanino, and **L. Z. Benet.** 1996. Acute dosedependent disposition studies of nicotinic acids in rats. Drug Metab. Dispos. 24(7): 773 - 779.

Knapp, D. R. 1979. Handbook of analytical derivatization reactions. John Wiley and Sons, New York, Chapter 3.

Manceau, F., M. Finiaux, and **A. Jecquin-Dubreuil.** 1992. High performance liquid chromatographic procedure for the analysis of tobacco alkaloids application to the evaluation of tobacco alkaloids in plants and cell suspension cultures. Phytochem. Anal. 3(2): 65 - 68.

Setliff, F. L. 1970. Some 2,5- and 5,6- dihalonicotinic acids and their precursors. J. Chem. Eng. Data. 4: 590 - 591.

Setliff, F. L. and **G. O. Rankin.** 1972. Some 2,5- and 5,6-dihalonicotinic acids and their precursors. II. J. Chem. Eng. Data. 17: 515 - 516.

Setliff, F. L. and **D. W. Price.** 1973. Some 2,5- and 5,6-dihalonicotinic acids and their precursors. III. J. Chem. Eng. Data. 18: 449 - 450.

Setliff, F. L. and **J. E. Lane.** 1976. Some 2,5- and 5,6-dihalonicotinic acids and their precursors. IV. J. Chem. Eng. Data. 21: 246 - 247.

Setliff, F. L. and **J. S. Greene.** 1978. Some 2,5- and 5,6-dihalonicotinic acids and their precursors. 5. J. Chem. Eng. Data. 23: 96 - 97.

Setliff, F. L. and **W. R. Huie.** 1981. Some 2,5- and 5,6-dihalonicotinates. J. Chem. Eng. Data. 26: 332 -333.

Zoellner, P. 1994. Utility of nicotinoyl derivatives in structural studies of mono- and diacylglycerols by gas chromatography mass spectrometry. Org. Mass Spectr. 29: 253 - 259.