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TRACE LEVEL ANALYSIS OF INDOLE-3-ACETIC ACID FROM LIGHT-GROWN, HIGHLY PIGMENTED LEAF TISSUE

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SUMMARY

A new chromatographic method has been developed for the isolation and quantitation of trace levels of the plant hormone, indole-3-acetic acid (IAA). The individual steps in the method were selected and adjusted specifically to remove the large amounts of interfering substances present in highly pigmented, light-grown plant tissue. This method employs a rapid extraction and preparative cleanup by open column chromatography, followed by preparative reversed-phase ion-pair high-performance liquid chromatography to remove the bulk of the interfering substances. The latter step maximizes the resolution of the IAA from its native matrix by interaction with the tetrabutylammonium (TBA) acetate ion-pair at the ideal pH for the quantitative formation of the TBA–IAA ion-pair. Two additional high-performance liquid chromatographic separations ultimately produce a baseline resolved IAA peak, which is quantitated by fluorescence and amperometric detection. Identity of the IAA peak was verified by capillary gas–liquid chromatography with nitrogen–phosphorus detection and by gas chromatography–mass spectrometry analysis. The method is illustrated here by the quantitation of IAA from 1 g fresh weight of highly-pigmented, light-grown cotton leaf tissue. The limit of detection is as low as 1 ng of IAA from 1 g fresh weight with average recoveries of around 50% at the quantitation step. Although the method was designed to quantitate IAA, the extraction and high-performance liquid chromatographic steps can be used to prepare purified IAA samples for esterification and gas chromatography–mass spectrometry analysis.

INTRODUCTION

The analysis of plant hormones by physicochemical methods involves special problems because the active compounds are present in trace amounts in a highly complex matrix, and they are often chemically labile[1–3]. The methods of choice are gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC)[1,4]. The mass spectrometer offers advantages in the accu-

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racy of identification and quantitation, while HPLC offers advantages in cost, effectiveness and speed. Recent advances in column materials[5–7] and detectors[8–12] have added to the versatility of HPLC for both the purification and the analysis of plant hormones.

Despite the rapid evolution of analytical technology, the analysis of plant hormones, and particularly the growth promoting substance indole-3-acetic acid (IAA), remains difficult. Surveys of the present literature in the plant hormone field[1–4,13,14] reveal that most of the data still are obtained from tissues that have relatively low levels of interfering compounds, such as seeds and etiolated tissue. Thus the inherent problems associated with the analysis of low levels of IAA in the presence of high levels of pigments and other interfering compounds found in light-grown plants are avoided. The method described in this paper has allowed us to remove the high levels of interfering compounds found in leaves of light-grown cotton plants leaving an IAA peak pure enough to be quantitated by paired fluorescence and amperometric detectors. The identity of the IAA peak was confirmed by gas–liquid chromatography (GLC) and GC–MS.

EXPERIMENTAL

Materials

All solvents were HPLC grade. Water was type I water oxidized by UV irradiation to remove the last traces of organic contaminants. Butylated hydroxytoluene (BHT) was used as antioxidant. [^{3}H]IAA was used as radiotracer; it was repurified by HPLC and the specific activity verified by independent analyses of IAA and ^{3}H content.

Precautions

During the extraction and subsequent analysis, all samples and fractions were protected from overhead fluorescent lights. BHT was replaced after every separation to inhibit oxidation. All glassware was silylated and clean of contaminants.

HPLC apparatus

The HPLC system consisted of: two Waters Model 6000A pumps, WISP Model 710 autosampler, Waters system controller and data module, ISCO fraction collector with an Angar isolation valve (P/N 368-3-12), Perkin-Elmer LS-4 fluorescence spectrometer, Waters Model 440 UV monitor, and a Bioanalytical Systems Model LC-4B amperometric detector.

Scintillation counting

Recovery of internal standard was monitored with a Beckman LS-1801 liquid scintillation counter. Aliquots of 0.1 ml were counted and corrected for quenching with curves developed from plant extracts.

Capillary GLC and GC–MS apparatus

The capillary GLC instrument was a Varian Model 3400 with the capillary on-column cryogenically cooled injector, electron-capture detector, and nitrogen–phosphorus detector.

The GC–MS instrument consisted of a Varian Model 6000 capillary GC interfaced to a V.G. Industries Model 70-70 double focusing mass spectrometer.

Extraction

Tissue samples to be studied are harvested, immediately frozen in liquid nitrogen, and ground to a fine powder in liquid nitrogen with a mortar and pestle. Samples of 1 g (fresh weight) are placed in cooled, pre-tared test tubes and stored at −70°C until extracted.

Our extraction procedure is similar to that developed by Thompson *et al.*[15] and employed by Law and Hamilton[12] and by Riov, Goren and Sagee (personal communication, 1984). Samples (1 g) are suspended in 5 ml of methanol–water (8:2), containing 0.1 *M* ammonium acetate and 10 μg/ml BHT; an appropriate amount of radioactive tracer is added, and the mixture is homogenized with a Brinkmann (7 mm diameter probe) Polytron at full speed for 30 s in an ice bath. Each homogenized sample is refrigerated in the dark (30 min) and centrifuged, and the supernatant fraction is saved. The pellet is resuspended in 5 ml of water and centrifuged, and the supernatant fraction is pooled with the supernatant fraction from the initial centrifugation.

Preparative cleanup

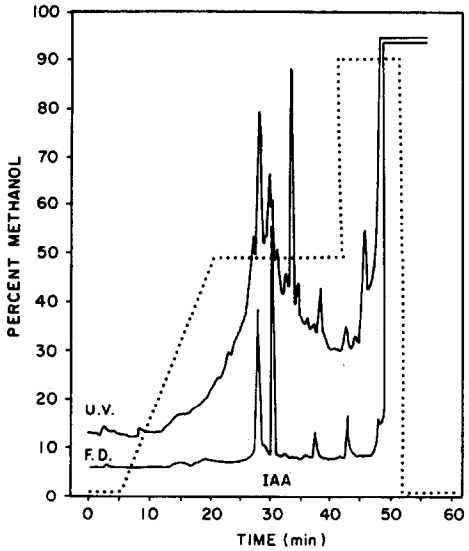
The highly pigmented extracts are subjected to three open-column liquid chromatography steps, yielding virtually colorless, partially purified extracts. Location and recovery of the IAA is monitored by the radioactive internal standard.

As described by Thompson *et al.*[15], the extracts are loaded on a polyvinylpyrrolidone (PVP) column developed with 0.01 *M* ammonium acetate. The active fraction is placed directly on a DEAE Sephadex (A-25-120) column developed with 0.01 *M* ammonium acetate. The active fraction is eluted from this column with 1 *M* acetic acid and applied to a Sep-pak (Waters Assoc.) prewashed with methanol. The excess acetic acid is eliminated by washing with 5 ml of water, and the active fraction is eluted with 5 ml of HPLC-grade methanol. This fraction is brought to dryness with a Buchler Evapornix test tube evaporator and later is dissolved in 4 ml of methanol–water (5:95) containing 0.1% BHT for subsequent HPLC analysis.

HPLC analysis

Aliquots (2 ml) of the samples are first purified on a C_8 column (3 μm, DuPont Golden Series, 8.0 cm × 6.2 mm I.D., with a 5 μm Supelguard LC-8 2.0 cm × 4.6 mm I.D. precolumn) by preparative reversed-phase ion-pair chromatography (RPIPC) using the tetrabutylammonium (TBA) cation. A gradient, as shown in Fig. 1, is employed with water and methanol–water (9:1), each containing 100 m*M* TBA, 100 m*M* Tris and adjusted to pH 8.0 with acetic acid (flow-rate 1.0 ml/min). The tetrabutylammonium acetate ion-pair is formed *in situ* in each solvent by titrating tetrabutylammonium hydroxide with acetic acid. The eluent is monitored by UV detection at 254 nm and by fluorescence detection at ex^{280}/em^{362}. The TBA–IAA ion-pair elutes at 31.8 min and is collected and reduced to 1 ml with the test tube evaporator. If samples are stored between steps the BHT is replaced, capped vials are covered with foil and placed in a 0°C freezer.

A 0.9-ml aliquot of the TBA–IAA fraction is rechromatographed on a C_8 column (3 μm Supelcosil LC-8 (15.0 cm × 4.6 mm I.D. with a 5 μm Supelguard



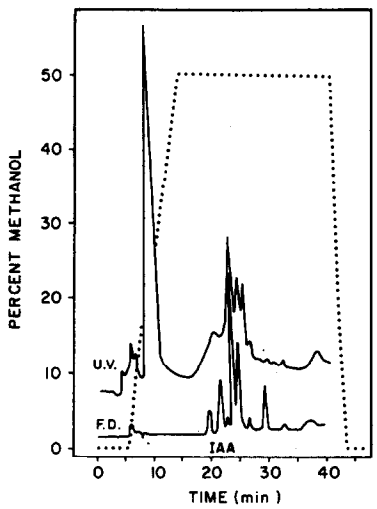


Fig. 1. Chromatogram of plant extract analyzed by RPIPC on a DuPont Golden Series 3 μm C_8 column. Retention time for TBA–IAA was 31.8 min. UV detection at 254 nm × 0.2 a.u.f.s., fluorescence detection at ex^{280}/em^{362} × 1 full scale. Dotted line is the gradient profile; full scale indicated by maximum traces at upper right. HPLC conditions described in text.

Fig. 2. Chromatogram of IAA containing fraction collected from RPIPC step (see Fig. 1) on a 3 μm Supelcosil LC-8 column. Retention time for IAA was 22.1 min. UV detection at 254 nm ×0.1 a.u.f.s., fluorescence detection at ex^{280}/em^{362} × 1 full scale. Dotted line is the gradient profile; major UV peak reaches full scale. HPLC conditions described in text.

LC-8 precolumn, 2.0 cm × 4.6 mm I.D.). The gradient shown in Fig. 2 is employed with water and methanol–water (1:1) each adjusted to pH 2.1 with phosphoric acid (flow-rate 0.7 ml/min). The eluent is monitored as above showing a retention time for IAA of 22.1 min. The IAA fraction is collected and reduced to 1 ml with the test tube evaporator.

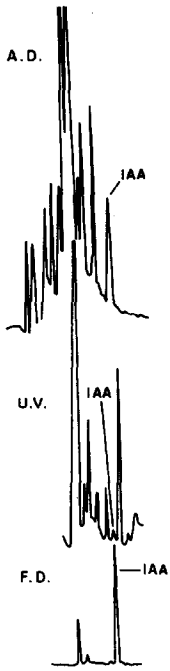
A 0.9-ml aliquot of the IAA fraction is rechromatographed on a C_{18} column (3 μm Supelcosil LC-18, 15.0 cm × 4.6 mm I.D., with a 5 μm Supelguard LC-18 precolumn, 2.0 cm × 4.6 mm I.D.). The mobile phase is acetonitrile–water (22:78) with the pH adjusted to 3.0 with acetic acid (flow rate 1.0 ml/min). IAA quantitated at this step by fluorescence (as above) and by amperometric detection (0.95 V with a glassy carbon electrode) has a retention time of 11.67 min (Fig. 3).

Quantitation

The baseline resolved peaks obtained from the last HPLC step are quantitated against authentic standards and normalized to reflect the total sample composition. This is done by taking into account the percentage of the recovered radioactivity, the amount of labeled IAA present at the quantitation step, and all the dilution factors.

Methylation

The pure endogenous IAA fractions obtained from the HPLC analysis were



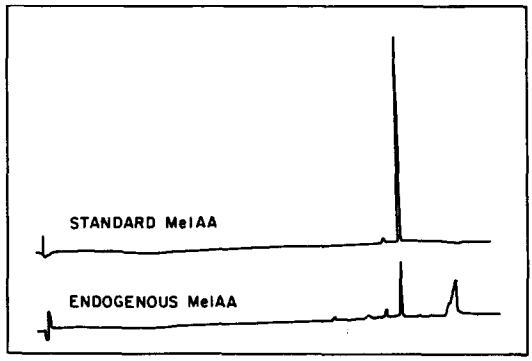


Fig. 3. Quantitation step for IAA. Chromatogram of IAA containing fraction collected from second HPLC step (see Fig. 2) on 3 μm Supelcosil LC-18 column. Retention time for IAA was 11.67 min. Detector sensitivities for full scale set at 200 nA (amperometric detection, 0.005 a.u. (UV) and $\times 5$ fluorescence detection. HPLC conditions described in text.

Fig. 4. Chromatogram of capillary GLC analysis of methyl IAA (MeIAA) from reagent (standard) or plant (endogenous) source. The retention time observed was 38.7 min. See text for conditions.

partitioned three times against diethyl ether and methylated by the dropwise addition of freshly generated diazomethane in ether[19]. With frequent stirring the reaction usually was complete after 20 min at 30°C. An aliquot was taken for liquid scintillation counting (LSC), the fractions were brought to dryness and dissolved in methanol–water (1:1) for subsequent HPLC purification. This was done by gradient elution from methanol–water (1:1) to 100% methanol in 5 min with a linear program on the C_{18} column used in step 3 (Fig. 3). The purified methyl IAA was collected, an aliquot was taken for LSC, and the methylation yield was calculated based on the amount of radioactivity recovered. Each sample was brought to dryness under a nitrogen stream and resuspended in hexane (Burdick & Jackson) for capillary GLC and GC–MS analysis.

Capillary GLC and GC–MS

The methyl IAA was subjected to capillary GLC on an SGE BP-10 bonded phase cyanopropyl silicone capillary column, 30 m $\times$ 0.33 mm I.D., with a 1.0-μm film thickness. The column flow-rate was 1.2 ml/min of helium, and the makeup flow-rate was 28.8 ml/min of helium. A thermionic specific detector for nitrogen and phosphorus was used at 280°C. Injection (2 μl) was isothermal at 190°C. The oven

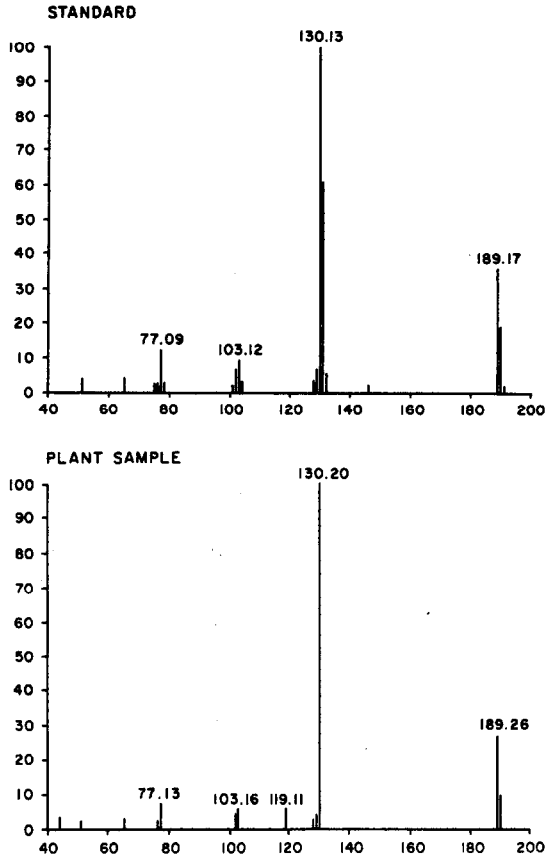


Fig. 5. GC–MS trace of fragmentation pattern of methyl IAA, reagent standard (above) and plant sample (below). See text for conditions.

program was: initial at 100°C (hold for 10 min); increase at 5°C/min up to 240°C; then hold for 10 min. The retention time for methyl IAA was 38.7 min (Fig. 4).

The same column/program combination was used for the GC–MS analysis, which gave only one total ion peak at 38.7 min, which had the appropriate fragmentation pattern for authentic methyl IAA, showing a parent ion peak at 189 and a base peak at 130 *m/z* (Fig. 5).

RESULTS AND DISCUSSION

After preparative cleanup by open-column chromatography the extracts are subjected to three HPLC separations at the end of which the total baseline resolution of IAA is accomplished for fluorescence detection and UV detection (Fig. 3). The bulk of this purification is done in the first HPLC step by the use of RPIPC. In this step the tetrabutylammonium acetate ion-pair is formed *in situ* by mixing the tetrabutylammonium hydroxide reagent with an appropriate amount of acetic acid in the mobile phase. Acetic acid is used since it has approximately the same pK_a as IAA,

therefore assuring quantitative TBA–IAA ion-pair formation, on-column retention and subsequent separation by programmed gradient elution. The separation of TBA–IAA from an extract of cotton leaf tissue is shown in Fig. 1. TBA–IAA elutes at 31.8 min followed by the bulk of the remaining material after 40.0 min. We estimate that *ca.* 90% of the remaining interfering material is eliminated at this step. The TBA–IAA-containing fraction is collected and reduced in volume for the next HPLC step.

The second HPLC step is designed to dissociate the TBA–IAA ion-pair upon injection into a second reversed-phase C_8 column running at a low pH at which IAA is not ionized. This can be accomplished quantitatively only if the acid chosen in this mobile phase has a lower pK_a than that of IAA. For that reason we chose to use phosphoric acid which has a pK_{a_1} around 2.1; this allows the H_2PO_4 ion to displace the IAA from its ion-pair, protonating it and retaining it in the column for subsequent elution by gradient programming. A typical separation is shown in Fig. 2; the new TBA–H_2PO_4 ion-pair elutes soon after the void volume, followed by the elution of a series of protonated acids. IAA elutes at *ca.* 22.1 min and is collected, reduced in volume and subjected to a third HPLC separation. During the first two HPLC steps a separate IAA peak is usually not observable, and IAA must be located by ^3H activity (Figs. 1 and 2).

The last HPLC step serves a dual purpose. First, it separates the IAA from the last trace of contaminants, yielding a baseline resolved peak in two of the three detectors. Second, since it is an isocratic separation, it helps to achieve a more accurate quantitation by providing gaussian shaped peaks at different concentration ranges. Also, it optimizes the fluorescence detection by keeping the solvent conditions constant, allowing the detector to be set at the maximum excitation and emission wavelengths at that particular solvent composition. Furthermore, it allows the use of amperometric detection downstream of the previous detector. With the present technology amperometric detection can be used only under isocratic conditions. Our recovery of the radioactive internal standard is routinely above 50% at this third HPLC step.

The excitation and emission wavelengths (ex^{280}/em^{362}) employed are the maxima for IAA under these conditions and when supplied as narrow bands give a degree of specificity to the fluorescence detector. The amperometric detector is operated at 0.95 V, the optimum voltage for IAA. By using dual detection we can use detector response ratios as a qualitative tool. If the IAA peak did happen to be contaminated at this stage, it is highly unlikely that the contribution of the contaminant to the fluorescence signal at the chosen wavelengths would equal its contribution to the signal in the amperometric detector at the selected potential. Law *et al.*[16] have shown that amperometric detection sensitivity for IAA and contaminating substances is not parallel with voltage and varies considerably at selected voltages. Therefore, the detector response ratio comparison becomes an effective tool to verify the purity of the IAA peak (Table I).

Table I shows an example of data from cotton leaf tissue; a detector response ratio of 1.002 was obtained for samples containing 38 ng/g fresh weight, while a ratio of 0.817 was obtained for samples in the 7–9 ng/g fresh weight range. We usually observe a sloping baseline with the amperometric detector (Fig. 3) which makes quantitation of the peak less accurate, especially with low amounts of IAA. This

TABLE I

LEVELS OF IAA DETECTED BY FLUORESCENCE DETECTION (FD) AND AMPEROMETRIC DETECTION (AD)

Samples were 1 g fresh weight leaf blades of 38-day-old cotton plants.*

Tissue	*FD IAA (ng/g)*	*AD IAA (ng/g)*	*Ratio FD:AD*
Youngest leaf, partially unfolded	38.02	37.94	1.002
Intermediate leaf, fully expanded	7.46	9.13	0.82

* Actual amounts quantitated by the detectors can be calculated by dividing data by 4.4 for this experiment.

error accounts for the detector ratio deviation shown in Table I; reagent IAA samples give linear responses and detector ratios of 1 over the range from 1 ng to several hundred nanograms per sample. Because of this situation, extreme sample purity and careful chromatography of the quantitation step are especially desirable for samples with low amounts of IAA (1–2 ng/sample as for intermediate leaves, Table I). Therefore, as a quality control standard we have adopted a minimum response ratio of 0.8 for samples containing small amounts of IAA. This criterion, plus co-chromatography with authentic standards in all three HPLC steps, plus confirmation by GC with nitrogen–phosphorus detection and GC–MS analysis, demonstrates that a high degree of confidence can be placed in our qualitative and quantitative analysis of IAA.

After experience with the method was accumulated, recoveries ranged from *ca.* 40 to *ca.* 60% (Table II). The higher values occurred predominantly with repetition. Initially we observed that disproportionately large losses were possible during the preparative cleanup stage at each step resulting in total losses of 40–60% prior to the first HPLC step. These losses were minimized by careful monitoring of the fraction containing the labeled internal standard and careful equilibration and prewashing of the columns. These adjustments lowered total losses during the preparative cleanup stage to 15–25%. During HPLC losses per step are uniform except that

TABLE II

PRECISION OF IAA ANALYSIS AND RECOVERY OF INTERNAL STANDARDS FROM DUPLICATE DETERMINATIONS OF TISSUES

Each sample was 1 g fresh weight from 38-day-old cotton plants; data are from fluorescence detector plus or minus one standard deviation.

*Tissue**	*IAA (ng/g ± S.D.)*	*Recovery ± S.D. (%)*
Youngest leaf	38.52 ± 0.71	37.41 ± 0.53
Fourth leaf	7.26 ± 0.29	37.81 ± 0.23
First leaf	9.33 ± 0.20	59.69 ± 6.93
Cotyledons	10.16 ± 2.71	58.46 ± 0.17

* Leaves numbered from base to shoot tip. Leaf four was just fully expanded; cotyledons are leaf-like storage organs below the first true leaf.

IAA is labile at pH 2.1, and in order to minimize losses samples should not be stored between the second HPLC step (Fig. 2) and the quantitative HPLC step (Fig. 3). With these precautions losses were a few percent for each step and recoveries of *ca.* 60% were achieved routinely (Table II).

This method was developed recognizing that precision and accuracy of analysis of IAA are not limited by the actual detection step but rather by the impact of extensive amounts of interfering substances in the plant tissues and the potential for contamination of the final peak with co-eluting substances. Accuracy of the quantitative step is assured by calibrating the detectors with a standard curve of reagent IAA which is linear for both detectors from tenths of a nanogram to several hundred nanograms. This calibration is performed for each set of samples to be analyzed in a continuous sequence. Precision and recovery for a series of duplicate samples are shown in Table II. Standard deviations (S.D.) of both amounts of IAA and recovery are small when compared with differences between samples. Another way to assess accuracy is to compare data from the independently calibrated fluorescence and amperometric detectors. For example the youngest leaf data from Table II were 38.52 ± 0.71 ng IAA/g fresh weight for fluorescence and 38.57 ± 0.53 for amperometric detection for duplicate, independently processed samples. The average S.D. for all four determinations was ±0.67 ng/g. These results indicate that the method is accurate and produces reproducable data given the level of sample purity (Fig. 3) and recovery (Table II) achieved here.

This method has several advantages. First, the high level of confidence for the correct identification and quantitation after extensive HPLC purification allows for the analysis of IAA without having to perform a GC–MS confirmation for each sample, therefore, allowing scientists without routine access to GC–MS instrumentation to accurately and confidently analyze IAA from plant tissue. This statement assumes an initial unequivocal identification of the IAA by MS and sufficient chromatographic purity to allow use of the fluorescence–amperometric detector response ratio to verify the purity of the putative IAA peak (Fig. 3). Secondly, this method maximizes the resolving power of HPLC to remove the high levels of interfering compounds found in extracts of leaves and other highly pigmented plant organs. Even if GC–MS is used to quantitate IAA, the present HPLC procedures represent an effective means to clean up highly complex extracts so as to allow analysis of uncontaminated methyl IAA in the MS detector.

Using the automated HPLC system described, twelve to twenty samples can be purified and assayed in a 3–4 day cycle. Extraction and the initial open-column chromatography cleanup require one person about a day for eight samples; thus, the procedure could handle roughly sixteen samples per week. Chromatographic conditions, flow-rates and steepness of gradients have been chosen to optimize chromatography, and in our hands changes to speed up the method resulted in less pure samples and greater losses.

RPIPC has been employed previously[17,18] for this type of analysis, but in these previous cases the ion-pair used was tetrabutylammonium phosphate, which in our experience is less efficient in the quantitative ion-pair formation with IAA than the tetrabutylammonium acetate. Also, quantitation was done by measuring the peak intensity of the TBA–IAA ion-pair as it was eluted by gradient programming[18]; however, this approach is more subject to error for reasons mentioned before.

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