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Indirect detection in capillary electrophoresis Comparison between indirect UV and indirect laser-induced fluorescence detection for the determination of isoprenyl pyrophosphates

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Abstract

Three different detection modes were compared for the determination of isoprenyl pyrophosphates by capillary electrophoresis, viz., direct UV, indirect UV and indirect laser-induced fluorescence (ILIF). The composition of the background electrolyte was varied accordingly. On the basis of separation efficiency ($N = 600\,000$) and limit of detection (ca. 0.5 μM), the ILIF method gave the best results. Separation was complete in less than 6 min.

1. Introduction

Indirect absorption and indirect fluorescence detection have been a boon in the determination of analytes possessing poor chromophoric properties [1,2]. Both techniques have been successfully applied to the determination of numerous analytes. Indirect detection was first applied in 1967 to electrophoresis by Hjertén [3]. Since that time, the technique has been extensively developed [4], especially in connection with organic and inorganic anions [5–17]. Indirect fluorescence was first applied to capillary electrophoresis (CE) by Kuhr and Yeung [18]. Both techniques operate on the principle of displacement of a visualization buffer component by the analyte to yield a decrease in the background signal. Of the two techniques, indirect absorption is the more commonly used. This can be attributed to the numerous indirect absorbance visualization agents that exist and because of the commercially available CE instruments having UV detectors. Indirect fluorescence is used less frequently because a laser is typically involved, which makes the technique expensive. At present, only one major manufacturer of CE instruments offers a laser-induced fluorescence (LIF) detector. Further, the number of fluorophores that serve as visualization agents is smaller than the number of indirect absorbance visualization agents.

Determination of isoprenyl pyrophosphates is important as these compounds are considered to

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be involved in the regulation of cell growth and division [19–24]. Common methods for the determination of isoprenyl pyrophosphates in samples of biological origin are TLC, isotope dilution–HPLC, enzymatic reaction–HPLC [25] and HPLC [26]. Preliminary results have indicated that the level of farnesyl pyrophosphates (FPP) in rat liver is of the order of 5–10 μM [27]. In general, the chromatographic methods have given relatively poor accuracy and precision for the determination of isoprenyl pyrophosphates. Further, the HPLC methods are time consuming [25]. The use of CE for the determination of isoprenyl pyrophosphates would be a logical progression. Compared with the other methods, its main advantages are speed of analysis and reduced sample consumption.

In this work, three modes of detection, direct UV, indirect UV and indirect LIF (ILIF) were evaluated based on separation efficiency and limit of detection.

2. Experimental

2.1. Instrumentation

The CE system was of in-house design and consisted of a 0–30-kV high-voltage power supply (Bertan, Hicksville, NY, USA), two platinum electrodes and a Plexiglas high-voltage isolation box. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) 360 μm O.D. × 26, 40, 50 and 75 μm I.D. and 150 μm O.D. × 20 μm I.D. were used.

Direct and indirect UV detection were accomplished with capillary on-column detector (CV-4; ISCO, Lincoln, NE, USA). Indirect fluorescence detection was performed according to Yeung and co-workers [28,29]. A He–Cd laser (Liconix, Sunnyvale, CA, USA) operating at 325 nm served as the excitation source. It was stabilized to within 0.05% of 2.5 mW with a laser power stabilizer (Model LS 100; Cambridge Research and Instrumentation, Cambridge, MA, USA). The stabilized beam was focused into the capillary by a 17-mm focal length biconvex lens (Melles Griot, Irvine, CA, USA). A 20 × microscope objective was used to collect the fluorescence and focus it on to the cathode of a photomultiplier tube (PMT) (R928; Hamamatsu, Upplands-Väsby, Sweden). Scattered light from the capillary was eliminated by using an aperture and two long-pass filters (KV 389; Schott Serving, Bromma, Sweden) mounted between the objective lens and the PMT. This removed at least 99% of the scattered light. Injection was at the anode and detection was at the cathode.

2.2. Buffers

Phosphate buffer of 10 mM (pH 7.0) was used in conjunction with direct UV detection. Three different buffers were used in the indirect UV method: 2.5 and 5 mM phthalic acid and 5 mM benzoic acid, the pH being adjusted to 6.5. Indirect fluorescence detection was performed using 1 mM salicylate buffer prepared by titrating salicylic acid (Aldrich, Steinheim, Germany) to the desired pH with NaOH. Water was distilled and deionized. Buffer solutions were degassed and filtered through 0.44-μm syringe filters (Supelco, Bellefonte, PA, USA).

2.3. Methods

Sample introduction was effected by an in-house designed vacuum injection system. Between sample introductions, the capillaries were rinsed with three capillary volumes of buffer. All experiments were carried out at ambient temperature. Reference substances were prepared according to Popják et al. [30]; the standards contained some salt residues emanating from the synthesis. Digital signal filtering was applied. The merits of such filtering in electrophoresis have been discussed recently [31].

3. Results and discussion

3.1. Direct UV detection

Direct UV detection of isoprenyl pyrophosphates depends on the number of isoprene units

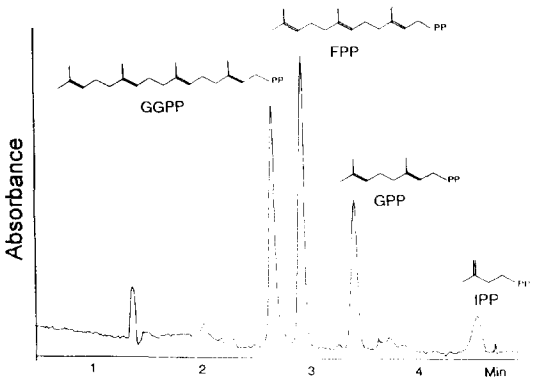

Fig. 1. Electropherogram (UV detection at 204 nm) of a standard mixture of isoprenyl pyrophosphates. Column, untreated fused-silica capillary, I.D. 50 μm, total length 60 cm, 50 cm to detector; 10 m*M* phosphate buffer (pH = 7.5); +30 kV applied for separation; current, 10 μA. Peaks: GGPP = geranylgeranyl pyrophosphate (all-*trans*), FPP = farnesyl pyrophosphate (all-*trans*), GPP = geranyl pyrophosphate (all-*trans*), IPP = isopentenyl pyrophosphate. Sample concentration, ca. 200 μ*M* of each component except 300 μ*M* for IPP.

that exist. Therefore, detectability in this mode will improve with increasing isoprenyl pyrophosphate chain length (Fig. 1). The limit of detection (LOD) for farnesyl pyrophosphate was ca. 50 μ*M*.

3.2. Indirect detection methods

Indirect absorbance and indirect fluorescence detection can yield linear ranges of several orders of magnitude when peak areas are plotted against concentration. Indirect fluorescence detection is more attractive at the lower concentration range because its lower concentration limit of detection, C_{lim}, is more sensitive to the visualization agent concentration, C_b, than that for indirect absorbance detection. To illustrate this point, assume that the detector used for indirect UV detection is flicker-noise limited. In this case, the dynamic reserve, D_r, which is the ratio of background signal to background noise, can be written as

$$D_r = \frac{A_b}{N_b} = \frac{\varepsilon b C_b}{N_b} \quad (1)$$

and the limit of detection can be defined as

$$C_{lim} = \frac{C_b}{RD_r} = \frac{N_b}{R\varepsilon b} \quad (2)$$

where A_b is the absorbance of the visualization agent, N_b is the baseline noise, ε is the molar absorptivity, b is the path length and R is displacement ratio (number of moles of visualization agent transferred by 1 mol of analyte). In this discussion, N_b is considered to be constant. This leaves C_{lim} independent of the buffer concentration. As an example of this, Fig. 2 shows that the C_b/D_r ratio remains constant while the concentration of phthalate is varied.

As for indirect fluorescence, the dynamic reserve is directly related to the intensity stability of the laser and, to a first approximation, independent of visualization reagent concentration. This results in C_b being retained in Eq. 2; therefore, C_{lim} can be decreased by lowering C_b. Fig. 3 shows the relationship of the C_b/D_r ratio to C_b for ILIF. Comparing the two detection systems, it is apparent from Fig. 3 that ILIF becomes attractive in terms of detection limits only when C_b is less than 2 m*M*. Of course, lowering C_b does not extend the linear range, but rather shifts it to the lower concentrations.

It is considered that, in order to minimize electromigration dispersion, the buffer-to-analyte concentration ratio should exceed 100 [32,33] or even 400 [34]. As shown above, a very low buffer concentration is required for the successful application of ILIF detection and

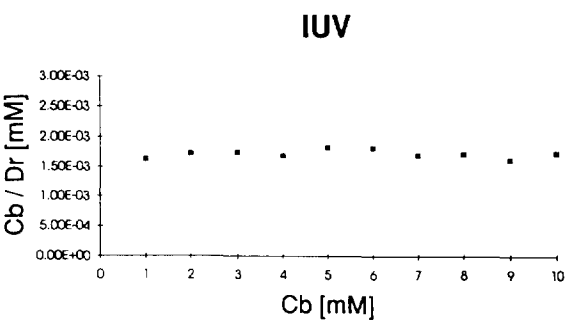

Fig. 2. Plot of C_b/D_r vs. C_b for IUV detection. Data from a untreated fused-silica capillary, total length 60 cm, 50 cm to detector, 40 μm I.D.

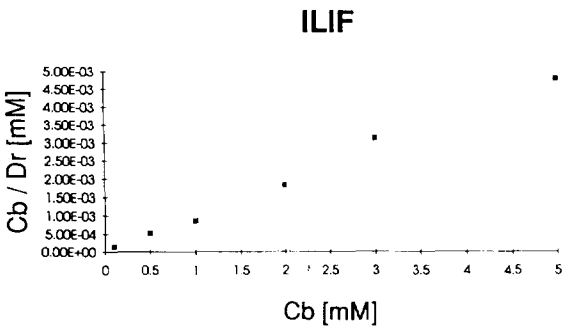

Fig. 3. Plot of C_b/D_r vs. C_b for ILIF detection. Data from a untreated fused-silica capillary, total length 60 cm, 50 cm to detector, 20 μm I.D.

when the sample contains a complex matrix, having a high ionic strength, the necessary high buffer-to-analyte ratio cannot be achieved. Such samples, which are often of biological origin therefore need to be desalted or, when detection is sufficiently sensitive, diluted prior to introduction into the separation capillary. This is an inherent limitation of the ILIF technique. In fact, it is recommended to use high-ionic-strength buffers when analysing samples derived from serum or urine [35]. It seems that the conductivity of the sample is less critical when IUV detection is employed. This is because much higher background electrolyte (BGE) concentrations can be used; however, the applicable concentration is limited by Joule heating and flicker noise from the UV detector. The traditional method for clean-up of liver samples for the determination of isoprenyl pyrophosphates includes the addition of large amounts of salt [25]. The previously used HPLC methods are largely immune to any deleterious salt effects while a background of large and variable concentrations of salt may be harmful to any electrophoretic separation [17]. A procedure for desalting samples intended for protein analysis by CE was recently presented [36]. The method is not applicable to small analytes however, as these would, to a large extent, be removed along with the salt.

3.3. Indirect UV detection

Detector cell length

The influence of detection cell length, b, was investigated. For this purpose, the performances of capillaries having different diameters were compared. As a consequence, the electrophoretic separation was also affected. However, in order to make the comparison realistic, a lower BGE concentration was applied for the widest capillary. Otherwise, convection made the results very poor on that capillary. Further, the noise has a different character in the three cases and therefore the electropherograms were filtered according to noise frequency. Table 1 shows D_r and I for the three column diameters evaluated, and the corresponding electropherograms are shown in Fig. 4. The dynamic reserve increased with increasing capillary diameter. This was obviously due to the increase in path length (Eq. 1). Dispersion due to Joule heat in electropherograms B and C is evident. Largely the same LOD, ca. 7 μM (at three times the background noise level), was obtained for the different capillary diameters (Fig. 4).

A further factor concerns the optical arrangement of the detector. The importance of optimization for different capillary diameters has been demonstrated by Bruin et al. [37]. With the detector used here, such optimization cannot be readily performed, and the same optical arrangement was therefore applied for the different capillary diameters. Detector specifications concern 75 μm I.D. capillaries, and it may be speculated that the conditions concerning the

Table 1
Influence of column diameter on current and dynamic reserve

Capillary diameter (μm)	Buffer concentration (mM)	Current (μA)	Dynamic reserve
26	5	3.4	740
40	5	8.2	1140
75	2.5	15	1340

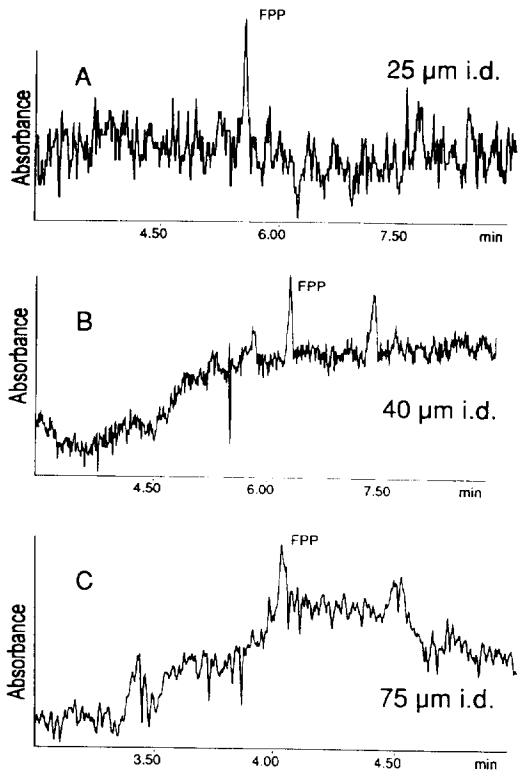

Fig. 4. Electropherograms (indirect UV detection at 228 nm) of a standard solution of farnesyl pyrophosphate. Columns, untreated fused-silica capillary, total length 60 cm, 50 cm to detector, I.D. (A) 25, (B) 40 and (C) 75 μm; background electrolyte, (A and B) 5 m*M* phthalic acid and (C) 2.5 m*M* phthalic acid (pH 6.5); +30 kV applied for separation.

signal-to-noise ratio were non-optimal for the smaller capillary diameters.

Analyte migration was most rapid in the 75 μm I.D. capillary. This can be explained by the lower C_b and the increase in capillary temperature [32,38]. The difference in migration rate between the 25 and 40 μm I.D. capillaries may be due to differences in the surface of the fused-silica tubing.

Background electrolyte

According to Eq. 2, the BGE should have a high molar absorptivity and a high displacement ratio in order to give a low LOD. It has been demonstrated that a high value of ε results in improved sensitivity [5,9]. The displacement ratio is more complex, since the Kohlrausch function has to be fulfilled throughout [11]. However, an equivalent-per-equivalent displacement ratio is achieved only when the sample ion has the same electrophoretic mobility as the background ion [39,40]. Generally, transfer ratios are decreased when the mobility of the analyte is lower than that of the BGE and increased when the analyte mobility is higher than that of the BGE [11,40]. Further, the mobility of the BGE should be similar to that of the analyte ions in order to maintain an approximately Gaussian type of distribution [5]. Gaussian peaks will give the highest sensitivity and resolution. Owing to electrophoretic band broadening, peaks eluting before the BGE show fronting whereas peaks eluting after the BGE show tailing [5,32]. This effect is illustrated in Fig. 5. Phthalate has a mobility greater than that of isopentenyl pyrophosphate (IPP), causing IPP to front. Another BGE, benzoate, has a mobility less than that of IPP, and here tailing of the IPP peak occurred. For the type of system applied here, it is therefore advantageous to select a BGE eluting after the last component of interest. This requirement is fulfilled by phthalic acid. Other types of visualization agents, e.g., trimelli-

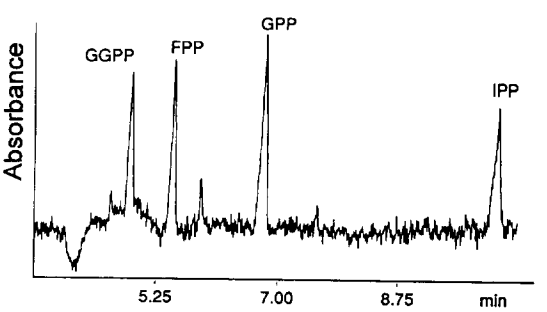

Fig. 5. Electropherogram (indirect UV detection at 228 nm) of a standard mixture of isoprenyl pyrophosphates. Columns, untreated fused-silica capillary, 26 μm I.D., total length 60 cm, 35 cm to detector; background electrolyte, 5 m*M* phthalic acid (pH 6.5); +30 kV applied for separation; sample concentration, 125 μM for each component; D_r = 740. Peaks as in Fig. 1.

tate and pyromellitate, migrate too slowly, whereas *p*-phenolsulfonate and *p*-hydroxybenzoate migrate too rapidly [39,40].

Application of benzoate as the BGE resulted in shorter migration times than when phthalate was employed. The differences in migration time can be explained by the differences in buffer ion strength. The reason for the increase in migration times for phthalate is thus the decrease in electroosmotic flow (EOF) due to compression of the double layer present at the capillary surface. The migration time is also influenced by the ionic strength of the injected sample [17]. This has to be considered when the samples contain variable concentrations of salt.

3.4. Indirect fluorescence detection

The use of a laser stabilizer was of crucial importance for this work. Without a stabilizer D_r was 80, and with a stabilizer values up to 1005 were obtained. Low electroosmotic flow resulted in a decrease in D_r. This could be due to photobleaching of the fluorophore [41]. Detection of 0.5 μM FPP in a 0.5 mM BGE by means of ILIF is illustrated in Fig. 6. The system peak corresponds to the effective mobility of the salicylate ions in the capillary, as discussed by Gross and Yeung [29].

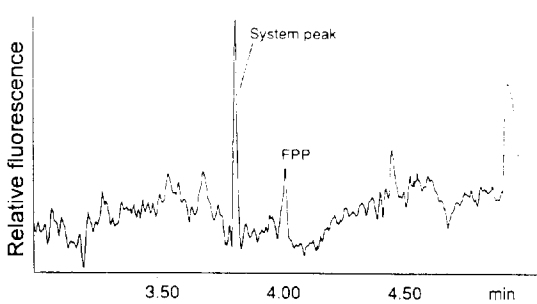

Fig. 6. Electropherogram (ILIF detection) of a standard solution of farnesyl pyrophosphate. Column, untreated fused-silica capillary, 20 μm I.D., total length 60 cm, 50 cm to detector; background electrolyte, 0.5 mM salicylic acid (pH 6.5); +30 kV applied for separation; current, 175 nA; sample concentration, 0.5 μM; D_r = 1005.

BGE concentration in ILIF

As mentioned above, C_{lim} can be decreased by a decrease in C_b. The buffer concentrations must not be too low, however. At very low buffer concentrations, capillary wall interactions can cause the separation to deteriorate. In addition, the buffering capacity is reduced, making pH difficult to control. Moreover, at very low levels of C_b, the choice of pH is restricted as the H^+ or OH^- ions begin to compete in the displacement process [1]. Further, as mentioned above, the buffer-to-analyte concentration ratio should be sufficiently high.

The influence of pH on separation and response is shown in Fig. 7. It was determined that pH 5 gave the best selectivity for the separation of *cis–trans* isomers. The number of ionized species increases with increase in pH; this explains the presence of an additional peak (U) in Fig. 7B. A plate number of 600 000 was determined for FPP in Fig. 8. Note that this is for 35-cm migration distance. Lower plate numbers, 50 000–200 000, were obtained in the indirect

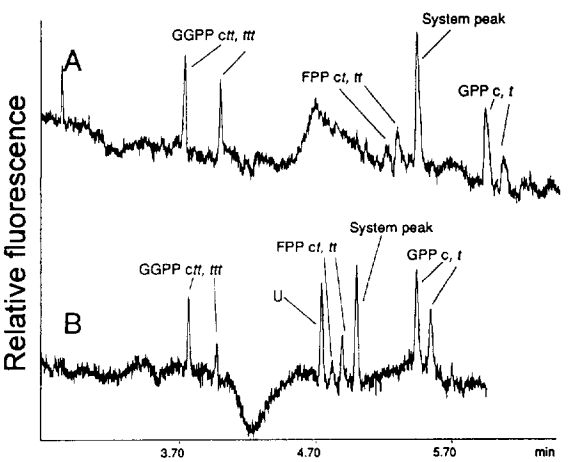

Fig. 7. Electropherograms (ILIF detection) of a standard mixture of isoprenyl pyrophosphates. Column, untreated fused-silica capillary; 20 μm I.D., total length 60 cm, 50 cm to detector; background electrolyte, 1 mM salicylic acid [pH adjusted to (a) 4.0, and (B) 5.0]; +30 kV applied for separation; D_r = (A) 846 and (B) 924; current (A) 208 nA and (B) 170 nA. Peaks: *cis,trans,trans*-GGPP; *trans,trans,trans*-GGPP; *cis,trans*-FPP; *trans,trans*-FPP; *cis*-GPP; *trans*-GPP; U = unknown. Abbreviations as in Fig. 1.

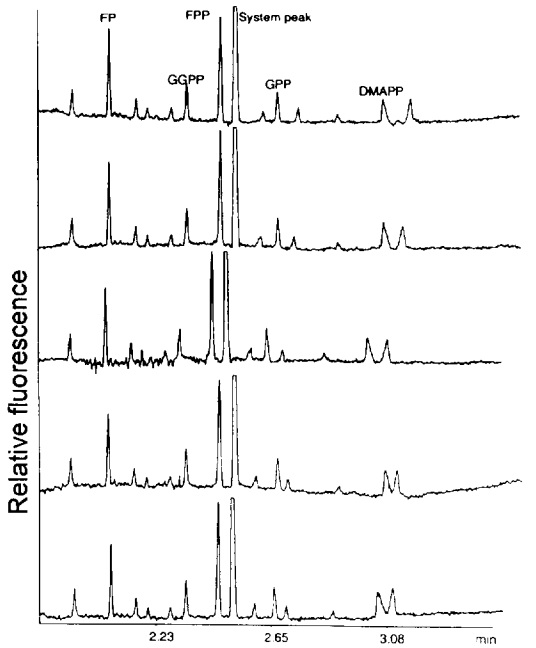

Fig. 8. Electropherograms (ILIF detection) of a standard mixture of isoprenyl pyrophosphates. Column, untreated fused-silica capillary, 20 μm I.D., total length 60 cm, 35 cm to detector; background electrolyte, 1 m*M* salicylic acid (pH 6.1); +30 kV applied for separation; sample concentration, 10 μ*M* of each; $D_r = 970$. Peaks as in Fig. 1; FP = farnesyl phosphate (all-*trans*); DMAPP = dimethylallyl pyrophosphate.

UV mode, and direct UV detection gave 10 000–50 000 plates. The observed difference in plate numbers is partly due to the greater width of capillary illumination when the UV detector is employed. This section is ca. 0.5 mm in the UV mode and about 10 μm in the fluorescence mode. Further, the low plate count obtained for the system used for direct UV detection can be explained as being a consequence of the relatively large difference in electromigration velocity of the phosphate buffer and the analyte in this case [42]. In Figs. 7 and 8, the system peak appears after FPP whereas it appears before FPP in Fig. 6, because of the difference in the applied pH.

In systems having two, almost equally large, velocity vectors acting in opposite directions, small shifts in electrophoretic mobility or electroosmotic flow can lead to large shifts in migration time. As indicated above, facts such as the ionic strength of the injected samples and the nature of the fused-silica capillary will affect migration times. Clearly, the system must be carefully optimized to allow the analyte having the highest electrophoretic mobility to arrive at the detector in a reasonable and reproducible time. However, when all factors are kept constant, highly reproducible migration times can be achieved. The relative standard deviations (R.S.D.) for FPP in five runs were time 0.33%, peak height 7.1% and peak area 7.0%. These are in the same range as the values obtained for the other isoprenyl pyrophosphates (Fig. 8). The relatively poor precision originates from the in-house constructed pressure system used for sample introduction. To achieve time-to-time reproducible systems, capillaries should be 50 cm or shorter (maintaining the applied voltage), the pH should be kept as high as possible and the BGE concentration should be kept low. All these factors ensure a high EOF. A low EOF, in this case, results in irreproducible performance and poor dynamic reserve.

4. Conclusions

ILIF was the most suitable detection method considering the LOD and separation efficiency. The LOD was, however, not low enough to permit dilution of samples emanating from rat liver, and for such samples desalting would have to be applied before electrophoresis. With the equipment used in this work, the LOD with IUV or UV detection was not low enough for our application.

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