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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PS2517

2. Project title:
   Infant Food Directive Analytes (Development and Validation of Methods Required to meet The EU Infant Food Directive)

3. Contractor organisation(s):
   Central Science Laboratory
   Sand Hutton
   York

   YO41 1LZ

4. Total Defra project costs: £

5. Project: start date ..... 01 April 2004

   end date ..... 31 March 2006

6. It is Defra's intention to publish this form.
   Please confirm your agreement to do so.....**YES** ☒ **NO** ☐

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   In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

   (b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Overview

The EU Baby Food Directive 2003/13/EC (amending Directive 96/5/EC) on processed cereal-based foods and processed foods for infants and young children came into force on 6th March 2004[1]. The Directive places emphasis on the control of pesticides or metabolites of pesticides with an acceptable daily intake lower than 0.5 µg kg^{-1} body weight. Pesticides are either designated as prohibited, and considered not to have been used if their residue does not exceed 3 µg kg^{-1}, or have maximum residue limits (MRLs) set between 4 - 8 µg kg^{-1}. Twelve of the pesticides and metabolites listed in the Directive are amenable to gas chromatography mass spectrometry (GC-MS) analysis; the remaining compounds, because of their physico-chemical properties, require analysis either by liquid chromatography mass spectrometry (LC-MS) or by specific single residue methods.

Analytical methods capable of achieving very low levels required by the EU Infant Food are urgently required to enable PRC to enforce the legislative requirements. The purpose of this project was to develop and validate, robust, low cost, multi-residue GC-MS/MS and LC-MS/MS 'fit-for-purpose' methods

Main objectives

To provide the PRC with methods, which will allow the enforcement of EU Infant Food Directive 2003/13/EC.

Summary of research findings

Development and validation of a GC-MS/MS multi-residue method

A method based on gas chromatography-tandem quadrupole mass spectrometry (GC-MS/MS) has been developed for the determination of twelve of the 'red list' pesticides and pesticide metabolites included in the EU Baby Food Directive 2003/13/EC. The compounds were aldrin, cadusafos, dieldrin, endrin, ethoprophos, fipronil, fipronil desulfinyl, heptachlor, heptachlor epoxide (trans), hexachlorobenzene, nitrofen and omethoate. The method was also suitable for the determination of dimethoate, a possible breakdown product of omethoate, which was included at the specific request of the Pesticides Safety Directorate. Samples of baby food were extracted with acetonitrile and co-extractives were removed by dispersive solid phase extraction using octadecyl and primary secondary amine sorbents prior to GC-MS/MS analysis. Extracts of baby food samples, spiked with pesticides at 1 to 8 μg kg^{-1}, yielded average recoveries typically in the range 60 - 110 % with relative standard deviations less than 20 %. The GC-MS/MS multi-residue method developed is simple, rapid and, with the exception of hexachlorobenzene, is suitable for the screening of 12 'red list' pesticides in fruit, fish and potato based baby food at 1 μg kg^{-1}, and to quantify and confirm the identity of these pesticides at their respective MRLs. If a residue of hexachlorobenzene is detected an alternative method providing more accurate quantification would be required.

Development and validation of a LC-MS/MS multi-residue methods

A method based on high performance liquid chromatography-tandem quadrupole mass spectrometry (HPLC-MS/MS) has been developed for the determination of 16 priority pesticides and transformation products (cadusafos, demeton-S-methyl, demeton-S-methyl sulphone, dimethoate, ethoprophos, omethoate, oxydemeton methyl, disulfoton, disulfoton sulphoxide, disulfoton sulphone, fensulfothion, fensulfothion oxon, fensulfothion sulphone, fensulfothion oxon sulphone, terbufos, terbufos sulfoxide, and terbufos sulphone) specified in the EU Baby Food Directive 2003/13/EC. Excellent results were also obtained for dimethoate.

Prior to HPLC-MS/MS or ultra performance liquid chromatography (UPLC)-MS/MS analysis, co-extractives were removed from acetonitrile extracts using dispersive solid-phase extraction with primary secondary amine. Extracts spiked with pesticides at 1 μg kg^{-1} yielded average recoveries in the range 85–119%, with relative standard deviations less than 17%. The HPLC–MS/MS and UPLC–MS/MS multi-residue methods developed are simple, rapid and suitable for the quantification and confirmation of the 16 priority pesticides in fruit-, potato- and cereal-based baby food at 1μg kg^{-1}. The major advantages of UPLC over HPLC are the speed of analysis (2.5 times faster than HPLC), the narrower peaks (giving increased signal-to-noise ratio) and improved confirmation for the targeted pesticides in the analyses of baby foods.

Conclusions

The overall outcome of the project is that all of the objectives were met fully. The finalised approach based on a single extraction determination by GC-MS/MS and LC-MS/MS places the PRC is in the unique position of being able to analyse baby foods for a total of 25 of the pesticides and transformation products included in the EU Baby food Directive 2003/13/EC. Further details have been published by Leandro et al.[2,3] PSD have previously funded the development of a single residue GC-MS method for the determination of haloxyfop and LC-MS/MS methods exist for the analysis of propylenethiourea in baby foods. Consideration should be given to the development of specific robust methods for the determination of the two remaining pesticides, fentin and propineb, included in the Directive.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:

- the scientific objectives as set out in the contract;
- the extent to which the objectives set out in the contract have been met;
- details of methods used and the results obtained, including statistical analysis (if appropriate);
- a discussion of the results and their reliability;
- the main implications of the findings;
- possible future work; and
- any action resulting from the research (e.g. IP, Knowledge Transfer).

Objectives:

(i) Development and validation of a GC-MS/MS multi-residue method

It is proposed to develop and validate a GC-MS/MS multi-residue method for the determination of omethoate (0.003 mg kg^{-1}), dimethoate (0.01 mg kg^{-1}), ethoprophos (0.008 mg kg^{-1}), cadusafos (0.006 mgkg^{-1}), HCB (0.008 mg kg^{-1}), fipronil (0.002 mg kg^{-1}), fipronil-desulphinyl (0.002 mg kg^{-1}), heptachlor (0.0015 mg kg^{-1}), *trans*-heptachlor epoxide (0.0015 mg kg^{-1}), aldrin (0.0015 mg kg^{-1}), dieldrin (0.0015 mg kg^{-1}), endrin (0.003 mgkg^{-1}), and nitrofen (0.003 mg kg^{-1}).

The proposed approach will be optimised and validated at the relevant limit, or below, in a fruit based baby food, potato-based baby food and /or a cereal-based baby food as appropriate.

(ii) Development and validation of an LC-MS/MS multi-residue method

The development of a multi-residue HPLC-MS/MS analysis of the same extract for GC will be attempted to include some of the other pesticides in the Infant Food Directive. The potential list includes demeton-S-methyl (0.002 mg/kg), demeton-S-methyl sulphone (0.002 mg kg^{-1}), oxydemeton methyl(0.002 mgkg^{-1}), disulfoton (0.001 mg kg^{-1}), disulfoton sulphoxide (0.001 mg kg^{-1}), disulfoton sulphone (0.001 mg kg^{-1}), fensulfothion (0.005 mg kg^{-1}), fensulfothion oxon (0.005 mgkg^{-1}) fensulfothion sulphone (0.005 mgkg^{-1}) fensulfothion-oxon sulphone (0.005 mg kg^{-1}) terbufos (0.001 mg kg^{-1}), terbufos (0.001 mg kg^{-1}), sulfoxide and terbufos sulphone (0.001 mg kg^{-1}).

The proposed approach will be optimised and validated at the relevant limit, or below, in a fruit-based baby food, potato-based baby food and /or a cereal-based baby food as appropriate.

Experimental

Extraction

Crude acetronitrile extracts of baby foods were not suitable for direct analysis using GC-MS/MS because of high levels of matrix coextractives. A number of different clean-up options to reduce the level of coextractives in extracts (1 ml) were evaluated. The effectiveness of different amounts (100 to 300 mg) of C_{18} sorbent with a constant amount (50 mg) of PSA sorbent was assessed by the quantity of co-extractives remaining after evaporation of the solvent, by the effect on the long-term chromatographic performance and recovery data. Increasing the amount of C_{18} sorbent used in the clean up reduced the recovery of a number of the pesticides but a minimum of 200 mg of C_{18} material was required to provide relatively clean extracts, improved signal to noise ratio and consistent response resulting in satisfactory calibration curves. Consequently, 50 mg of PSA and 200 mg of C_{18} sorbent were used for all GC-MS/MS analyses.

The C_{18} sorbent reduced the recovery of some LC-MS/MS analytes, notably oxydemeton methyl, and fensulfothion oxon. Therefore, the extracts were cleaned up with primary secondary amine

(PSA, 50mg) and diluted 10:1 with water prior to LC-MS/MS analysis.

The finalised extraction and cleanup procedure is described below;

A sub-sample (10 g) was weighed into a centrifuge tube (40 ml) and an appropriate volume of standard solution added to give a fortification level of 1–8 µg kg^{-1}. Acetonitrile (10 ml), anhydrous magnesium sulfate (4 g) and sodium chloride (1 g) were added and shaken immediately, thus preventing coagulation of MgSO4. [Volumetric standard (delta-hexachlorocyclohexane) was added (100 µl of a 1 µgml^{-1} solution) – for use in GC-MS/MS]. The sample was centrifuged at 4300×g for 5 min.

For GC-MS/MS an aliquot (1 ml) of the supernatant was transferred to a microcentrifuge vial containing PSA sorbent (50 mg), anhydrous MgSO4 (150 mg) and C_{18} sorbent (200 mg). The vial was vortexed for 30 s, centrifuged at 5000×g for 1 min, and the supernatant was analysed by LVI–GC–MS/MS.

For LC-MS/MS an aliquot (1 ml) of the supernatant was transferred to a microcentrifuge vial containing PSA sorbent (50 mg) and anhydrous MgSO4 (150 mg). The contents were vortex mixed for 30 s, centrifuged at 5000×g for 1 min, and the supernatant was analysed by LC–MS/MS after dilution with water (1:10).

Further details of the extraction and clean-up are given by Leandro et al. [2,3]

GC-MS/MS

Determination was performed using a Varian GC–MS/MS system comprising a CP-3800 gas chromatograph with a 1079 PTV injector, a CP-8400 autosampler and a 1200 tandem quadrupole MS (Varian, Walnut Creek, CA, USA). Data acquisition and processing were performed using Varian Star Workstation software (version 6.2). A fused-silica capillary column (Zebron ZB-50, 50% phenyl–50% methylpolysiloxane, 30 m×0.25 mm i.d., 0.25 µm film thickness; Phenomenex, USA) was used and protected by a 7 mm CarboFrit insert in the GC liner. The injection volume was 8 µl.

The mass spectrometer was operated in electron ionisation (EI) mode. The MS/MS detector interface temperature was set at 200 °C, the source temperature at 300 °C, electron energy at 70 eV, filament current at 150 µA and the detector voltage at 1700V.

A number of instrumental parameters including; electron ionization, MS/MS transitions, collision energy, number of transitions per time segment and the number of data points across the peak were evaluated. The most intense, highest mass precursor ions were selected from full scan spectra and then product ion spectra were acquired by collision-induced dissociation (CID) with argon gas. Precursor ions were examined at collision energy (CE) voltages of 10, 20, 30 and 40 V (potential on quadrupole 2) and the most intense product ions were selected for each precursor ion. Two transitions were selected for each analyte with a maximum of four transitions programmed into each time segment to maintain sufficient response. A scan rate of 0.3 seconds $datapoint^{-1}$ was set to collect a minimum of 7 to 10 points across a chromatographic peak.

LC-MS/MS

HPLC analyses were performed using a Waters Alliance 2795 Separations Module (Waters, Milford MA, US) equipped with a quaternary solvent delivery system, autosampler, and column heater. HPLC separation was achieved using a Sunfire C18 column (100 mm×2.1 mm, i.d., 3.5 µm particle size (Waters), maintained at 40 ◦C and fitted with a Sunfire C_{18} Guard column (10 mm×2.1 mm, i.d., with a mobile phase flow rate of 0.3 ml min^{-1}. The HPLC operating pressure was 2500 psi at initial gradient conditions. The injection volume was 50 µl.

The mobile phase contained 90% water, 10% methanol and ammonium acetate at a final concentration of 20 mmol l^{-1} (A) and 10% water, 90% methanol and ammonium acetate at a final concentration of 20 mmol l^{-1} (B). Gradient elution was employed, starting at 0% B and rising linearly to 100% B over 13 min. The composition was held at 100% B for a further 4 min before being returned to the initial conditions, followed by re-equilibration for 8 min, giving a total run time 25 min.

UPLC analyses were performed using a Waters Acquity Ultra Performance LC system (Waters). UPLC separation was achieved using an Acquity UPLC BEH C18 column (100 mm×2.1 mm, i.d., 1.7 µm particle size (Waters), maintained at 40 ℃, with a mobile phase flow rate of 0.3 ml min^{-1}. The Acquity system operating pressure was 6000 psi at initial gradient conditions. The mobile phase contained 90% water, 10% methanol and ammonium acetate at a final concentration of 20 mmol l^{-1} (A) and 10% water, 90% methanol and ammonium acetate at a final concentration of 20 mmol l^{-1} (B). Gradient elution was employed, starting at 0% B and rising linearly to 100% B over 5 min. The composition was held at 100% B for a further 2 min before being returned to the initial conditions, followed by re-equilibration for 3 min, giving a total run time 10 min. The injection volume was 50µl.

Determination was performed using a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray source in positive mode. The □onization source parameters were: capillary voltage 3.5 kV; sample cone voltage (optimized for each analyte), source temperature 120 °C; and desolvation gas temperature 350 °C at a flow rate of 1.4×104 ml min^{-1} (N_2).

Multiple reaction monitoring (MRM) conditions were optimized for each pesticide during infusion. Data acquisition and processing were performed using MassLynx 4.0.

Results

GC-MS/MS

Calibration curves were linear over the range 0.0005 – 0.01 µg ml^{-1} (equiv. 0.5 – 10 µg kg^{-1}) with correlation coefficients > 0.98 for all analytes.

The validation results are summarised in Table 1.

With the exception of hexachlorobenzene, satisfactory recoveries (63-113 %, n=7) and % CV s (generally <20 %) were obtained for all the pesticides spiked at 1 and 3 µg kg^{-1} levels in 3 different baby foods (main ingredients comprising fruit, rice, fish, pasta and potato). Quantification of ethoprophos, heptachlor and endrin was not reliable at 1 µg kg^{-1} level in at least one of the test matrices. At 3 µg kg^{-1} level (MRL) there was an improvement in recovery and precision for endrin. The relatively low recoveries (approximately 60%) for hexachlorobenzene, even after correction by the volumetric internal standard (δ-HCH), suggest that hexachlorobenzene is preferentially retained by the C_{18} sorbent. Accurate quantification of any potential residues, especially hexachlorobenzene, will require use of internal standards matched more closely to the lipophilic analytes or an alternative clean-up method.

In addition to the results given in Table 1, the method was used to determine pesticides in 10 different baby food samples containing a variety of ingredients. The losses of the pesticides at the clean-up step were matrix dependent; consistent across the individual sample types but variable between different sample types. To achieve accurate results it proved necessary to prepare matrix matched calibration standards for each individual sample type. The different matrices tested did not affect the ability of the method to correctly identify the target pesticides at the MRL levels. This is attributable to the excellent selectivity of GC-MS/MS and is discussed by Leandro et al. [2]

The large volume injection GC-MS/MS multi-residue method developed is simple, rapid and suitable for the screening of 12 ‘red list’ pesticides in fruit-, fish- and potato-based baby food at 1 µg kg^{-1}, and to quantify and confirm the identity of these pesticides at their respective MRLs, with the exception of hexachlorobenzene. If a residue of hexachlorobenzene is detected an alternative method providing more accurate quantification would be required. Regardless, the

extraction and clean up method presented is still a practical solution to the challenge set by EU Directive. It is possible to include the analysis of other GC-amenable pesticides to allow for any future amendments to the Directive. To enforce the EU Baby Food Directive, parallel multi-residue analysis for LC-MS amenable pesticides is also required.

Table 1: Summary of GC-MS/MS results (n=7)

Baby Food Main Ingedients →			Apple/Pear & Rice		Salmon/Spinach				Potato/Pork	
Fortification level →			1 µg kg^{-1}		1 µg kg^{-1}		3 µg kg^{-1}		1 µg kg^{-1}	
Compound ↓	tR	MRL*	Rec	RSD	Rec	RSD	Rec	RSD	Rec	RSD
	(min)	µg kg^{-1}	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
ethoprophos	8.07	8	66	28	101	6	78	9	90	5
cadusafos	8.24	6	69	5	85	12	83	26	65	6
HCB	8.95	3	66	15	57	11	65	5	60	12
omethoate	9.22	3	63	13	71	7	71	5	70	9
fipronil-desulphinyl	11.04	2	87	8	106	4	102	3	102	12
dimethoate	11.33	10	91	14	99	11	100	7	113	10
heptachlor	11.38	1.5	84	12	90	5	80	5	92	23
aldrin	12.73	1.5	93	10	81	9	73	6	94	13
fipronil	14.10	2	82	14	100	4	94	5	87	10
heptachlor-epoxide (*trans)*	14.69	1.5	75	19	88	10	90	7	99	19
dieldrin	15.78	1.5	80	11	88	10	88	8	78	18
endrin	16.32	3	79	10	79	23	84	4	81	25
nitrofen	16.48	3	80	8	87	5	89	4	81	11

Note; * For multi-component MRLs the target levels are equivalent to the MRL level divided by the number of individual components.

All of the results presented in Table 1 have been entered into the PSD methods compendium.

LC-MS/MS

Calibration curves were linear over the range 0.0005–0.01 µg ml^{-1} (equivalent to 0.5–10 µg kg^{-1}) with correlation coefficients >0.99 for all analytes both for HPLC–MS/MS and for UPLC–MS/MS.

The validation results are summarised in Table 2.

Excellent method recoveries were obtained for all the pesticides spiked at the 1 µg kg^{-1} levels in three different baby foods (HPLC–MS/MS 85–113%, RSD < 17% and UPLC–MS/MS 92–119%, RSD < 11%). Although recoveries obtained by HPLC and UPLC are very similar, the precision obtained by UPLC is improved, especially for disulfoton and terbufos. A detailed comparison of the results of HPLC and UPLC–MS/MS has been given by Leandro et al. [3]

Table 2: Summary of HPLC-MS/MS results (n=7)

Baby Food Main Ingedients →			Apple/Pear & Rice		Potato/Pork		Oats/Cream	
Fortification level →			1 µg kg^{-1}		1 µg kg^{-1}		1 µg kg^{-1}	
Compound ↓	tR	MRL*	Rec	RSD	Rec	RSD	Rec	RSD
	(min)	µg kg^{-1}	(%)	(%)	(%)	(%)	(%)	(%)
omethoate	3.63	3	98	2	90	2	97	3
Oxydemeton-S-methyl	5.37	2	105	6	101	4	110	3
Demeton-S-methyl sulphone	5.56	2	104	4	105	6	103	3
dimethoate	7.16	10	107	2	106	7	104	4
Fensulfothion-oxon	8.86	0.75	106	2	102	5	103	4
Fensulfothion-oxon sulphone	9.02	0.75	109	4	102	4	105	4
Demeton-s-methyl	9.97	2	113	5	104	4	98	5
Disulfoton sulphoxide	10.85	1	105	4	106	4	103	3
Disulfoton sulphone	10.99	1	106	6	103	1	102	3
Fensulfothion	11.40	0.75	107	5	98	5	106	3
Fensulfothion sulphone	11.54	0.75	105	4	102	5	104	2
Terbufos sulphone	12.05	1	108	4	103	5	107	5
Terbufos sulphoxide	12.10	1	110	3	99	4	106	4
Ethoprophos	13.28	8	107	2	103	3	103	4
Disulfoton	14.44	1	89	17	100	13	106	13
Cadusafos	14.63	6	105	4	100	4	105	2
Terbufos	15.23	1	108	14	85	10	98	6

Note; * For multi-component MRLs the target levels are equivalent to the MRL level divided by the number of individual components.

All of the results presented in Table 2 have been entered into the PSD methods compendium.

Conclusion

The overall outcome of the project is that all of the objectives were met fully. The finalised approach based on a single extraction determination by GC-MS/MS and LC-MS/MS places the PRC is in the unique position of being able to analyse baby foods for a total of 25 of the 30 or so pesticides and transformation products included in the EU Baby food Directive 2003/13/EC. Further details have been published by Leandro et al. [2,3] PSD have previously funded the development of a single residue GC-MS method for the determination of haloxyfop and LC-MS/MS methods exist for the analysis of propylenethiourea in baby foods. Consideration should be given to the development of specific robust methods for the determination of the two

remaining pesticides, fentin and propineb, included in the Directive.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

REFERENCES

[1] Commission Directive 2003/13/EC of 10 February 2003 amending Directive 96/5/EC on processed cereal-based foods and baby foods for infants and young children, Official Journal L41/33.

[2] Leandro C C , Fussell, R J, Keely, B, (2005). Determination of 'priority' pesticides in baby foods by gas chromatography tandem quadrupole mass spectrometry; J. Chromatogr. A., 1085, 207-212.

[3] Leandro C C , Hancock P, Fussell, R J, Keely, B, (2006). Comparison of ultra-performance liquid chromatography and high-performance liquid chromatography for the determination of priority pesticides in baby foods by tandem quadrupole mass spectrometry. J. Chromatogr. *A*., 1103, 94-101.