Automated detection of fecal contamination of apples by multispectral laser-induced fluorescence imaging

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Animal feces are a suspected source of contamination of apples by disease-causing organisms such as *Escherichia coli* O157. Laser-induced fluorescence was used to detect different amounts of feces from dairy cows, deer, and a dairy pasture applied to Red Delicious apples. One day after application, detection for 1:2 and 1:20 dilutions was nearly 100%, and for 1:200 dilutions (<15 ng of dry matter) detection was >80%. Detection after apples had been washed and brushed was lowest for pasture feces; detection for 1:2, 1:20, and 1:200 dilutions of feces was 100%, 30%, and 0%, respectively. This technology may encourage development of commercial systems for detecting fecal contamination of apples.

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1. Introduction

Foodborne illnesses resulting from the introduction of bacterial pathogens into the food chain are an important issue for human health.[1–4] In recent years, a number of cases of serious illness, including some that have led to death, have been attributed to the presence of hemolytic *Escherichia coli* bacteria, particularly the O157:H7 strain, in unpasteurized apple juice.[5–9] Animal feces are thought to be the primary source of contamination of foods, including apple juice, with coliform bacteria.[10–13] The federal government has issued directives in attempts to reduce fecal contamination of animal carcasses[14] and fruit juices.[15] In previous studies, the present authors and others demonstrated that fecal contamination of apples can be detected by use of either fluorescence[16] or reflectance[17] methods but that sensitivity is greater with fluorescence. To further investigate the ability to detect fecal contamination of produce, the authors employed a system that uses a pulsed laser and a gated-intensified camera that was developed to measure fluorescence emissions from large objects under ambient lighting conditions.[18]

In this study we tested the ability and sensitivity of laser-induced fluorescence to detect fecal contamination of apples by applying serial dilutions of feces, from dairy cows, deer, and a pasture formerly used to house dairy cows, to Red Delicious apples. Fluorescence responses were determined by a multispectral laser-induced fluorescence imaging system[18] 1 day and 7 days after application and subsequently after washing, or washing and brushing, of the apples. The specific objectives were to determine the effectiveness of the detection system, whether elapsed time after application of feces affects detection, and how washing affects detection. In addition, to test the ability to discriminate fluorescence responses caused by contamination invariant to background fluorescence, we selected control apples to incorporate the extreme range of coloration across the surfaces of individual apples.

2. Materials and Methods

Serial dilutions of feces were applied to Red Delicious apples. Two series of trials were conducted. The first series used feces from dairy cows. The second series used feces from deer and from a pasture formerly used by dairy cows.

A. Feces

Fresh feces from individual cows were collected at the Beltsville (Maryland) Agricultural Research Center

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Table 1. Comparison of Dry-Matter Content of Feces by Source

Source	Dry Matter (%)
BA	15.2
JUM	14.3
Deer	51.2
Pasture[a]	16.4

[a]Pasture feces were collected after a period of rainfall.

dairy (BA) and at the University of Maryland Clarksville dairy (UM). Feces were collected in 50 mL polypropylene vials and stored at 4 °C for as long as 2 days before use. Deer feces were collected from fresh droppings at the BA. Pasture samples were obtained from a fallow pasture that had not been used to house animals for more than 6 months. To determine the dry-matter content of the feces we dried three samples of feces from each source at 65 °C until a constant weight was obtained (Table 1). The dry-matter content of the pasture feces was similar to that of the wet feces because the sample was obtained after a period of rainfall. With one exception, fecal samples were serially diluted 1:2, 1:20, 1:200, and 1:2000 by weight with doubly distilled water. Because of the dryness of the deer feces, the first dilution for deer feces was 1:10 instead of 1:2. Two separate 20-μL drops of each of the four dilutions were applied to individual apples in an X pattern (Fig. 1). To mark the locations of application sites, a black dot was drawn at the center of the X pattern. To accommodate particulates in the dilutions, ~2 mm of the end of the pipette tip was cut off with a razor blade. Undiluted samples of feces were not applied to apples because applying them was difficult and because they did not adhere well to the apples.

B. Apples

The Red Delicious apples used in the study were hand picked at the Rice Fruits Company (Gardners, Pa.) orchards. The apples were stored under commercial conditions in an apple refrigerator maintained at 3 °C. Apples for treatment were selected randomly from crates of apples based on two criteria: that the apples were not damaged and that the flesh of an apple was firm with no sign of rot. BA and UM feces were applied to sets of 20 apples. Subsequently, deer and pasture feces were applied to sets of five apples. In addition, to examine confounding factors caused by extremes in variation of color and shading we selected 20 control apples for maximum potential variation in autofluorescence. A total of 80 apples was used in the study.

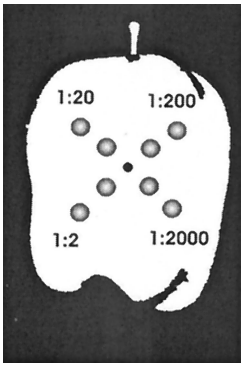

Fig. 1. Locations where duplicates of serial dilutions of feces were applied to an apple relative to a single black dot drawn near the center of the apple's surface. For deer feces, a 1:10 dilution was substituted for the 1:2 dilution.

C. Treatments

The cheek of each apple with the flattest surface was selected for application of fecal dilutions. After application of the feces, apples were placed on cardboard trays and returned to the apple refrigerator for 24 h. Subsequently, fluorescence emissions in response to laser excitation (see below) were measured. The apples were returned to the apple refrigerator and stored for 7 days; then emissions were measured again. The BA and UM treated apples were then divided into two groups. One group of 10 apples was only tumble washed, whereas the second group of 10 apples was tumble washed, brushed, and washed a second time.

To tumble wash the apples we placed 10 apples in a rectangular bucket containing clean water at 10 °C. The apples were manually tumbled for 60 s before being moved to a second bucket kept under running water. The running water caused the apples to tumble naturally. Again after 60 s, the apples were removed, held for a moment to allow most of the water to drip off, and then placed on clean cardboard trays. For washed and brushed apples, brushing was applied after the apples were removed from the first bucket and before they were placed in the second bucket. The apples were brushed with a shoe-polish applicator brush to remove any visual sign of feces and for at least three strokes over each area of feces application. It took ~30 s for two persons to brush 10 apples. Each wash cycle included apples from BA and UM, or deer and pasture, treatment groups. Washed apples were returned to cold storage for 24 h before emissions were measured again. Because results were similar and consistent for BA and UM treated apples, particularly in terms of the effects of washing, the number of apples per treatment used for the deer and pasture trial was reduced from 20 to 5, and all the apples were washed and brushed. The control apples were not washed.

D. Measurements

Fluorescence emissions from whole apples were acquired by use of a multispectral laser-induced fluorescence imaging system designed to capture multispectral images simultaneously from a large target area without regard to ambient light. The imaging system consisted of a pulse laser, a beam expander, a lens, a common-aperture adapter, and a fast-gated intensified camera. A complete description of the system can be found in a companion paper.[18] The common-aperture adapter converts a

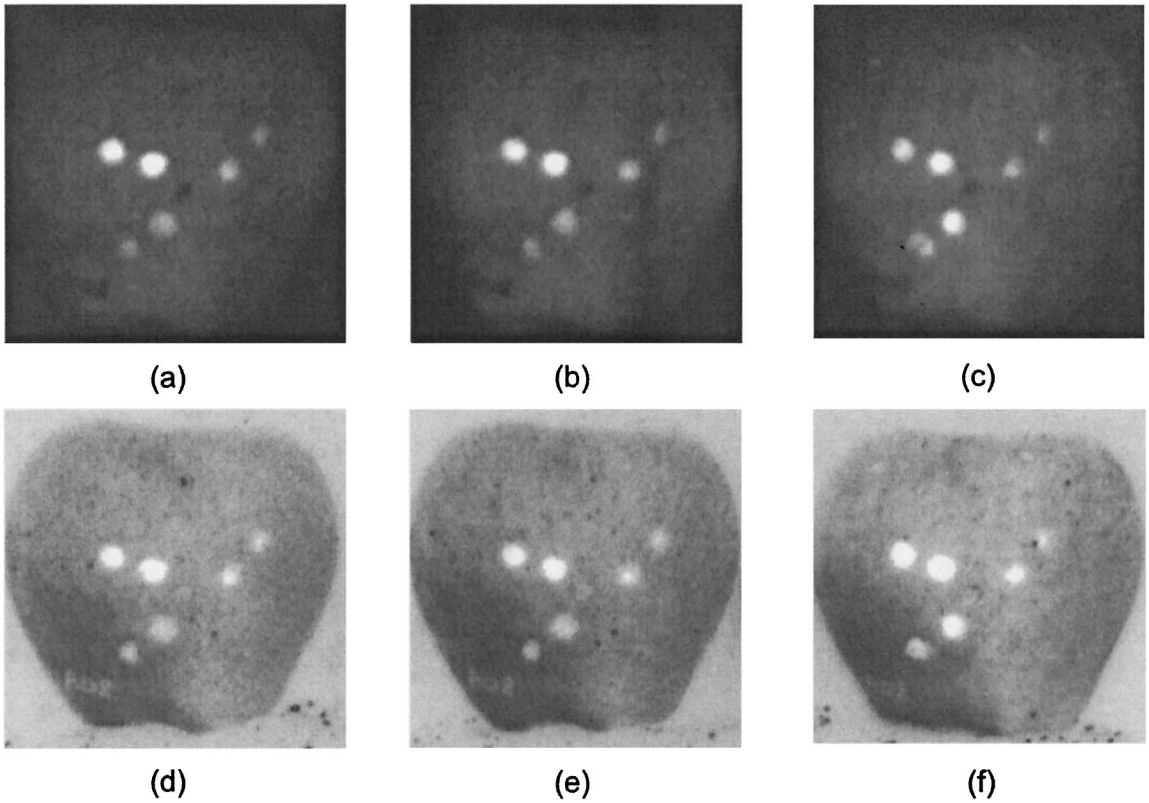

Fig. 2. (a)–(c) Red-band and (d)–(f) red–blue ratio images of an example apple 1 day (a), (d) and 7 days (b), (e) after dilutions of BA dairy feces have been applied and after washing and brushing of the apple (c), (f).

target area into four equal-sized (640 × 512) images and includes a four-filter holder. Filter parameters used for this study were 30 nm FWHM at 450 nm (blue band), 40 nm FWHM at 550 nm (green band), 22 nm FWHM at 678 nm (red band), and 10 nm FWHM at 730 nm (far-red band).

E. Analysis

A number of image-processing techniques were used in attempts to detect and quantify the fecal contamination. The method selected for detailed analyses was based on the observed steep and relatively wide gradient in fluorescence surrounding contamination sites. From earlier studies[17,18] it was expected that the maximum fluorescence emission would occur in the red-band images because of the chlorophyll *a* and chlorophyll-related compounds in the feces. Thus the red-band images were analyzed first. Each raw 12-bit image was scaled to an 8-bit gray-scale image. The 8-bit image was then subjected to a 3 × 3 Sobel filter.[19] We used the histogram of the filtered image to determine the intensity level that included the brightest 1.5% portion of that image. This 1.5% level was next used as the basis of a binary threshold filter. The binary image was then subjected to two repetitions of shrinking (removal of 1 pixel from the edges) and smoothing. The Sobel filter produced a ridge around contamination sites as well as some localized, scattered response areas. The selection of the threshold and the number of smoothing iterations were based on the criteria of maximizing the response to contamination sites while removing nonspecific responses, i.e., noise. To permit illustration of the detected regions, the binary image was overlaid upon the original image in black. A computer algorithm was developed to allow the number of black pixels surrounding each point of contamination to be identified and counted.

Earlier studies indicated that ratios of different band images helped to normalize the effects of heterogeneous illumination and that detection of fecal contamination was enhanced when a red-band image was divided by the corresponding blue-band image.[16,17] Ratios of red to blue images were calculated from raw 12-bit image data. The resultant ratios were then scaled to an 8-bit gray-scale image with the ratio value of 6.0 corresponding to an intensity of 255. With a few bright fluorescence responses, peak ratios sometimes approached 8; however, scaling to ratios higher than 6 reduced the contrast of most images, resulting in reduced sensitivity of detection. Ratios greater than 6 were assigned the maximum value of 6. The ratio images were treated in the same manner as the red-band images, with the exceptions that the histogram level used for the threshold was 20% instead of 1.5% and that the binary images were subjected to only a single repetition of edge shrinking and smoothing.

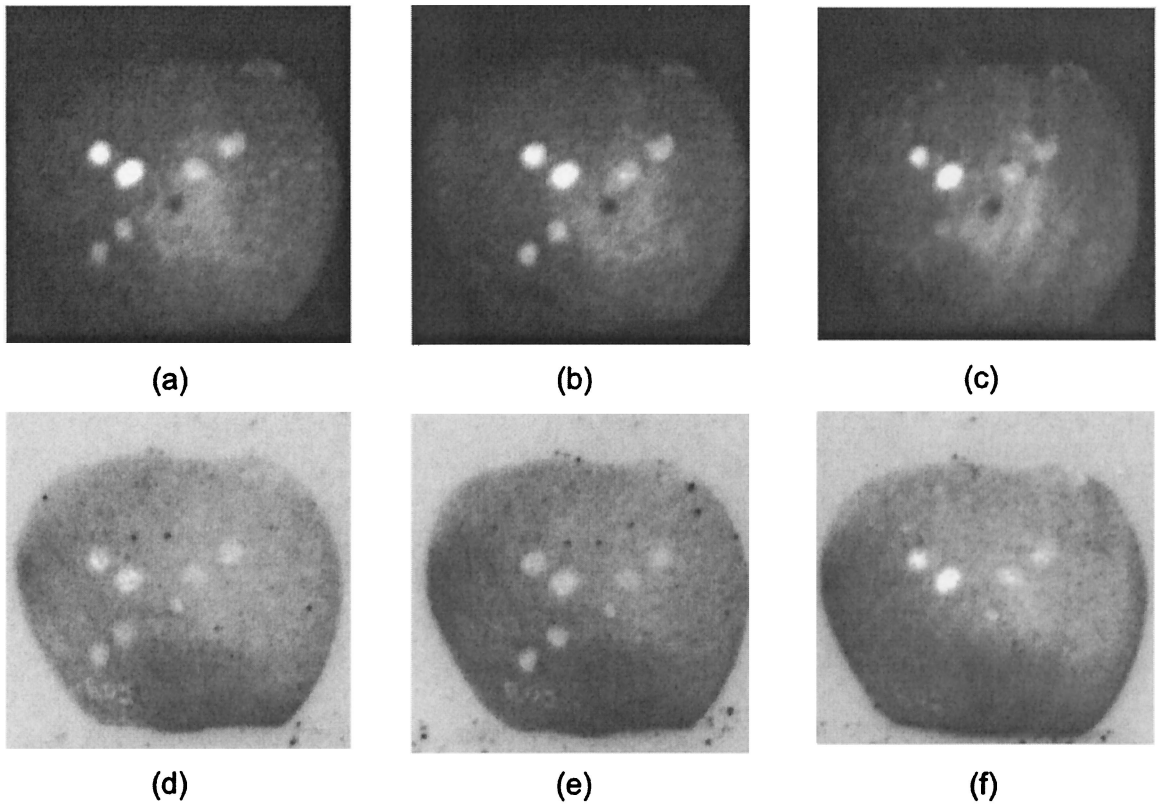

Fig. 3. (a)–(c) Red-band and (d)–(f) red–blue ratio images of an example apple 1 day (a), (d) and 7 days (b), (e) after dilutions of UM dairy feces have been applied and after washing and brushing of the apple (c), (f).

3. Results and Discussion

Representative examples of red-band and red–blue-ratio images are shown in Figs. 2–6. The bright circles represent areas of fecal contamination. The dark specks in some ratio images are due to fluorescent responses of dust particles in blue-band images. Intensities of circles within fecal type and dilution were similar 1 and 7 days after application of feces. Following washing, or washing and brushing, the intensities of the circles were generally altered. Whether intensities were increased or decreased depended on the source of the feces, the dilution, and interactions with individual apples. Six additional phenomena are of particular interest. First, the feces from UM, particularly the 1:2 dilution, tended to flake off the apples. This flaking was particularly apparent 1 day after fecal application. Second, the intensities of circles for 1:2 dilutions tended to be much less than the intensities of circles associated with 1:20 dilutions. Third, except for deer feces and for one instance of UM feces, the 1:2000 dilutions were not detectable. Fourth, for apples treated with pasture feces, washing and brushing tended to distribute evidence of fecal contamination across wide areas of the apple surfaces (Fig. 5). Fifth, for apples treated with deer feces, washing and brushing tended to increase the relative intensities of 1:2 and 1:20 dilution circles. Sixth, ratio images provided greater sensitivity for detecting 1:2 dilutions, whereas red-band images provided greater sensitivity for detecting 1:200 dilutions.

Table 2 lists the number of black pixels associated with detection of fecal contamination in red-band images by fecal type, dilution, and treatment. For BA, UM, and deer feces, the numbers of black pixels were greatest for the 1:20 dilutions. When black pixel data were used with a cutoff threshold of 10 pixels, 69% of 1:2, 92% of 1:20, and 73% of 1:200 treated spots could be detected. The 10-pixel cutoff was based on a desire to reduce the number of identified false positives detected at the edges of some control apples (see below). When detection information from both red-band and ratio images was combined by use of a Boolean OR function, detection was improved to 85%, 96%, and 79%, respectively. The basis for improved detection when information from red-band and ratio images were combined is clearly demonstrated in Fig. 6. In the red-band image, the 1:2 dilution is barely visible, whereas the 1:20 and 1:200 dilutions are clearly visible. In contrast, the 1:200 dilution is barely visible in the ratio image, but the 1:2 and 1:20 dilutions are clearly visible. The reduced fluorescence responses of the 1:2 dilutions in the red-band images may be due to quenching by self-adsorption.[18] Visible air gaps that existed between the feces and the apple surface for 1:2 dilutions may have enhanced the quenching effects. In support of the importance of reduced adhesion is the

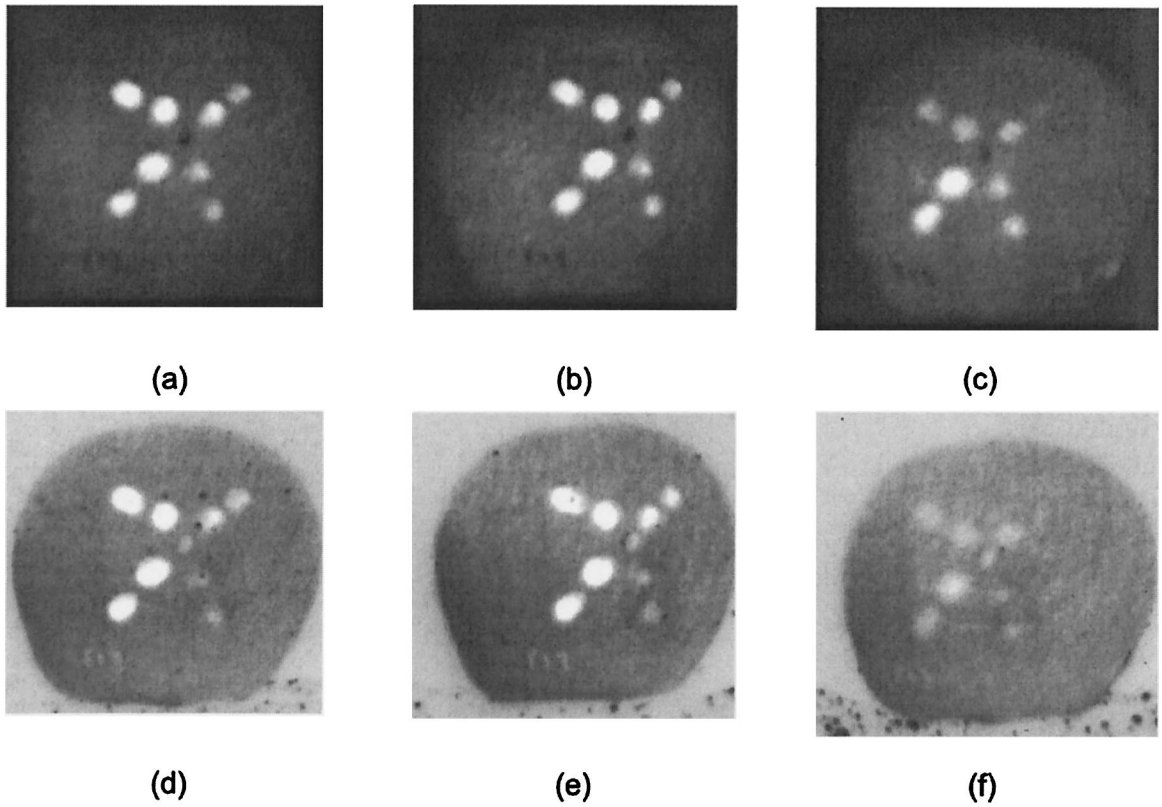

Fig. 4. (a)–(c) Red-band and (d)–(f) red–blue ratio images of an example apple 1 day (a), (d) and 7 days (b), (e) after dilutions of deer feces have been applied and after washing and brushing of the apple (c), (f).

finding that, after washing, responses for 1:2 dilution contamination sites often were still less than responses for 1:20 dilution sites.

Table 3 lists the detection percentages obtained with combined detection results from red-band and ratio images by treatment, dilution, and source. Again, only contamination of deer feces could be reliably detected at 1:2000 dilution. Deer feces were the easiest source of contamination to detect. All deer contamination spots were detected, except after washing and brushing for one spot treated with the 1:200 dilution. One reason for the enhanced detection of deer feces is probably related to relative dry-matter contents; deer feces were more than three times as concentrated as the other sources of feces (Table 1). Thus the 1:2000 dilution of deer feces is essentially equivalent to a 1:600 dilution of the other sources of feces. Also, visual observation of the deer feces showed a higher content of green materials than in the other feces. This enhanced green content occurred even though the deer feces were collected in February when limited green vegetation was available for foraging. During seasons when green plants are more readily available, it should be even easier to detect contamination from deer feces.

Detection percentages for 1:2 and 1:20 dilutions of feces were similar for BA, UM, and pasture sources at 1 and 7 days. Detection percentages for 1:200 dilutions were slightly lower. The detection rate for UM feces at the 1:2 dilution was also reduced because of a tendency of the applied feces to flake off the apples. The influence of washing and brushing on detection percentages depended on the source of the feces. Generally, washing spread the pasture feces across large areas of the apple surfaces, thus hindering detection. The 1:2 dilution of pasture feces was 100% detectable after washing and brushing; however, the number of black pixels in red-band images was severely reduced (Table 2). At the 1:20 dilution only 30%, and at the 1:200 dilution 0%, of treated spots were detectable. Feces from BA was detectable in most cases with some decrease in detectability with increasing dilution and washing. Feces from UM were slightly less detectable than feces from BA, primarily because of problems associated with flaking. The flaking problem was also apparent during washing, when contaminated spots would often lift off an apple as soon as the apple touched the water.

A. Control Apples

Fluorescence responses of the untreated control apples were the primary reason why a gradient method was used for detection as opposed to a simple threshold. The control apples were selected to provide the greatest potential for detection of false positives; i.e., the apples were selected based on previous experience of the color characteristics of apples that would be expected to yield the most variation in fluorescence responses across individual apple surfaces. Analyses of images of these control apples forestalled use of

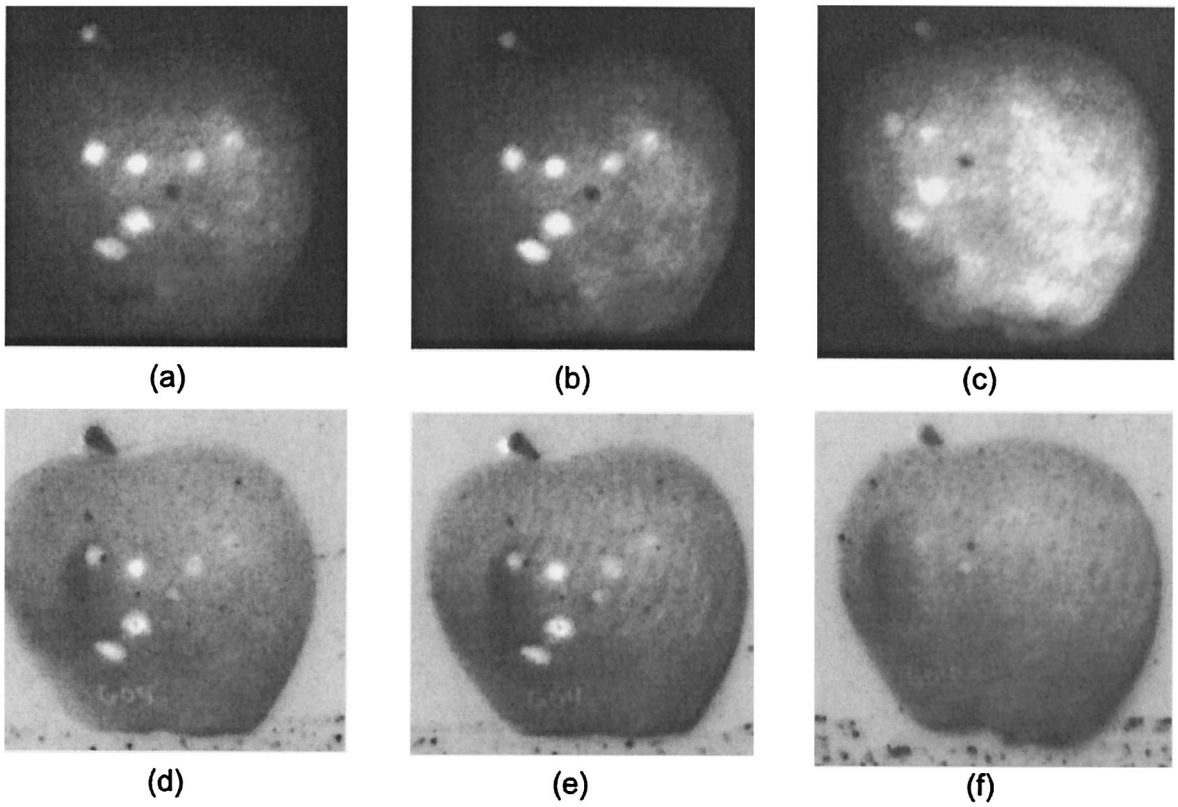

Fig. 5. (a)–(c) Red-band and (d)–(f) red–blue ratio images of an example apple 1 day (a), (d) and 7 days (b), (e) after dilutions of pasture feces have been applied and after washing and brushing of the apple (c), (f). Note how washing resulted in smearing of the contamination across the surface in (c).

simple thresholds for detection of fecal contamination. For example, any single threshold that permitted detection of contamination sites in red-band images of contaminated apples also detected large surface areas of many of the control apples. Most of these false positives could be eliminated by use of red–blue ratio images in conjunction with red-band images. However, elimination of these false positives required using the Boolean AND function. As was indicated above, maximum sensitivity was obtained when data from both red and red–blue ratio images were combined by use of a Boolean OR function. Fortunately, the fluorescence responses of the control apples exhibited either sharp borders or gradual changes across surfaces; hence, most potential false positives could be excluded by use of gradient detection methods with smoothing.

The second potential problem addressed by control apples involved variegated surfaces with sharp edges in coloration. This variation, combined with illumination effects caused by curvature of the apples near apple edges, resulted in detection of numerous false positives. In 8 of the 20 control apples, ridges in coloration near the edges of the apples were detected as contamination when the images were analyzed. The implications of this problem are discussed in Subsection 3.B below.

The control apples also showed evidence of natural contamination. In four control apples, intense fluorescence responses were seen in stem wells. The fluorescence was independent of color variation in the wells and was not likely due solely to illumination effects at the edge of the apples. Because of the location and shape of the stem area, the relative occurrence of natural contamination in this area would be expected to be high. Five of the twenty control apples also showed evidence of contamination on smooth surfaces. Two apples showed small circular areas, which yielded about 150 black pixels when their red-band images were subjected to the detection algorithm; the other three apples yielded fewer than fifty black pixels.

Problems with detection near the edges of apples and examples of potential natural contamination were also evident in a small number of the feces-treated apples. However, as cross contamination during application of the feces could not be eliminated as the source of these responses, spots that were not directly due to treatment were excluded from analyses of treated apples.

B. Practical Considerations

The results of this study clearly demonstrate the sensitivity of laser-induced fluorescence imaging for detection of fecal contamination of apples. Detection of contamination spots for 1:200 dilutions of feces generally exceeded 80%. At this dilution, with a dry-matter content of 15%, the 20-μL application vol-

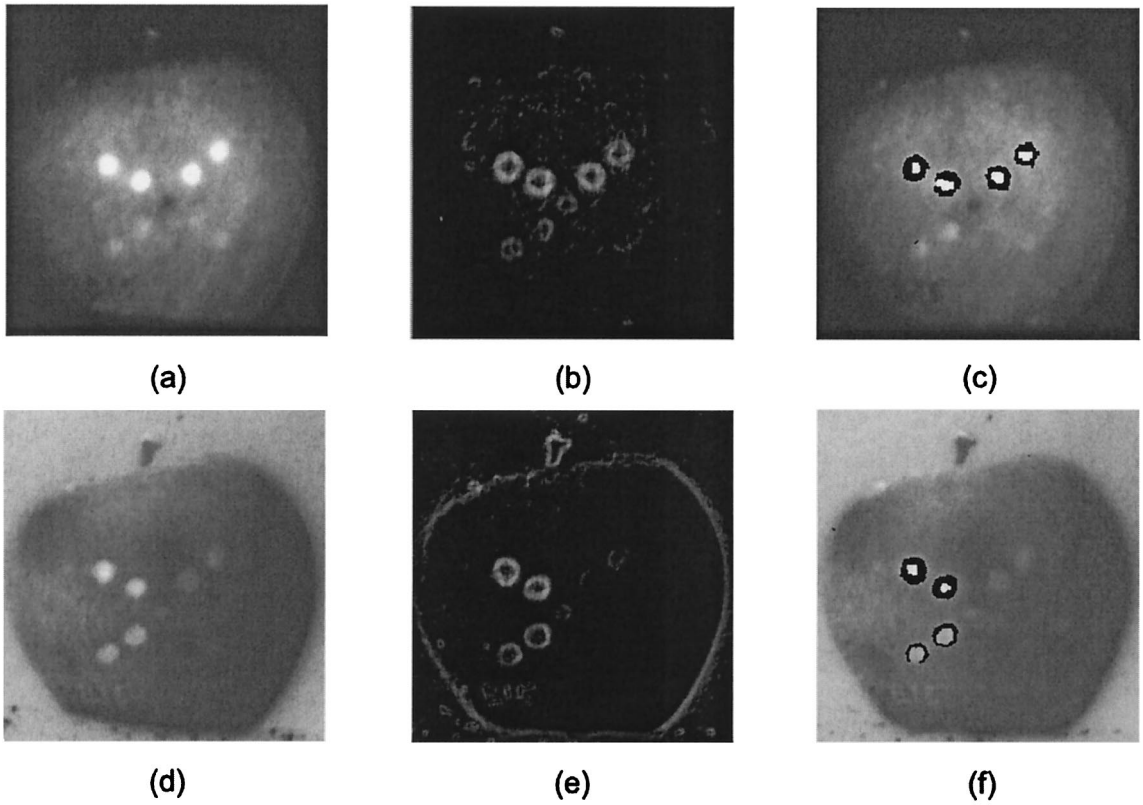

Fig. 6. Comparison of detection of fecal contamination by use of (a)–(c) red-band and (d)–(f) red–blue ratio images. Original images (a) and (d) were subjected to edge detection. Edge images (b) and (d) were filtered primarily by threshold filter to produce the black pixels shown in (c) and (f) overlaid upon the original images. Detection of the edge of the apple in ratio image (e) was due to the relatively high ratio value of the background that resulted from differences in the background values in the red and blue images used to produce the ratio image.

Table 2. Number of Black Pixels in Red-Band Images detected with Machine Vision, According to Source of Feces, Dilution, and Time after Application and Treatment[a]

Source	Time After Application (days)/ [Treatment]	Number of Detected Pixels for Dilutions of Applied Feces of			
		1:2[b]	1:20	1:200	1:2000
BA	1	83.4 ± 13.0	338.2 ± 23.6	142.8 ± 19.4	
	7	61.5 ± 9.9	312.7 ± 23.0	138.1 ± 22.1	
	[Washed]	161.9 ± 35.9	321.4 ± 38.7	125.6 ± 29.0	
	[Washed and brushed]	162.6 ± 41.4	182.3 ± 28.9	51.6 ± 14.6	
UM	1	72.9 ± 11.0	313.4 ± 16.2	109.8 ± 15.2	
	7	52.0 ± 12.2	246.7 ± 17.1	104.6 ± 19.0	
	[Washed]	33.1 ± 14.5	213.1 ± 35.7	98.2 ± 28.8	
	[Washed and brushed]	26.1 ± 14.3	147.8 ± 39.0	78.9 ± 22.7	
Deer	1	250.8 ± 54.5	312.4 ± 61.8	382.4 ± 43.1	305.0 ± 77.5
	7	242.1 ± 50.5	250.2 ± 43.4	361.2 ± 30.6	314.9 ± 74.6
	[Washed and brushed]	411.0 ± 78.5	303.7 ± 30.3	279.2 ± 41.8	256.1 ± 70.7
Pasture	1	272.1 ± 51.4	243.7 ± 34.4	86.9 ± 33.2	
	7	248.5 ± 55.0	184.1 ± 39.7	69.1 ± 27.8	
	[Washed and brushed]	13.8 ± 5.0	23.9 ± 28.8		

[a]Treatment includes washing or washing and brushing.
[b]Dilution for deer feces, 1:10.

Table 3. Percentage of Feces-Application Spots Detected by Use of Combined Information from Red-Band and Red–Blue Ratio Images According to Source of Feces, Dilution, and Time after Application, and Treatment[a]

Source	Time After Application (days)/ [Treatment]	n[b]	Feces-Application Spots (%) Detected for Dilutions of Applied Feces of			
			1:2[c]	1:20	1:200	1:2000
BA	1	40	97.5	95.0	85.0	0.0
	7	40	97.5	100	80.0	2.5
	[Washed]	20	80.0	80.0	65.0	0.0
	[Washed and brushed]	20	100	100	80.0	0.0
UM	1	40	95.0	100	85.0	0.0
	7	40	85.0	100	77.5	0.0
	[Washed]	20	35.0	65.0	45.0	0.0
	[Washed and brushed]	20	30.0	100	70.0	0.0
Deer	1	10	100	100	100	100
	7	10	100	100	100	100
	[Washed and brushed]	10	100	100	90.0	100
Pasture	1	10	100	100	80.0	0.0
	7	10	100	100	70.0	0.0
	[Washed and brushed]	10	100	30.0	0.0	0.0

[a]Treatment includes washing or washing and brushing. washing and brushing.
[b]n is the number of apples tested.
[c]Dilution for deer feces, 1:10.

ume contained 15 ng of solid material. Detection for deer feces diluted 1:2000 was essentially 100%; the 20-μL application volume contained 5 ng of solid material. Further evidence of sensitivity was the ability to detect contamination on apples after they had been washed and brushed even though careful visual scrutiny of the apples failed to detect any evidence of the contamination. These results suggest that laser-induced fluorescence imaging is a practical technology for use in commercial systems to detect contaminated apples destined for juice production or the fresh produce market.

Issues that need to be addressed to develop a practical system for detecting fecal contamination include (1) improved performance with use of different excitation wavelengths, (2) trade-offs among selectivity, sensitivity, cost, and computational efficiency, (3) false positives near the edges of apples caused by illumination, and (4) the relevance of the source of the feces. The present authors recently demonstrated that excitation of dairy feces at the adsorption maximum at 418 nm resulted in the maximal fluorescence response and produced the greatest contrast between responses of feces and tested substrates, which included meat but not apples.[20] These results suggest that it would be worth investigating the effect of excitation wavelength on the ability to differentiate fluorescence responses of feces from those of apple surfaces.

Another consideration, in terms of both cost and functionality, is related to the ultimate goals of a practical detection system. The analytical methods used in this study were biased by a desire to detect and quantify numerous, known sites of contamination. In practice, it may be necessary to identify only a single site of contamination and, depending on detection goals, there may be no reason to attempt to quantify the contamination. Speed and sensitivity could also be enhanced if false positives were tolerated. In the current study, detection parameters were set such that, when edges were excluded from analyses, there were no false positives.

The problem of detection of false positives at the edges of apples could be addressed by improvement of illumination to make it more nearly uniform in terms of both intensity and incident angle. Alternatively, the effects of apple curvature and nonuniform illumination could be dealt with mathematically.[21] Finally, the problem could be addressed in the design of the measurement system by addition of sufficient additional imaging perspectives such that the entire surface of the apple could be represented without the need to consider apple edges in individual perspectives.

The final issue involves the relative detectability of feces from different sources. The present authors have demonstrated that green plant content in the diet is related to fluorescent responses of resulting feces.[20] Detectability of deer feces in this study was probably the result of higher roughage content than in the tested cow feces. Another aspect of detectability concerns adhesion of feces to apple surfaces. During the development of the experimental protocols for this study, we found it difficult to make undiluted feces adhere to apples. With the adopted protocols, adhesion problems were still evident in results for the 1:2 dilution of UM feces. Concerns for the influence of adhesion on detectability are somewhat mitigated by the finding that contamination could still often be detected after the applied feces

were removed by washing and brushing. However, washing and brushing had different effects on detectability, depending on the source of the feces. Pasture feces, likely candidates to be sources of natural contamination, were difficult to detect after washing and brushing. Additional research is needed to elucidate the role of adherence in naturally occurring contaminations.

4. Conclusion

Laser-induced fluorescence imaging was found to be a highly sensitive method for detecting fecal contamination of apples. Diluted feces obtained from dairy cows, deer, and a dairy pasture could by detected at very low levels (<15 ng). In many cases, the artificial contamination could still be detected after the apples were cleaned by washing and brushing. Results suggest that this technology may encourage the development of commercial systems to detect fecal contamination of apples.

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