FINAL PROJECT REPORT

Project Title: Validation of Analytical Method for Analysis of Morpholine on Apples

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Cooperators: Mike Willett, Northwest Horticultural Council

Other funding sources

Agency Name:
Amount awarded:
Notes:

Total Project Funding:

Budget History:

Item	Year 1:	Year 2:	Year 3:
Salaries	$8,900		
Benefits	$2,800		
Wages			
Benefits			
Equipment Operation	$3,000		
Supplies	$2,500		
Travel			
Plot Fees			
Miscellaneous	$1,000		
Total	$18,200		

OBJECTIVES

1. Validation of an analytical method developed by Dr. Michelangelo Anastassiades at the EU Reference Laboratory in Stuttgart, Germany (termed the QuPPe-Method) for morpholine residue analysis in/on apple.
2. Upon successful validation of the analytical method, a third laboratory will evaluate apple samples spiked with known amounts of morpholine to establish that the methodology is reasonably rugged and reproducible, lending credence to the reproducibility, accuracy and precision of results as well as establishing a minimum level of quantitation that every laboratory must meet.
3. Publication of the method in a peer-reviewed venue, possibly the Journal of AOAC International or the Journal of Agricultural and Food Chemistry.

SIGNIFICANT FINDINGS

1. Completed.
    - The analytical method to determine morpholine residues in/on apples was successfully validated by both testing facilities at the UC Davis Trace Analytical Laboratory (TAL) and the Pacific Agricultural Laboratory (PAL).

2. Omitted.
    - Given the excellent correlation of validation data between TAL and PAL, the need for a third laboratory to provide testing was unnecessary.

3. Revising
    - The manuscript for publication has been reviewed by the Journal of Agricultural and Food Chemistry. Reviewer evaluations were mostly positive and the manuscript is being revised to incorporate reviewer comments and suggestions.

RESULTS & DISCUSSION

Objective 1. Validation of EU Reference Laboratory QuPPe-Method

Method Background. During the initial phases of method development, one of the main goals was to have very simple and rapid sample workup that could provide a target limit of quanititation (LOQ) of 0.01 ppm. This target was based on the default threshold which some governmental agencies, such as the EU, typically set at 0.01 ppm on compounds that do not have established tolerances (*1*). Another goal was to have a method which utilized a relatively small amount of solvent and did not require the use of expensive and often difficult to obtain labeled internal standards. Gros et al. reported an LC-MS/MS based method for the determination of morpholine on pineapples which was able to achieve an LOQ of 0.01 ppm (*2*). While this method achieved the target LOQ, the procedure was not favorable as it required a dual solid phase extraction cartridge system which adds additional time and cost to the analysis. An unpublished method by Anastassiades et al described a procedure for very polar, non-QuEChERS-amenable compounds in foods which utilizes acidified methanol extraction and determination by LC-MS/MS (*3*). This method met our needs in terms of simple and rapid sample workup but only showed recovery data on lime at 1.0 ppm and extrapolated LOQs on apples at 0.01 ppm (*3-4*). We therefore adopted a similar extraction procedure utilizing acidified methanol as in the Anastassiades method as a starting point for our method development. During early instrumental analysis fortified extracts were compared to external standards prepared in solvent to assess possible ion enhancement/suppression. It was

observed that the response from the fortified extracts was suppressed greater than 20% relative to the standard in solvent. As a result of these observations, the researchers chose to prepare matrix-matched standards to correct the suppression effects.

Method Validation. In addition to the goals above, the method needed to be rugged and transferrable to other laboratories. Therefore, method validation was conducted at both the PAL and TAL facilities. All control samples used for the validation studies showed no significant morpholine residues above the calculated method detection limits (MDL) for apple. Recovery data generated at the PAL facility for apple ranged from 86–120% over three levels of fortification (0.2, 0.04 and 0.01 ppm) with all standard deviations ≤ 15%. The TAL facility produced recovery data for apple which ranged from 97–112% over the same fortification levels listed above. As with the PAL good precision was observed with all standard deviations ≤ 15%. Typically standard deviations of 20% or less are considered acceptable, but as can be seen in Table 1, the majority of the standard deviations are ≤6%. This speaks to the excellent precision of the method in both facilities. In the case of calculated MDLs, the value is directly related to the standard deviation of the recoveries at the target LOQ. Some variability between laboratories is to be expected and the factor of 2 difference between TAL and PAL is acceptable. Overall, the results from apple method validation correlated very well between the two laboratories (Table 1).

As a result of the research conducted, a rapid, rugged and selective method was developed for the screening of morpholine in apples. With the method presented herein, 50 samples can be extracted in ~ 8 hours and analyzed by LC-MS/MS in approximately 20 hours (overnight).

Table 1. Method Validation Results

	Fortification Level			
Laboratory	0.01 ppm	0.04 ppm	0.2 ppm	Calculated MDL ppm[1]
TAL	104 ± 6%	100 ± 2%	107 ± 1%	0.0018
PAL	108 ± 13%	104 ± 2%	94 ± 4%	0.0040

1. Calculated MDL for n=7: Standard Deviation at 0.01 ppm fortification level × Student t_{99} (n-1)

LITERATURE CITED

1. European Union Pesticide Database. http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=commodity.selection (Accessed May, 2010).
2. Gros, P.; Matignon, F. and Plonevez,S. Determination of Morpholine in Fruits Using LC-MS/MS. *Falsifications de l'Expertise Chimique et Toxicologique*. **2011**, 974, 17–22.
3. Anastassiades, M.; Kolberg, D. I.; Mack, D.; Sigalova, I.; Roux, D. and Fügel, D. Quick Method for the Analysis of Residues of Highly Polar Pesticides in Foods of Plant Origins Involving Simultaneous Extraction with Methanol and LC-MS/MS Determination (QuPPe-Method). EU Reference Laboratories for Residues of Pesticides. Version 6, 2011. http://www.eurl-pesticides.eu (Accessed October, 2012).

4. Anastassiades, M.; Kolberg, D. I.; Mack, D.; Wildgrube, Chr.; Sigalova, I.; Roux, D. and Fügel, D. Quick Method for the Analysis of Residues of Highly Polar Pesticides in Foods of Plant Origins Involving Simultaneous Extraction with Methanol and LC-MS/MS Determination (QuPPe-Method). EU Reference Laboratories for Residues of Pesticides. Version 7.1, 2013. http://www.crl-pesticides.eu/library/docs/srm/meth_QuPPe.pdf (Accessed November, 2013).

Objective 2. Third –Party Method Validation

During initial discussions on the method validation of morpholine there was interest in identifying third-party laboratories to evaluate morpholine spiked apples using the validated method. However, once the validation data was tabulated and compared from PAL and TAL, the project PIs, in conjunction with Mike Willett, decided that this objective was no longer necessary. The decision was based on how well the validation correlated between the two facilities. This shows the overall robustness of the method and how the method was successfully transferred from one laboratory to another in which different analysts, equipment and instrumentation were utilized.

Objective 3. Publish Peer-Reviewed Method

Upon completion of method validation, a manuscript was prepared for submission to the Journal of Agricultural and Food Chemistry in November, 2013. By late December we received the response back from the Editor that our manuscript requires some revising with inclusion of some of the reviewer's comments. Overall, the reviewer's comments were positive and related how useful this procedure would be to regulatory agencies and laboratories, as well as, how these entities appreciate a validated method which has been optimized and streamlined.

Currently, the manuscript is being revised and will be re-submitted in early February, 2014.

EXECUTIVE SUMMARY

With the detection of morpholine (a food additive not approved in the EU but used in most U.S. fruit coatings) on apples in January 2010, U.S. apple exports to the EU have declined precipitously from 1.3 million cartons in 2008-2009 to 604,855 boxes in the 2012-2013 crop year. Since that time, detections of morpholine upon arrival by our industry's customers in Europe have resulted in numerous rejections and market shyness. Private laboratories in both the EU and the U.S. are performing morpholine analysis using in-house developed proprietary methods which have not been validated or even shared with other organizations causing disparity in the analytical results and violations upon the product's arrival in the EU. The loss to the industry from this restriction on morpholine is estimated to be in the range of $20-$25 million. To address this trade barrier issue, the Northwest Horticultural Council proposed the development of a standardized analytical method to determine morpholine residues in/on apples. Researchers from the Trace Analytical Laboratory at UC Davis (TAL) and the Pacific Agricultural Laboratory (PAL) were contacted to validate a method currently being utilized in Europe which looks for several compounds, including morpholine, in agricultural commodities.

Significant progress has been made in the development and validation of the morpholine analytical method. To date, the analytical method developed by Dr. Michelangelo Anastassiades was successfully modified and validated in two separate facilities (TAL and PAL). Each facility validated the method on apples down to a level of 0.01 ppm with excellent accuracy and precision. The 0.01 ppm level was chosen as many governmental agencies set a default maximum residue limit (MRL) for compounds which do not have an established MRL or tolerance. This includes the EU which does not have a MRL established for morpholine on apples.

In addition to the successful validation of the morpholine method, a manuscript was prepared and submitted for publication in a peer reviewed journal. The Journal of Agricultural and Food Chemistry was chosen for its high prestige and logical fit for this type of research. The response from the journal was positive and the manuscript is currently being revised for submission. Once published, the analytical method can serve as a starting point for a domestically accepted method and eventually become an internationally accepted method.