DETERMINATION OF THE RESIDUES OF BRANCHED DECANOIC ACIDS IN THE PRESENCE OF SOME NATURALLY OCCURRING CARBOXYLIC ACIDS BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Neo-decanoic acid (NDA) is a mixture of di-α-branched decanoic acids which is being evaluated for use as a cotton harvest aid. The objective of the work being reported was to develop a method capable of determining NDA residues on cottonseed and products derived from it.

Separation of aliphatic carboxylic acids by thin-layer chromatography has been the subject of many publications in recent years[1–9]. The overall consensus seems to be that due to the overwhelming properties of the carboxyl group, it is difficult to separate their homologues by adsorption chromatography. Conversion of the carboxylic acids to derivatives such as methyl ester[10–12], hydroxamic acid[13], or 2,4-dinitrophenylhydrazine derivatives[14–16] and subsequent separation by TLC has been employed effectively. Methods have been described for the separation of the olefinic from the paraffinic carboxylic acids by silver nitrate or mercuric chloride impregnation of the sorbent which leads to the formation of adducts with the olefinic acids[10]. Partition paper chromatographic methods have been described as successful in the separation of homologues of carboxylic acids[17–24].

These methods were not applicable to detection and measurement of very small residues of neo-decanoic acid in cottonseed, in the presence of large amounts of saturated and unsaturated carboxylic acids that occur naturally in cottonseed (linoleic 44 %, oleic 33 %, palmitic 21 %, stearic 2 %)[25]. A very low limit of detection was required, which is not obtainable with the conventional acid-base indicators that are commonly used with carboxylic acids as the revealing agents[26, 27]. The method also had to be simple enough to render itself useful in the field.

The present method is capable of determining as little as 1 p.p.m. NDA in cottonseed and cottonseed oil. It involves the extraction of the seeds with diethyl ether (or dissolving the oil in diethyl ether), extracting the diethyl ether solution with an aqueous caustic solution, acidifying the aqueous phase and extracting it with ether. This step extracts all the carboxylic acids quantitatively. At this point, a selective esterification procedure is carried out, using boron trifluoride–methanol[3, 28], which quantitatively esterifies all but the sterically hindered neo-decanoic acid. The mixture is then chromatographed on a thin layer of silica gel, which leads to the separation of NDA from the esters. Using phosphomolybdic acid as the revealing agent, the TLC procedure has a sensitivity of 1 μg NDA. The size and density of the spots are compared to those of known standards in the proper concentration range,

either visually or by densitometry in order to determine the level of NDA. These standards are prepared by spiking the cottonseed or its oil, in the laboratory, and taking them through the procedure described above. Recovery of the NDA, selectivity of the esterification procedure and sensitivity of the method were carefully checked and proven appropriate. The method was also proven appropriate for measuring the NDA residues in the soil samples taken from the sprayed acreage.

EXPERIMENTAL

Preparation of standards

Five batches of control cottonseed or cottonseed oil were spiked with 0, 1, 2, 5 and 10 μg NDA/g of sample respectively. NDA was added in the form of iso-octane solutions, in concentrations such that 10 % (v/w) solution was added to the material being spiked. In the case of the seed standards, the NDA solution was sprayed onto the seeds which were then stored in capped jars within 10 min.

Procedure

50 g portions of the field samples or standards were packed in individual glass columns (2.5 × 40 cm). With the stop cocks closed, 100 ml ether was added to each column to cover the material and allowed to soak for 5 min. The flow rate was then adjusted to about 5 ml/min and the eluate collected in a 500 ml separatory funnel. An additional 100 ml of ether was used to elute each sample. In the case of the oil samples and standards, 50 ml portions were dissolved in 200 ml ether in 500 ml separatory funnels. All ether solutions were then extracted with 5 % aqueous sodium hydroxide (3 × 30 ml).

The combined caustic extracts of each sample were neutralized with 6 *N* HCl and then acidified with 5 ml of excess acid. After cooling, each sample was extracted with ether (3 × 30 ml). The combined ether extracts of each sample were evaporated on a steam bath, under a nitrogen jet, to about 10 ml. Each sample was transferred to a 50 ml flask using chloroform to wash. After adding 5 ml of boron trifluoride–methanol reagent (14 % w/v Applied Science Laboratories) to each flask, they were refluxed under a water-cooled condensor for 20 min. After cooling, each sample was transferred with chloroform into a 100 ml separatory funnel and then washed with 30 ml distilled water. The chloroform layer was then evaporated to 2 ml in a graduated centrifuge tube, on a steam bath under a nitrogen jet.

100 μl of each of the 2 ml samples were spotted with a Hamilton syringe, on a 20 × 20 cm Silica Gel G plate of 250 μ thickness (precoated by Analtech), using an air blower to keep the diameter of the spots below 1 cm. Up to 12 spots could be spotted on a single plate. The plates were then developed in a chamber containing the solvent mixture (iso-octane–acetone–acetic acid, 83:15:2), dried in a 140° oven for 5 min (to remove acetic acid), then sprayed with a 5 % ethanolic solution of phosphomolybdic acid and heated in the oven for 10 min. NDA appeared as dark blue spots on a light yellow background at an R_F of about 0.35 while the esters appeared as a larger spot at an R_F of about 0.6. The intensity of the NDA spot of each sample was compared to those of the standards visually or by densitometry.

Densitometric measurements were made using a Photovolt densitometer model 530 equipped with the automatic scanning stage and a tungsten light source, coupled

with a multiplier photometer model 520-A, Variacord recorder model 42-B, and Integraph integrator model 49. The plates were scanned only along the line corresponding to the R_F of the NDA spots and thus all the NDA spots on one plate were measured in a single scan.

DISCUSSION

In order to obtain positive proof of the selectivity of the esterification procedure a synthetic mixture of the acids naturally present in cottonseed, an extract of cottonseed and a sample of pure NDA were esterified separately under the conditions listed in the experimental procedure. Another sample of NDA was esterified with diazomethane[29,30] Portions of each sample before and after esterification were then chromatographed on the same plate. Comparison of the resulting spots proved that esterification of the naturally occurring acids was complete with the boron trifluoride–methanol reagent (no spot at R_F 0.35) and no esterification had occurred with NDA (no spot at R_F 0.6). However, diazomethane had reacted with NDA to produce the methyl ester which showed as a spot at R_F 0.6 (Fig. 1). Varying the time of reflux by $\pm$ 50% did not seem to alter these results. The limit of detection for NDA was established by spotting successively smaller amounts of NDA on a plate and de-

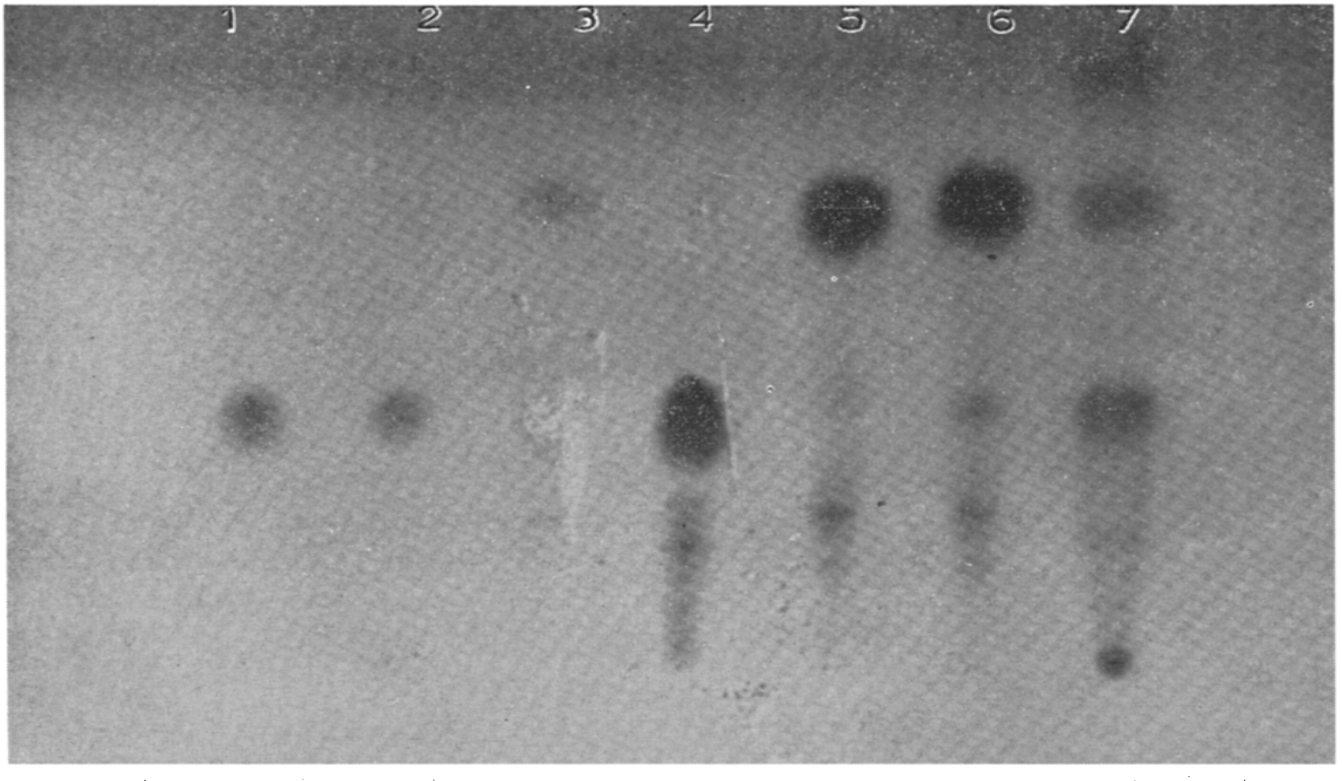

Fig. 1. Selectivity of the esterification procedure. (1) 0.1% NDA (10 μl); (2) 0.1% NDA refluxed with boron trifluoride–methanol reagent for 30 min (10 μl); (3) 0.1% NDA esterified with diazomethane (10 μl); (4) composite of 0.43% linoleic, 0.33% oleic, 0.20% palmitic and 0.03% stearic acids (10 μl); (5) No. 4 esterified with boron trifluoride–methanol (10 μl); (6) (No. 4 + 0.01% NDA), esterified with boron trifluoride–methanol (10 μl); (7) extract of NDA-spiked cottonseed, esterified with boron trifluoride–methanol (2 p.p.m. NDA).

veloping and revealing it in the manner described above. Although 0.5 μg NDA was detectable, the limit of "convenient detection" was taken as 1 μg.

The recovery of the NDA in this procedure was determined by comparing the intensities of the spots produced by the standards with those of spots produced by direct application of equal volumes of equivalent concentrations of NDA in chloroform (Fig. 2). The recovery determined in this manner was about 75% and quite reproducible. As long as the residues are determined by comparing the samples to the standards treated in like manner and spotted on the same plate, any reasonable level of recovery should be satisfactory. In order to determine whether any loss of NDA was occurring in any of the evaporation steps, several 20 ml portions of a 0.005% solution of NDA in iso-octane were evaporated on a steam bath under a jet of nitrogen

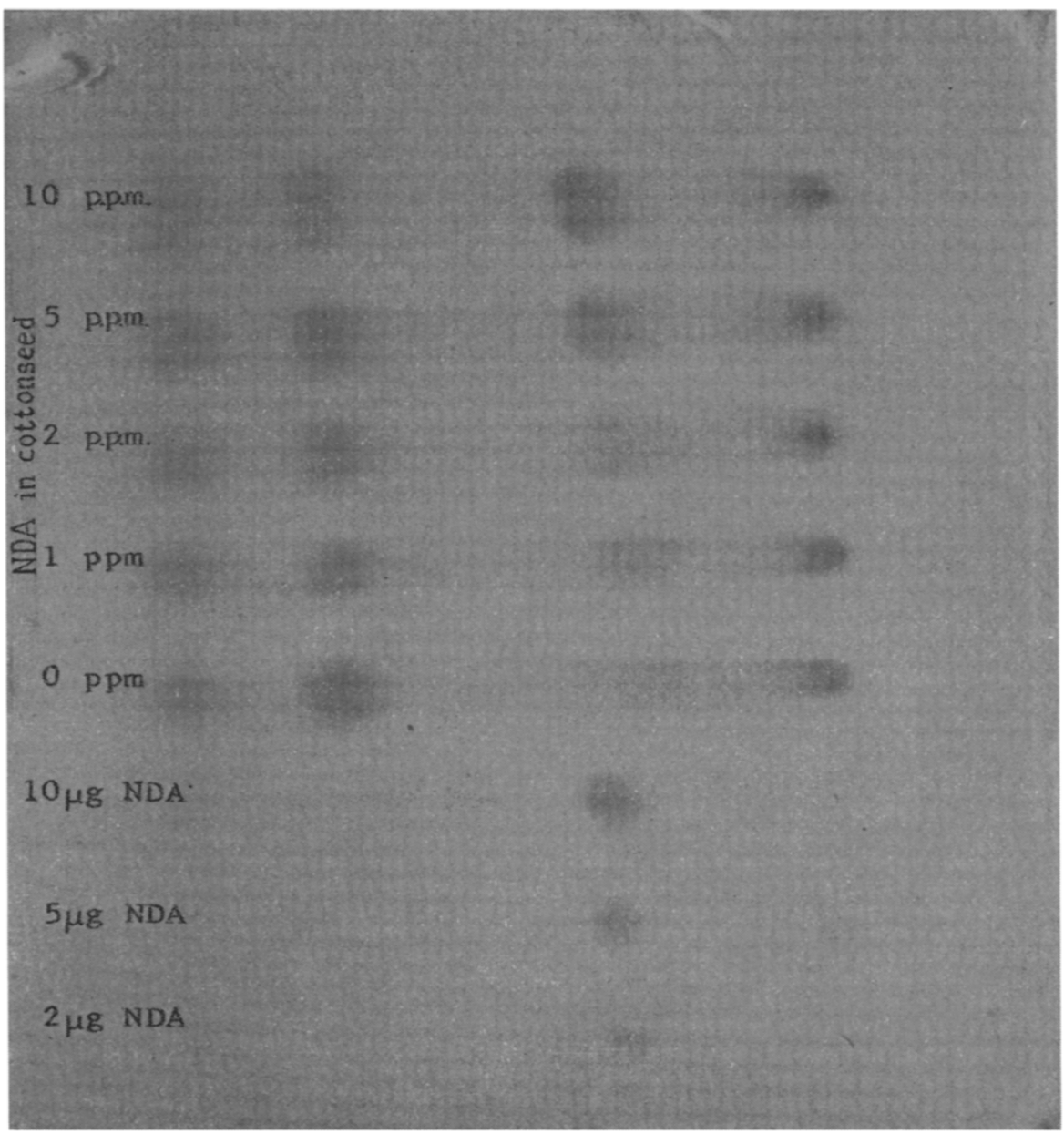

Fig. 2. Recovery of NDA.

to 1/2, 1/4, 1/10, 1/20, 1/40 of the original volume and one was evaporated down to dryness and redissolved in 1 ml solvent. Appropriate volumes of the original and each of the evaporated solutions (calculated to contain 5 μg NDA each) were chromatographed and compared. The results showed that in all but the last case the intensity of the spots produced was equal to that of the original solution. Evaporation to dryness had resulted in almost total loss of NDA. Appropriate precautions were taken, therefore, to prevent the evaporation to dryness in any of the evaporation steps.

It was also discovered that air drying of the spiked cottonseed, before the extraction, for more than 10 min resulted in a gradual loss of NDA. Proper precautions were then taken in storing the seed samples and standards to prevent such losses.

No loss of NDA was encountered as a result of drying the plates in the oven, prior to revealing the spots. This was tested by comparing the intensity of a spot produced by a given volume of an NDA solution on an undeveloped plate, with one that was developed in the usual manner and dried in the oven before revealing.

It was noted that exposing the finished chromatograms to ammonia vapors for a few minutes eliminated the light yellow background. However, the intensity of the spots seemed to diminish slightly as a result.

Although freshly prepared phosphomolybdic acid solution produced the desired spots, much superior intensity was obtained with solutions that were aged at room temperature for one week or longer.

When comparing the spots visually, differentiation was possible only between the spots that have a difference of at least 0.5 μg NDA. Such differentiation was only possible in the range 1–5 μg and became increasingly difficult as the NDA content increased. For example, it was much easier to differentiate between a 1 μg and 2 μg spot than it was to differentiate between a 5 μg and a 6 μg spot. In visual evaluation of the spots containing more than 5 μg NDA, better accuracy is obtainable if such samples are diluted by a known factor (to place the concentration in the 1–5 p.p.m. range) and then respotted and re-evaluated, keeping the dilution factor in mind.

When using densitometry, usually a calibration curve was obtained by plotting the peak areas corresponding to the standards (a measure of size and intensity of spots) *versus* the μg of NDA in that standard. Using the peak area obtained for each sample, the corresponding NDA concentration was then determined from the calibration curve. Results obtained in this manner were generally much more reproducible and accurate than those obtained by visual comparison. Differences of 0.5 μg NDA were detectable in the range 1–10 μg. The area of the spots was obtained with the integrator attached to the recorder of the densitometer. Some problems were encountered in this respect where the background was nonuniform due either to the nonuniformity in the thickness of the sorbent layer or unevenness in the application of the revealing agent. In such cases, a nonlinear base line was obtained and the areas measured by the integrater were not usable. Therefore, either the areas were measured from an appropriately drawn base line by triangulation, or the recorder response was adjusted to zero prior to scanning of each spot. In most cases superiority of the densitometry to visual comparison was quite evident. In those cases, however, where the NDA spots were superimposed on a variable background, due to a component in the sample, the visual comparison remained to be the only method of evaluation.

SUMMARY

Neo-decanoic acid (NDA) is a mixture of di-α-branched decanoic acids which is being evaluated for use as a cotton harvesting aid. To determine the residue on the seeds of the treated plants and in the products derived from such seeds, a quantitative thin-layer chromatographic procedure has been developed which is capable of detecting 1 μg NDA in the presence of much larger amounts of saturated and unsaturated carboxylic acids that are naturally present in cottonseed. Since these acids interfere with the determination of NDA, a procedure has been developed that selectively esterifies only the naturally occurring acids, leaving the sterically hindered NDA intact. The results obtained by visual comparison are compared to those obtained by thin-layer chromatographic densitometry. Evidence is presented to establish that: (1) the naturally occurring acids are completely esterified; (2) the NDA remains completely intact in the esterification step; (3) recovery of NDA is appropriate and reproducible.

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