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Analysis of Maleic Hydrazide in Potatoes by Competitive Inhibition Enzyme-Linked Immunosorbent Assay

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ABSTRACT

A competitive inhibition enzyme-linked immunosorbent assay (ELISA) based on two monoclonal antibodies specific for the synthetic plant growth regulator maleic hydrazide (MH) has been evaluated for application to the measurement of MH in two potato varieties. This ELISA method requires minimal sample preparation and much less time and effort than the current analytical procedure. The limit of detection of the homologous ELISA systems used was approximately 1 ppm MH. The effect of the matrix on the assay depended upon the potato variety, the method of sample preparation, and the antibody used for analysis. Variability of recovery of a 10 ppm spike was lowest within blanks and highest among samples, indicating excellent reproducibility of the method, but also suggesting significant sample variability. The data presented demonstrate the ability of the ELISA to measure MH in potatoes at levels typically found in domestic potatoes.

INTRODUCTION

Maleic hydrazide (MH), a synthetic plant growth regulator, is the sprouting inhibitor of choice for storage potatoes in the United States. Despite more than three decades of study, uncertainty about its chronic toxicity to non-target organisms remains due to conflicting research results (Swietlinska & Zuk, 1978; USDA, 1979; Ponnampalam *et al.*, 1983; Johnson, 1983). Typical residue levels have been estimated at 10–40 ppm for fresh potatoes and 80 ppm for potato chips, while respective tolerances are set at 50 and

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160 ppm (USDA, 1979). Dietary intake has been estimated at 20 μg/kg per day for the average American adult (USDA, 1979), primarily via treated potatoes.

Gas and liquid chromatographic methods for MH have been developed (Haeberer & Chortyk, 1974; Newsome, 1980; King, 1983), but a modified colorimetric method (Wood, 1953; Ihnat *et al.*, 1973) remains the official and most widely used method (AOAC, 1980). Analysis for MH is not routinely performed on food crops despite the existence of legal tolerances, largely because of the laboriousness and expense of these methods, thus restricting knowledge of human exposure to MH. Numerous immunoassay methods have previously been applied to the analysis of low molecular weight contaminants in foods (Newsome, 1986; Pestka, 1988; Fukal & Kas, 1989). A monoclonal antibody-based ELISA method of MH residue analysis has been described (Harrison, 1987; Harrison *et al.*, 1989*b*) which allows faster and easier analysis. Application of this method for broader monitoring of crops for MH should enhance our understanding of the hazard of the continued use of MH. We present here the application of this assay to the analysis of MH in field treated potatoes, demonstrating its utility and effectiveness.

MATERIALS AND METHODS

Colorimetric analysis

All colorimetric analyses were performed using the official method of analysis (AOAC, 1980) by Biospherics, Inc. (Rockville, MD) on field-treated potato samples provided by Uniroyal (Naugatuck, CT). This method requires hydrolytic reduction of the MH ring in concentrated NaOH, steam distillation to remove the liberated hydrazine, and colorimetric analysis of the recovered hydrazine. Coarsely ground potato samples, approximately 5 lb each, were stored frozen after colorimetric analysis. Subsamples of 300–500 g were analyzed by immunoassay 1 year later.

Sample preparation for ELISA analysis

The preparation of fresh potatoes for ELISA analysis is summarized in Fig. 1. Potatoes were obtained already coarsely ground as noted above or fresh potatoes grown without MH were ground to the same consistency using the coarse plate of a Hamilton Beach food grinder. Freshly ground or previously ground and frozen 10 g potato subsamples were homogenized with an equal weight of water for 60 s at 10 000 rpm in a Sorvall Omni-mixer.

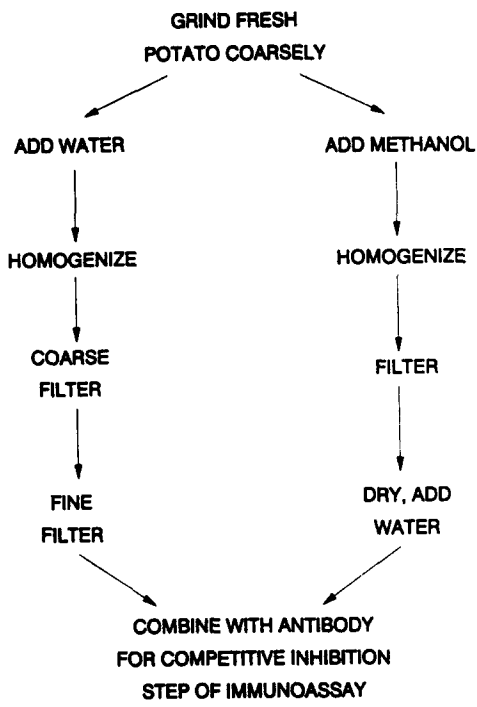

Fig. 1. Sample preparation for the ELISA analysis of MH in potatoes.

Large particles were removed by filtration through three layered polypropylene or aluminum screens (top to bottom) of 1·2, 0·4 and 0·1 mm mesh sizes. The first filtrates were clarified by passing through a double or triple layer of Whatman No. 1 filter paper; as particles accumulated on the top layer of filter paper, it was removed to maintain the filtration rate. The final filtrates were stored at −20°C.

Methanol extracts of potato were made by homogenization (as above) of coarsely ground 5 g potato samples with 20 ml of methanol. The homogenates were filtered through Whatman No. 1 paper, but did not require pre-filtration or removal of clogged filter paper, as did aqueous extracts, because of the lower content of particulate matter. The filtrates were evaporated to near dryness at 50°C, reconstituted to 5 ml with water, and stored at −20°C for later use. ELISA analysis was performed by adding final filtrates from the water method or reconstituted filtrates from the methanol method directly to the competitive inhibition step of the ELISA.

ELISA and competitive inhibition ELISA

Immunoassays were performed as described by Harrison *et al.* (1989*b*), using anti-MH monoclonal antibodies IH9 and IIC7. A summary of the

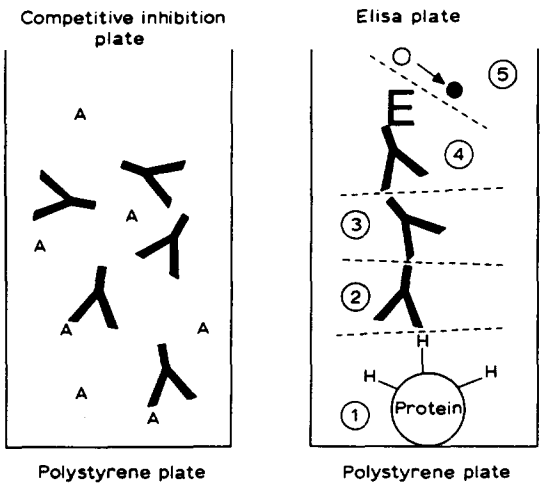

Fig. 2. Summary of procedures for competitive inhibition ELISA analysis of MH. Mouse anti-MH antibody is mixed with sample or standard (represented by A for analyte) in an uncoated polystyrene plate for competitive inhibition step (left). The sequence of steps for performing ELISA is given on the right, starting from the bottom. Each dotted line between steps represents washing to remove excess unbound reagent from the previous step. (1) The hapten–protein conjugate, non-specifically adsorbed to the plate. (2) Mouse monoclonal anti-MH antibody (for competitive inhibition ELISA, this step is actually the addition of the mixture of antibody and analyte from the competitive inhibition plate on the left). (3) Rabbit antibody against mouse κ light chain protein. (4) Goat anti-rabbit antibody conjugated to alkaline phosphatase. (5) Colorless alkaline phosphatase substrate, *p*-nitrophenyl phosphate, enzymatically converted to yellow product, *p*-nitrophenol, in inverse proportion to concentration of analyte (A) in the competitive inhibition plate on the left.

competitive inhibition ELISA procedure is given in Fig. 2. For the ELISA step, 96-well microplates (Nunc #442404) containing the coating antigen dissolved in coating buffer were sealed with adhesive plate sealers to prevent evaporation and incubated 14–20 h at 4°C to adsorb the antigen to the wells. The competitive inhibition step of the assay was performed for 1 h at 20–23°C in untreated 96-well microplates, also sealed with adhesive plate sealers. Quadruplicate 50 μl aliquots were transferred from the competitive inhibition plate to the antigen coated ELISA plate (after washing). Anti-MH antibody bound to the solid phase was quantitated by sequential incubations with rabbit antibody against mouse κ light chain protein, goat anti-rabbit antibody conjugated to alkaline phosphatase, and alkaline phosphatase substrate. All other incubations and washes were done at 20–23°C in buffer containing 0·05% Tween 20 (except substrate buffer). Assay response was measured by reading the absorbance at 405 nm with an MR 600 microplate reader (Dynatech). Results were recorded and analyzed using an Apple II + computer and the Immunosoft 1·4 data acquisition and

analysis program (Dynatech). Inhibition curves were analyzed by an Apple II + computer using a weighted four parameter logistic curve fitting procedure (Rodbard, 1983) which calculated IC_{50} values (inhibitor concentrations giving 50% inhibition), confidence limits, and sample MH concentrations.

RESULTS AND DISCUSSION

Choice of homologous ELISA system

In previous work on ELISA for MH (Harrison *et al.*, 1989*b*), heterologous systems were shown to be more sensitive than homologous systems for the analysis of MH in buffer. However, this advantage did not extend to the analysis of MH in potato extracts. Mean IC_{50} values for heterologous standard curves in potato extracts were approximately half of those for homologous standard curves, for both IH9 and IIC7 antibodies. However, the differences were not statistically significant for either antibody ($n = 5$ or 6, data not shown) because variability of IC_{50} values was greater for heterologous systems than for homologous systems. Susceptibility of heterologous ELISA systems to several non-specific factors also has been shown to be greater than for homologous systems (Harrison *et al.*, 1989*b*). Thus, only the more rugged (i.e. matrix tolerant) homologous systems were used here. It is important to note that the sensitivity to MH in buffer of homologous systems using both IH9 and IIC7 antibodies is adequate for the concentrations of MH expected in this study and is more than sufficient to identify samples exceeding the tolerances noted above.

All analyses used the same plate-coating antigen concentration of 1 μg/ml and anti-MH antibody concentrations of 0·75 μg/ml for IH9 and 0·25 μg/ml for IIC7. These optimized concentration values (Harrison *et al.*, 1989*b*) require 100 ng of plate coating antigen and 45 ng IH9 or 15 ng IIC7 antibody per ELISA well, based on 100 μl/well plate coating volume and 240 μl/well competitive inhibition volume. These numbers demonstrate the tremendous potential of ELISA for performing many analyses with small amounts of reagents. This is an important consideration in standardization of the method, such as for collaborative study. Analysis of MH could be performed on a national scale for several years without exhausting the modest amounts of reagents developed for this study.

ELISA quality control

A critical determinant of ELISA performance is the quality of the solid-phase adsorbent used (Shekarchi *et al.*, 1984). Selection of the 96 well

microplates for this study was made based on the results of a similar study of intraplate variability and antigen binding capacity (Harrison, 1987). Plates were treated uniformly under the conditions of the zero dose control (maximum absorbance and platewise mean absorbance values, coefficients of variation, and absorbance ranges were compared. The need for such a test is obvious upon examination of the results. The range of mean absorbance values was 1·279–0·160, with 7 of the 10 plate types clustered at the upper end (over 1·000). This range indicates grossly unacceptable binding of hapten–protein conjugate by some of the plates tested. More importantly, the means of platewise coefficient of variation values ranged from 39% to the 2·8 ± 0·5% value ($n = 6$) for the Nunc 442404 plates ultimately chosen for further work. Similarly, the range of individual wells as a per cent of the mean absorbance was 93 ± 2% to 107 ± 2% ($n = 6$) for the Nunc plates, but typically spanned 30% or more for most of the other nine types of plate tested. Since this value is roughly analogous to the baseline noise of chromatographic methods, it is highly desirable to minimize noise by the selection of the best plate available. We cannot overemphasize the critical nature of this type of quality control when using solid phase immunoassays for quantitative residue analysis.

A second critical aspect of quality control was microplate reader accuracy, specifically the ability of the reader to position the plate accurately in the light path and minimize interwell differences in path length due to misalignment. Errors of this type have been shown to introduce plate location dependent biases during the reading of 96 well microplates (Harrison & Hammock, 1988), a potentially serious source of systematic error when using standardized templates. Initial experiments to measure recovery of 0–50 ppm spikes were performed using such a standardized 96 well template. Using this template for the analysis of triplicate extracts on each plate (each extract analyzed in quadruplicate wells), we observed systematic overestimates of the MH concentration at the 0 and 1 ppm spike levels. The bias toward high concentration was quantitatively dependent on which quadrant of the plate contained the samples. Testing of the microplate reader by the method of Harrison and Hammock (1988) showed this bias to be due to misalignment of the reader, which was corrected by the manufacturer. Subsequently, periodic quality control checks were performed on plates and readers to verify the reliability of these components of the ELISA method. Several other aspects of routine quality control, described by Harrison *et al.* (1989*a*), were also found to be valuable.

MH standard curves

The competitive inhibition ELISA demonstrated the ability to measure MH

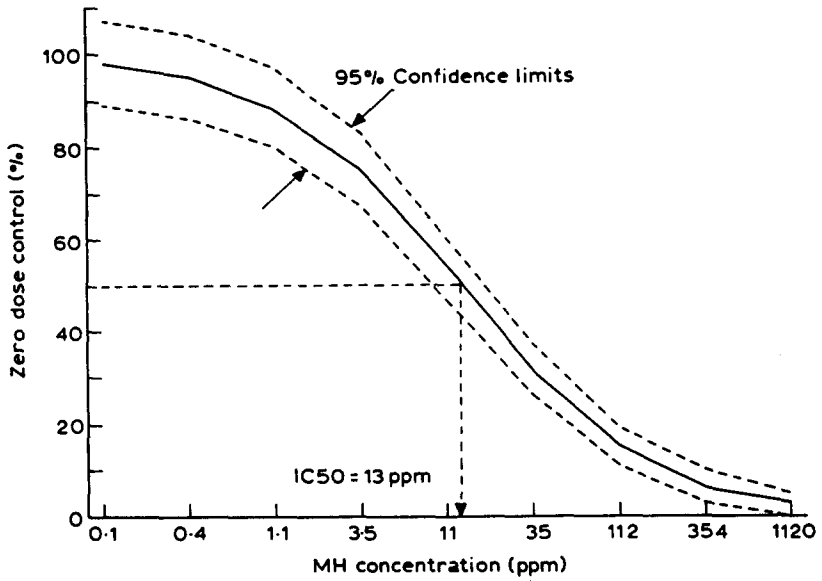

Fig. 3. Standard curve of MH in aqueous juice of MH-free Norchip potato. Competitive inhibition ELISA was performed using 0·45 μg/ml IH9 antibody and 1 μg/ml plate coating antigen. The limit of detection is the MH concentration for the point on the response curve corresponding to the *y* value of the upper asymptote of the lower confidence limit (1·0 ppm). Confidence limits are 95%.

at relevant concentrations in potato samples with minimal preparation. Typical standard curves of MH in aqueous and methanolic potato extracts are shown in Figs 3 and 4. Estimates of the limit of detection (LOD) were calculated as the *x* value (MH concentration) corresponding to the value of the four parameter logistic function at the estimated upper asymptote of the lower confidence limit. A response below this value represents a greater than zero concentration value, within the 95% confidence limits of the curve (Rodbard, 1981; Harrison *et al.*, 1989*b*). The LOD values determined by this method for the curves of Figs 3 and 4 were both near 1 ppm. The IC_{50} values and limits of detection were similar for all combinations of antibody, sample preparation method, and potato variety.

Matrix effects

The effect of the potato matrix alone on the ELISA was tested by combining MH free potato samples, prepared as shown in Fig. 1, and anti-MH antibodies in the manner of the zero dose control of the competitive inhibition ELISA, as shown in Fig. 2. Performance of this test is valuable for indicating potential unsuitability of an antibody, preparation method, or

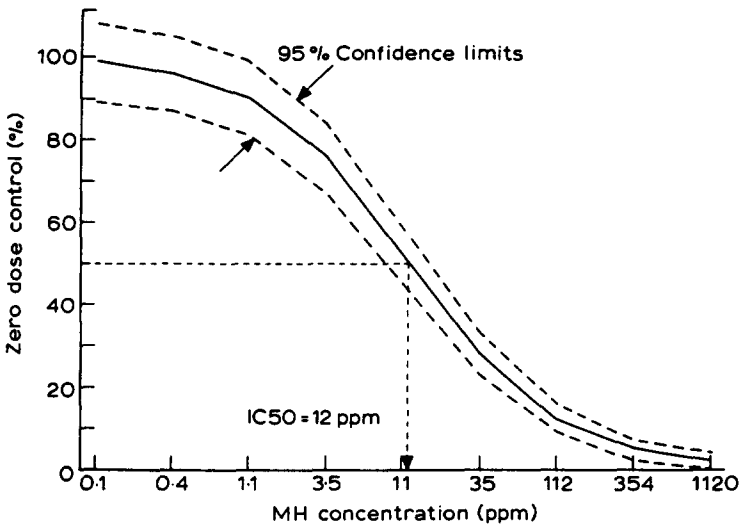

Fig. 4. Standard curve of MH in methanol extract of MH-free Russett Burbank potato. Competitive inhibition ELISA was performed using 0·15 μg/ml IIC7 antibody and 1 μg/ml plate-coating antigen. The limit of detection is the MH concentration for the point on the response curve corresponding to the y value of the upper asymptote of the lower confidence limit (1·2 ppm). Confidence limits are 95%.

potato variety. The results for the various combinations of variety, sample preparation method, and antibody are summarized in Fig. 5.

Effects were observed for all three variables. The effect of the matrix on the assay was consistently greater for IH9 antibody than for IIC7 antibody, regardless of variety and sample preparation method. Based on this difference IIC7 antibody appears better suited for the analysis of MH in field treated potatoes than IH9 antibody. The effect of the Norchip matrix was consistently slightly greater than for the Russet Burbank matrix. In only one case was a difference observed between sample preparation methods for the same antibody and variety (Russet Burbank and IH9 antibody). Because the matrix effects on IIC7 antibody were significant in only one of four cases, this antibody appears suitable for the analysis of most of the matrices tested.

Spike recovery

Two approaches were used to determine spike recovery by competitive inhibition ELISA. Analyses were performed to determine the recovery of 1, 3, 10, 25 and 50 ppm MH spikes from samples of untreated potato (aqueous method only, two determinations at each level for each antibody for two varieties). Individual level means and standard deviations for antibodies and varieties pooled were $2{\cdot}7 \pm 1{\cdot}6$ (1 ppm spike), $3{\cdot}1 \pm 1{\cdot}8$ (3 ppm), $11{\cdot}7 \pm 5{\cdot}9$

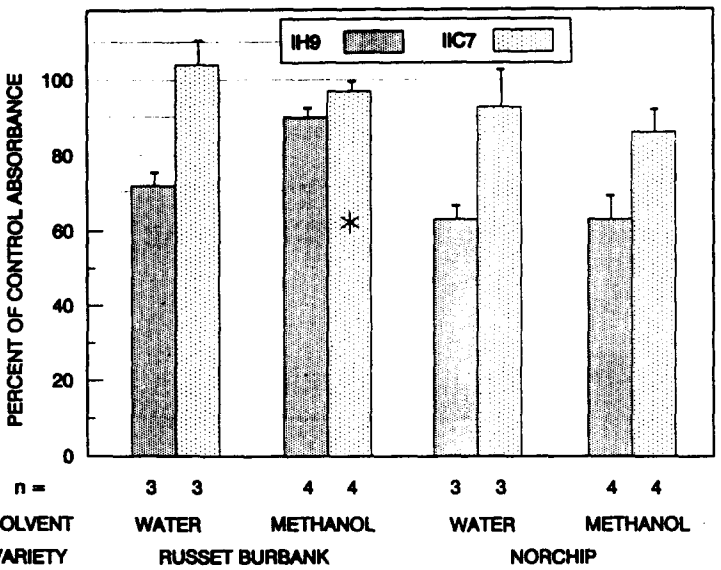

Fig. 5. Matrix effects for two potato varieties, using two anti-MH antibodies and two sample preparation methods. MH-free samples prepared according to Fig. 1 were combined with anti-MH antibodies as for competitive inhibition ELISA according to Fig. 2. Results are expressed as per cent of the control, which used buffer rather than potato extract. The asterisk denotes the system subsequently compared to the colorimetric method in Fig. 7.

(10 ppm), 23·8 ± 7·0 (25 ppm), and 45·8 ± 27·8 (50 ppm), and were similar for both antibodies and both varieties. The aberrantly high mean for the 1 ppm spike (2·7 ppm) is due to the plate-location-dependent bias described in the section on ELISA quality control. The high relative standard deviation at that level (1·6) reflects the fact that not all of the replicate determinations at the 1 ppm level showed this bias. The relative importance of this bias at the other spike levels was markedly decreased because of the higher MH concentrations and because the bias was less at the other positions on the plate.

Competitive inhibition ELISA analyses were also performed to determine the recovery of a 10 ppm MH spike from all of the Uniroyal potatoes, field treated and untreated (two sample preparation methods, three or four determinations for each method per sample, 12 samples per variety, for each of two antibodies). For all combinations of antibody and matrix, the mean amount of MH recovered was 11·0 ± 4·2 ppm, and the range was 6·5–19 ppm, primarily attributable to the variability of spike recovery among samples. Variability of recovery of this spike was analyzed according to source and was lowest within blanks and highest among samples (Fig. 6). These data indicate excellent reproducibility of the method, but also suggest that sample variability may contribute significantly to overall method variability.

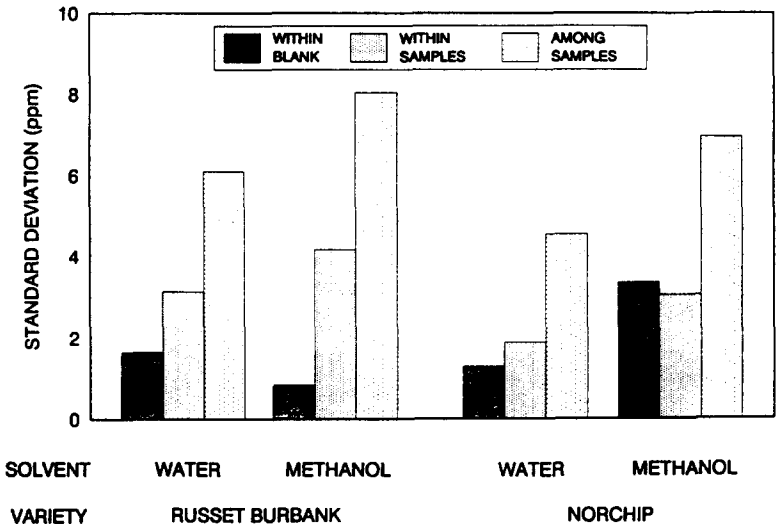

Fig. 6. Variability of recovery of a 10 ppm spike of MH added to various potato extracts. Values are means of pooled data for both IH9 and IIC7 antibodies. Standard deviations within blanks, 3 or 4 determinations per antibody; standard deviations within samples, 3 or 4 determinations per sample for 12 samples per antibody; standard deviations among samples, 3 or 4 determinations per sample for 12 samples per antibody.

Comparison of results from colorimetric and ELISA methods

The analyses for MH in both varieties of field-treated potatoes were performed using both antibodies and sample preparation methods. Correlation between ELISA and colorimetric methods was best ($r = 0{\cdot}92$) for the analysis of methanolic extracts of Russet Burbank potatoes with IIC7 antibody (Fig. 7). Correlations for the eight combinations of antibody and sample preparation methods ranged from 0·92 to 0·73 for Russet Burbank and from 0·77 to 0·55 for Norchip.

It is instructive to compare ELISA results for the two anti-MH antibodies for both sample preparation methods. The correlation between ELISA results for IH9 and IIC7 (both potato varieties, $n = 24$ for each comparison) was 0·99 for the analysis of methanol extracts and 0·97 for the analysis of aqueous extracts. In contrast, the correlation between aqueous and methanol sample preparation methods was 0·70 for IH9 and 0·69 for IIC7. These results indicate that variety and sample preparation method were more significant in the determination of assay performance than the anti-MH antibody used. However, as is often the case in the development of immunoassays, the usefulness of the method comparison data is limited by the lack of replication for the established method, which is determined largely by its difficulty and cost. The similarity of results for the two

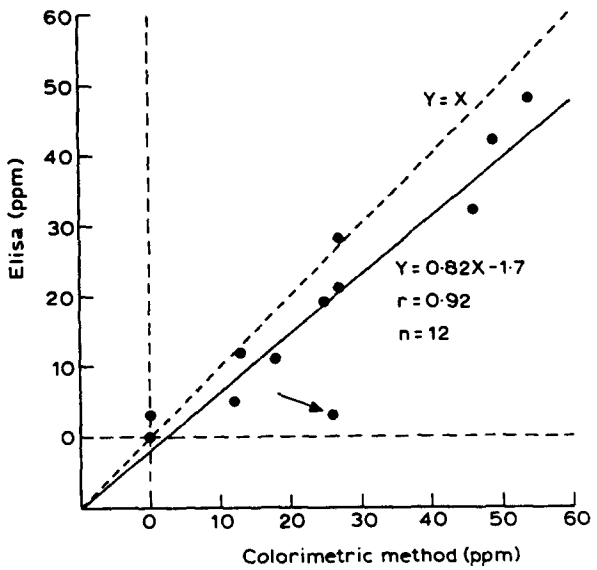

Fig. 7. Correlation between MH concentrations determined by the colorimetric method and the ELISA method (methanol extraction) for 12 samples of field treated Russet Burbank potatoes, corrected for recovery of a 10 ppm MH spike. Colorimetric ppm values are for single determinations. The ELISA standard deviations for duplicate analyses (each analysis the mean of quadruplicate wells) averaged 3·4 ppm. The arrow indicates the sample discussed in the text of the Results and Discussion section.

antibodies, which are demonstrably different in their responses to defined non-specific factors (Harrison *et al.*, 1989*b*) implies that the ELISA data may be more accurate than the unreplicated colorimetric data.

Two individual samples are worth discussion because of the possibility that their results reflect interferences with the colorimetric method not found in the ELISA method. This observation is important because the lack of replication in the colorimetric analyses prevents the identification of possible outliers attributable to errors of analysis. The colorimetric method values for these two samples were 26 and 19 ppm, while the immunoassay means and standard deviations were, respectively, 3 ± 2 and 3 ± 1 ppm (two or three determinations for each of two antibodies and two sample preparation methods). The point representing the first of these results is indicated by the arrow in Fig. 7. It should be noted that in competitive inhibition ELISA of low molecular weight analytes, false negative results are typically much less common than false positive results. This is due to the fact that false positives (erroneously decreased absorbance values) are frequently caused by excessive matrix effects, while false negatives generally require that the analyte actually be rendered unavailable to the antibody by modification or sequestering, a much less likely event.

Conclusions

The data presented above demonstrate the ability of the competitive inhibition ELISA method to measure MH in field treated potatoes. These data confirm the suitability of this method for use such as in a trial program to screen Russet Burbank potatoes for violative samples. Extension of the validation data presented above would allow large-scale screening of many potato varieties for MH residues. Further refinement of the ELISA method for MH should emphasize the estimation of false positive and false negative rates, with the most rigorous possible confirmatory analysis of ELISA results. In subsequent studies special attention should be given to increased replication of the established method to allow firmer conclusions to be drawn in the comparison of methods.

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