Ethyl formate, formic acid and ethanol in air, wheat, barley and sultanas: analysis of natural levels and fumigant residues

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Abstract: Ethyl formate and ethanol in air, eg in fumigant studies, were readily detected by gas chromatography (GC) (flame ionisation). Residues in wheat, barley and sultanas were analysed by GC, after extraction in polar solvents (eg methanol, aqueous propanol). Both natural levels and levels resulting from fumigation with ethyl formate were measured. Formic acid was extracted from commodities with polar solvents (eg methanol, water) and analysed by GC after esterification. Solvent extracts of commodities were concentrated after addition of disodium hydrogen orthophosphate, and an aliquot added to acidified alcohols (several combinations of acids and alcohols were tested) in a sealed container. Formic acid esters were determined by GC, from headspace sampling over the esterification solvent. Esterification was faster with strong acids than with boric acid. However, esterification with boric acid/butanol gave the least interference of all tested methods. Product identity was confirmed by GC/mass spectrometry. High natural levels of formic acid, and low natural levels of ethyl formate and ethanol, presented problems in identifying residues arising from fumigation. These natural levels are relevant to food regulations for ethyl formate, especially those based on 'total formic acid, free and combined'. Polar columns (eg FFAP, carbowax) were useful for measurement of formic acid esters, which eluted before the alcohols used for esterification or extraction, whereas elution followed the molecular mass on non-polar columns, such as GS-Q or DB-624.
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Keywords: ethyl formate; formic acid; ethanol; analysis; residues; fumigant

1 INTRODUCTION

Although ethyl formate is widely used as a fumigant for dried fruit,[1,2] there are few data on the fate of ethyl formate during fumigation. It was judged desirable to obtain data on residues arising from such practices, and also to evaluate the potential of ethyl formate as a fumigant for cereals. This is part of a wider programme that seeks alternatives for methyl bromide, because of the phasing-out of that compound as an ozone-depleting substance. Jewell[2] states that ethyl formate 'disappears within a few hours' during fumigation of dried fruit in unlined containers, but 'more slowly' with linings 'as a result of hydrolysis to formic acid and ethyl alcohol.' However, no evidence is given to support this claim of hydrolysis to formic acid. Certainly, hydrolysis of ethyl formate to formic acid is readily achieved in the laboratory, although this hydrolysis is catalysed by alkaline or acid conditions, and proceeds relatively slowly at neutral pH. Muthu *et al*[3] measured ethyl formate vapour by GC in sorption studies; that is, the concentration of ethyl formate in the air over fumigated commodities was measured. The quantity of ethyl formate in the dried fruit (the residue) was measured by heating the fumigated commodity in an air stream for 30 minutes at 80 °C, trapping the released ethyl formate and determining its concentration by GC. Released formic acid was also trapped, and its concentration was determined by change in pH. No evidence was given to show that the method of residue determination was valid, eg that all the ethyl formate and formic acid was desorbed from the commodity. With regard to formic acid, in particular, it is unlikely that airing would fully desorb this polar compound from a commodity and it is certain that change in pH is not specific for formic acid, but applies to other volatile acids, such as acetic acid.

Residues of formic acid, as well as of ethyl formate, are important because a regulation[4] governing the use of ethyl formate on dried fruits states that 'total formic acid present, free and combined, in the finished product shall not exceed 250 parts per million'. This regulation would seem to be based on the assumption that natural levels of 'free and combined' formic acid are very much less than 250 parts per million (ppm or mg kg^{-1}). However, this assumption may not be valid,

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Contract/grant sponsor: Dried Fruit Research and Development Council
Contract/grant sponsor: Stored Grain Research Laboratory Agreement
(*Received 25 March 1998; revised version received 17 September 1998; accepted 24 April 1999*)

as formic acid (or formate ion, depending on pH) is an essential component of plant systems, and natural levels of formic acid can be very high, eg up to $4500\,mg\,kg^{-1}$ in corn roots.[5]

In addition to possible natural levels of formic acid, both ethyl formate and ethanol occur naturally in many foodstuffs. For example, ethanol has been detected as a volatile component of all tested cereals,[6] including wheat, maize, rice and barley. Ethyl formate is also present in many commodities, including rice,[7] barley products such as beer[8] and wheat products such as bread.[9]

The presence of natural levels of compounds that can also arise from fumigation would generate problems in identifying levels resulting from fumigation. This is especially true when natural levels approach, or even exceed, levels arising from fumigation. An official procedure[10] for analysis of the fumigant HCN in grain exemplifies one approach to this problem. In this approach, HCN and/or interfering substances are determined in both fumigated and unfumigated commodity. Residues of HCN are calculated from the amount of HCN and/or interfering substances in fumigated commodity less that in the unfumigated commodity.

There appears to be no validated procedure for determination of residues arising from the use of ethyl formate. There are also no quantitative data on the natural levels of ethyl formate, ethanol and formic acid in wheat, barley and sultanas. We therefore evaluated several procedures, and report here on the results. Analytical procedures were evaluated on sultanas, because of commercial fumigation of dried fruit with ethyl formate, and on wheat and barley, as part of a background study on the potential use of ethyl formate for grain fumigation. According to protocols[11] for determining 'aged' residues, of unknown amount, it is important to evaluate a variety of techniques as a protection against systematic errors from any single procedure. Ideally, different techniques should give identical results. Accordingly, concentrations of ethanol and ethyl formate in air were assessed using different GC conditions (columns and detectors); methods of determining ethanol, ethyl formate and formic acid residues used both different chromatographic conditions and different extraction procedures. These included both different solvents and sampling from both the solvent and from the headspace over the solvent. Headspace sampling was tested both to increase the conditions studied and because it causes less column contamination than does injection of solvent extracts.

2 EXPERIMENTAL METHODS

2.1 Materials

Analytical grades of ethanol, methanol, propanol, butanol, octanol, formic acid, ethyl formate, boric acid, sulfuric acid, nitric acid, and disodium hydrogen orthophosphate were used. Water was glass-distilled. Sultanas (4-crown), barley (malting grade) and wheat (Australian Standard White) had not been previously treated, post-harvest, with any chemical.

Commodities were extracted in 100-ml flasks equipped with Mininert valves (Alltech, Cat No 66121). Esterification was performed in 10-ml microflasks equipped with Mininert valves (Alltech, Cat No 9551). Measured volumes, which always exceeded nominal volumes, were used in calculations.

2.2. Replication and checks on reagent purity

All quantitative work was performed with at least three replicates, and all semi-quantitative or confirmatory tests were performed at least in duplicate. In studies on wheat and barley, results from different analytical procedures were obtained over a five-week period during which the rate of loss of these compounds was measured (*cf* Section 2.5.4). This procedure enabled results from different procedures to be compared over the range of residues that would be expected to result from commercial fumigations. The procedure also enables a comparison between different methods to be based on 'complete replication', in the sense that results were obtained at different times, with different standards, with different values of residues and with different intervals after application of ethyl formate.

With any new batch of any reagent, the total analytical procedure (excluding extraction of commodity) was performed to test for trace impurities that might interfere with the analysis.

2.3. Conditions for GC and GC/MS

Formic acid was analysed on a Varian 3600 GC, equipped with a thermal conductivity detector, after separation on a megabore FFAP column (J&W 125–3212). Column temperatures were 50 °C for 3 min, followed by temperature programming to 120 °C at $20\,°C\,min^{-1}$, and holding at this temperature for 5 min. In addition, the effluent gas from the thermal conductivity detector was passed into a flame ionisation detector, and the relative response from each detector was used as a check on the identity of the peak analysed, at the correct retention time.

Analyses of formate esters and of ethanol were conducted on a Varian 3600 GC, equipped with a flame ionisation detector, and after separation on megabore capillary columns. Four columns were used, with the choice of column dictated by the need to avoid interferences from commodity extracts. These columns were DB FFAP (J&W 125–3212) and FFAP (Alltech, Econocap, No 19688), BP-624 (SGE 71830) and GS-Q (J&W 115–3432). Column conditions were isothermal for analysis of formate esters, on the polar FFAP columns. Temperatures were 36 °C, for methyl and ethyl formate, 60 °C for propyl and butyl formate and 80 °C for octyl formate. Column conditions were isothermal at 130 °C for analysis of ethanol on GS-Q and involved a temperature programming, from 100 to 140 °C at $10\,°C\,min^{-1}$, for analysis of ethanol and ethyl formate on BP-624. In

addition, analysis of ethyl formate or ethanol in commodity extracts required further temperature programming, to 220 °C with a hold time of 15 min, to elute co-extractives from the column.

The identity of ethyl formate was also confirmed on a Shimadzu GC-6AM GC, after separation at 80 °C on a 2 m × 3 mm ID glass column packed with 20% carbowax on Chromosorb W 100–120 mesh.

Formic acid, after esterification to methyl formate, was also detected by gas chromatography/mass spectrometry, using a Finnigan Ion Trap Mass Spectrometer, after separation on a capillary column, DB-624 (J&W 122–1334). GC conditions were isothermal at 40 °C for 2 min, followed by temperature programming at 50 °C min^{-1} to 140 °C, at which temperature the column was held for 2 min. Quantification was performed from the intensity of characteristic ions at *m/z* 60 and 61. As a confirmatory check, formic acid was also derivatised to the propyl ester, which was identified by the molecular ion (*m/z* 88).

2.4. Analysis of ethyl formate and ethanol in air

Known quantities of ethyl formate or ethanol (each 1–10 µl) were injected through a septum into Erlenmeyer flasks (500–1000 ml, nominal capacity) containing several glass beads. The flasks were shaken. Dilutions of these standards were obtained by serial dilution into similar flasks of capacity 120–1000 ml. Air was sampled with an air-tight syringe (20 µl) and injected into the GC. Dose-response curves were prepared from six concentrations, including the control of no added compound. 'Sorption' studies (eg Muthu *et al*[3]) were based on a similar procedure, that is, relating concentrations of ethyl formate in the headspace over commodities to standards of ethyl formate in air.

To avoid possible confusion, it is useful to note that the word 'headspace' has two meanings in fumigant usage. In one meaning, the air above commodities during fumigation, eg the air at the top of a silo, is called the 'headspace'. In another usage, residues of volatile chemicals in commodities are often determined from the 'headspace' over commodities plus solvent, ie from the vapour phase concentration in equilibrium with that in the liquid phase (*cf* Section 2.5.3). In each usage of the 'headspace', compounds are measured in the vapour phase.

2.5. Ethyl formate and ethanol residues in commodities

2.5.1. Extraction conditions and fortification studies

Sultanas or wheat or barley (30 g) were extracted in sealed flasks for periods of 24, 48 and 72 h, in 60 ml of solvent, either methanol, propanol, propanol + water (80 + 20 by volume) or water. Standards were prepared by injecting known quantities (eg 1 or 2 µl) of ethyl formate or ethanol into the solvent, after the flasks had been sealed. For lower levels of fortification, the compound to be determined was first diluted in the extraction solvent. For example, where ethyl formate was extracted from commodities with methanol, a standard of ethyl formate was prepared by diluting 1 ml of ethyl formate with 9 ml of methanol, and aliquots of this diluted mixture (eg 3 µl) were injected into a sealed flask containing commodity plus solvent. Fortified samples were prepared at the same time (± 1 h) as solvent was added to samples for analysis. In GC determinations, replicates of fortified samples and unknowns were interspersed, to enable equivalent extraction conditions for unknown and fortified samples.

2.5.2 Analysis of extract

Ethyl formate and ethanol were determined by GC, after injection of an aliquot (1 µl) of solvent extract. Residues were calculated from the peak area, with reference to peak areas in a dose-response curve prepared from fortified samples.

2.5.3 Headspace analysis

An aliquot of the headspace over the extraction solvent (25–50 µl) was injected directly into the GC. Peak areas were compared with those in the headspace of the standards in Section *2.5.2*.

In analysis either by headspace sampling or by injection of solvent, fortified samples were prepared by injection of compound into the solvent. Residues were determined from both injection of the liquid and of the headspace at 24 and 48 h after addition of compound, in order to assess whether equilibrium partitioning of compounds between the liquid and vapour phases had occurred after 24 h.

2.5.4. Preparation of 'aged' residues

Ethyl formate was added to filter paper on top of commodity (wheat, barley or sultanas) in a sealed flask, at rates varying between 60 and 1000 mg kg^{-1}. Containers were glass vials, of capacity 120 ml, sealed with a Mininert valve, or Erlenmeyer flasks, of capacity 270 ml, capped with a ground-glass adaptor fitted with a septum. For each of the cereals, decay of ethyl formate was determined on commodity of different moisture contents (in the range 9–13%, w/w, wet weight basis) and stored at either 15, 25 or 35 °C. This procedure resulted in 'aged' samples similar to those which could cause residue problems in commercial usage. At timed intervals, the contents of flasks were analysed for residues, until residues of ethyl formate had decayed to low values (2–5 mg kg^{-1}). This time varied from less than one week (for sultanas) to five weeks (for wheat of 9% moisture content, wet weight, stored at 15 °C). Comparison of results from different methods of residue determination provided 'complete replicates', as discussed in Section 2.2.

2.6. Formic acid residues

2.6.1 GC analysis of formic acid

One method tested was similar to that used for ethyl formate in this paper. After extraction of commodities in either methanol or water, unpurified extract (1 µl)

was injected into the GC. Formic acid was determined from the response of a thermal conductivity detector, after separation on DB-FFAP. The response from the flame ionisation detector was simultaneously determined.

2.6.2 GC analysis after esterification

Another tested method involved derivatisation, after concentration, as described below. In experiments designed to measure only formic acid, wheat or barley (10 g) was ground in a Buehler mill with 80 ml of water, and shaken over a period of 4 h. Sultanas were cut in two, rather than ground, because of the difficulty in transferring ground sultanas from a grinder into a flask. The solvent extract was filtered, disodium hydrogen orthophosphate (0.1 g) was added to 50 ml of filtrate, which was concentrated to 1–3 ml (but not boiled dry), transferred to a 5 ml volumetric flask and diluted with distilled water to 5 ml. Sultana extracts were concentrated to 10 ml, because the large amount of extracted sugar prevented concentration to 5 ml. Aliquots (0.3 ml) were added to Microflasks, the liquid was evaporated in an oven at 130 °C, and an acidified alcohol (1 ml) added. Acidified alcohols tested were 2% v/v strong acid (sulfuric, hydrochloric or nitric) in an alcohol (methanol, propanol, butanol or octanol) or boric acid (40 g litre^{-1}) in butanol. After sealing, the Microflask was kept at 60 °C for at least 4 h and samples (10 µl) were injected into the GC from flasks at this temperature. The GC response was compared with that from standards made from derivatising formic acid, eg by adding 10, 20, 40 and 80 µl of a solution of 5 mg ml^{-1} of formic acid in 0.2 M disodium hydrogen orthophosphate to flasks and drying and derivatising as described for residue analysis. Standards were prepared and analysed with the unknowns.

To determine whether the procedure would detect ethyl formate as formic acid, 1 ml of methanol containing 200 µg of ethyl formate was substituted for 1 ml of solvent extract, and the procedure otherwise followed as described.

2.6.3 Confirmation of esterification by GC/MS

After esterification of sultana extracts with methanol and, separately, with propanol, as described in *Section 2.6.2*, the headspace was injected into a GC/MS system. Retention times and mass spectra from commodity extracts were compared with those from methyl and propyl formate.

2.6.4 Semi-quantitative colorimetric test for formic acid

Formic acid was also determined by an adaptation of a procedure of Feigl.[12] Commodity extract in propanol (5 ml) plus disodium hydrogen phosphate (5 ml) was boiled dry, and residue was dissolved in 2 M hydrochloric acid (5 ml). An aliquot (2 ml) was heated at 60 °C for 10 minutes with 12 M sulfuric acid (4 ml), plus a little chromotropic acid, to detect formaldehyde and other interferences. Magnesium (approximately 0.1 g) was added to aliquots (0.2–2 ml) of the residue in 2 M hydrochloric acid, and left until the evolution of nascent hydrogen had ceased. These solutions were then heated at 60 °C for 10 min. with chromotropic acid in 12 M sulfuric acid (4 ml). Quantification was by visual comparison of the violet colour with that formed from formic acid standards in water, which were diluted into 2 M hydrochloric acid immediately prior to assay.

2.7 Recoveries from spiked samples

Ethanol or ethyl formate (32, 16, 8, 4 and control of 0 µl) were added to wheat or barley or sultanas (30 g) in sealable containers, corresponding to residues in the commodities in the approximate range 0–1000 mg kg^{-1}. Solvent, eg methanol (60 ml) was added, the container sealed and shaken, and left for 24 h before analysis. A dose-response curve was drawn and compared with that of standards in extraction solvent.

2.8 Studies on completeness of extraction

Two procedures, based on the criteria of Sandall[11] for 'aged' residues, were used to test completeness of extraction. In one procedure, used for unground commodity, the amount of chemical in the extraction solvent was determined after extraction periods of 4, 8, 24 and 36 h, to determine the time required for complete extraction. For determination of extraction of formic acid into water from ground wheat or barley, extraction periods were studied over the interval 0.5–28 h. In another procedure, residues were determined using different methodologies, and results were compared. For example, residues of ethyl formate were determined after extraction into both methanol and aqueous propanol, and from determination of amounts in both the headspace and in the supernatant. Residues of formic acid were determined after extraction in methanol and extraction in water.

3 RESULTS AND DISCUSSION

3.1 Analysis of ethyl formate and ethanol in air

The limits of detection of ethyl formate in purified air were measured on three detectors, a flame ionisation detector, a thermal conductivity detector and an ion trap detector, using the molecular ion. The limits of detection were lowest with the flame ionisation detector at <1 µg litre^{-1}. The response of the flame ionisation detector to ethyl formate, methyl formate and ethanol in air was linear over the tested range of 0–500 mg litre^{-1}, after separation on either DB-FFAP, BP-624 or GS-Q. The order of elution differed, with ethanol having a shorter retention time than ethyl formate on BP-624 and GS-Q but a longer retention time on each of the polar FFAP columns. The limit of detection was set by background interferences and/or natural levels, but was less than 0.1 mg litre^{-1}.

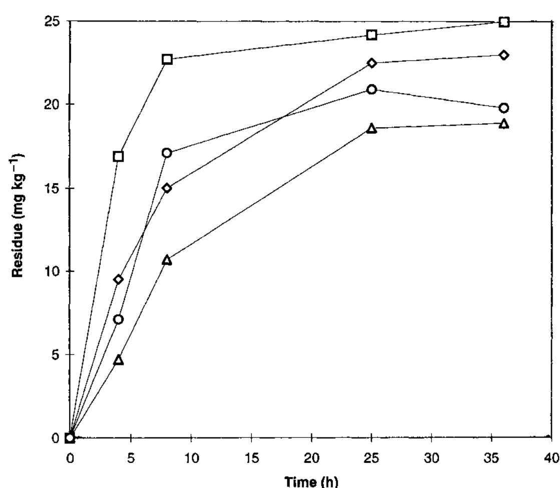

Figure 1. Time to extraction of ethyl formate: (◇) from wheat with MeOH; (□) from wheat with aqueous PrOH; (△) from sultanas with MeOH; (○) from sultanas with aqueous PrOH.

3.2 Analysis of ethyl formate and ethanol residues

3.2.1 Time to complete extraction of ethyl formate in three solvents

Time to complete extraction was tested in three solvents, methanol, water and propanol + water (80 + 20 by volume). Some solvents, such as aqueous acetone, which is used in an AOAC multi-residue procedure,[13] could not be tested because of interference. The choice of 80 + 20 propanol + water, rather than propanol, as an extraction solvent was based on previous work that showed that propanol extracted less residues of ethyl formate than did methanol. The effect of extraction period on amount of residues of ethyl formate extracted from wheat and sultanas is shown in Fig 1. Residues were determined from the headspace concentrations over each of three solvents. Residues were calculated from the ratio of concentrations in unknowns to those in fortified samples. Each organic solvent extracted similar amounts of ethyl formate, eg 22–25 mg kg^{-1} on wheat (Fig 1).

To test the stability of ethyl formate in different solvents, headspace concentrations over samples of wheat or barley or sultanas freshly fortified with 32 mg kg^{-1} were also compared with those over solvents (50 ml) fortified with the same mass of ethyl formate, but not containing commodity. The ratio of headspace samples over commodity plus aqueous propanol to that over pure solvent remained constant (±5%) over the tested period (4–48 h after fortification). However, ethyl formate slowly degraded in pure methanol, with an average loss of 14% over a period of 48 h at room temperature, whereas it was stable in commodity extract, ie in the supernatant liquid after extraction of unfumigated commodity in methanol. The headspace of ethyl formate over sultanas in water also remained constant, relative to that over water. However, the headspace of ethyl formate over wheat or barley plus water declined relative to that over water. The probable explanation for this loss is enzymatic activity in the aqueous extract of wheat. This is analogous to a previous study[14] which showed that ethyl formate was stable in water, but rapidly decayed in human blood *in vitro*.

In summary, similar results were obtained from each solvent if results from unknowns were standardised against fortified samples, but not if they were standardised against solutions of ethyl formate in pure solvent, except where that solvent was aqueous propanol (or water, in the case of sultanas).

Table 1. Ratio of residues of ethyl formate from five extraction conditions to residues from headspace analysis after 48 h extraction[a]

Commodity	*Solvent*	*Extraction period (h)*	*Method*	*n*	*Ratio (mean)*	*Ratio (SD)*
Wheat	MeOH	24	Headspace	14	0.80	0.14
Wheat	MeOH	48	Headspace	[b]	1.00[b]	[b]
Wheat	MeOH	24	Liquid	14	0.76	0.14
Wheat	MeOH	48	Liquid	14	0.96	0.13
Wheat	80% PrOH	24	Headspace	4	0.91	0.01
Wheat	80% PrOH	48	Headspace	4	0.99	0.04
Barley	MeOH	24	Headspace	9	0.99	0.14
Barley	MeOH	48	Headspace	[b]	1.00[b]	[b]
Barley	MeOH	24	Liquid	9	0.97	1.04
Barley	MeOH	48	Liquid	9	1.04	0.04
Sultanas	MeOH	24	Headspace	4	0.92	0.06
Sultanas	MeOH	48	Headspace	[b]	1.00[b]	[b]
Sultanas	80% PrOH	24	Headspace	4	0.93	0.08
Sultanas	80% PrOH	48	Headspace	4	1.04	0.09

[a] Residues were in the range 7–134 mg kg^{-1} on wheat, and 17–52 mg kg^{-1} on barley and were determined on wheat 7–56 days after dosing with ethyl formate. Each replicate was fully independent, ie determined on different days, with different standards. Residues on sultanas were 19 mg kg^{-1}, two days after fumigation.

[b] The ratio is unity, by definition and the number, *n*, and the standard deviation are tabulated under each method of analysis, related to the reference method.

3.2.2 Results from headspace and liquid samples, and from different extraction periods

The amount of ethyl formate obtained from different extraction conditions is summarised in Table 1, where the amount extracted by one procedure is expressed as a ratio of the amount extracted by another procedure. This comparative methodology enables results obtained over a period of five weeks to be summarised. The reference procedure (given a value of unity) was arbitrarily chosen as headspace sampling over methanol, after an extraction period of 48 h. Extraction of ethyl formate in methanol or aqueous propanol for 24 h resulted in residues close to, but less than, those obtained after 48 h extraction (e g ratios of 0.80, 0.76 for wheat, 0.99, 0.97 for barley; Table 1, methanol as solvent). Determination of ethyl formate by analysis of the headspace gave results almost identical to those obtained by analysis of the liquid, for the same extraction period (Table 1).

The amount of ethanol extracted from commodities with propanol after 24 h averaged 85%, standard deviation 6%, of that obtained after 48 h. Analysis of ethanol from injection of the solvent was found to be more sensitive than analysis from headspace sampling, presumably because of the high solubility of ethanol in propanol. Methanol was not suitable for determination of low residues of ethanol, because of interference in the GC. Water was also not suitable, because of fermentation which produces ethanol.

It is concluded that extraction in methanol or aqueous propanol for at least 24 h, and preferably for 48 h, is required to maximise extraction of ethyl formate and that 48 h extraction in propanol is required to maximise extraction of ethanol. Injection of a sample from either the headspace or the solvent is suitable for residue analysis of ethyl formate, but injection of a sample from the solvent is required for determination of low amounts of ethanol.

3.2.3 Dose-response curves and recovery from fortified samples

Dose-response curves for ethyl formate and ethanol added to commodity, either wheat or barley or sultanas, plus solvent were linear ($r^2 = 0.99$) for the tested range of 0, 15, 30, 60, 120 and 240 mg litre^{-1}. This concentration range corresponds to residues in the range 0–120 mg kg^{-1} (for the case of 30 g of commodity extracted in 60 ml of solvent). Although the curves were linear, they did not pass through the origin and there were apparent natural levels and/or interferences corresponding to residues in the range 0.006–4 mg kg^{-1}. These ‘interferences’ are discussed in Section 3.2.4.

Recovery of freshly added ethanol and ethyl formate from wheat and sultanas (‘spiked samples’), at the tested level of 100 mg kg^{-1}, and averaged from five replicates, was in the range 98–102 mg kg^{-1}, with standard deviations in the range 2.2–3.1 mg kg^{-1} (Table 2).

Table 2. Recoveries of ethanol and ethyl formate on sultanas and wheat, added at 100 mg kg^{-1}

	Recovery on wheat		*Recovery on sultanas*	
Chemical	*Mean*	*SD*	*Mean*	*SD*
Ethanol	101	2.2	99	2.2
Ethyl formate	102	3.1	98	2.8

3.2.4. Limits of detection, ‘interfering substances’, and in-situ esterification

Analysis of ethyl formate and ethanol on unfumigated wheat, barley and sultanas revealed natural levels of these compounds (and/or interfering substances). It was possible to estimate these natural levels from the peak area in unfumigated grain with that in grain fortified with known amounts of compound. Thus, if the peak heights of fortified and unfortified samples are P_f and P_u respectively, the fortification mass is f and the natural mass is n', the natural mass of compound in a given sample of commodity was calculated from the equation $P_f/P_n = (f+n)/n$. The apparent natural levels, calculated as mg kg^{-1}, are summarised in Table 3.

Natural levels of ethanol and/or interfering substances were low in wheat (<0.01 mg kg^{-1}), barley (<0.01 mg kg^{-1}) and sultanas (0.1 mg kg^{-1}). Because wet cereals and sultanas can ferment, one would expect higher natural levels on occasions. The low natural levels (Table 3) establish the current limit of quantification by the analytical procedure.

Apparent natural levels of ethyl formate and/or interfering substances were higher after extraction in methanol than in other solvents, even though the same solvents gave comparable results for higher residues of ethyl formate resulting from fumigation (Fig 1). In addition, the amount of apparent ethyl formate increased continuously with extraction in a manner different from that shown in Fig 1. We postulated that the higher levels from extraction with methanol were due to an artefact, methyl acetate, formed from the reaction of the solvent with acetic acid, extracted from the commodity. We were unable to separate methyl acetate from ethyl formate in any tested GC system, and the two esters have identical molecular masses and similar polarities. To test the possible formation of methyl acetate, acetic acid (0.5 ml) and also formic acid (0.5 ml) were added to methanol, and the headspace sampled over a 48-h period. There was an increase in peaks with the same retention times as methyl formate and methyl acetate/ethyl formate, on two tested GC systems (FFAP and Carbowax). In addition, wheat, barley and sultanas (each 30 g) were extracted in 60 ml of a solution of 5 g litre^{-1} phosphoric acid in methanol + water (95 + 5 by volume). The quantities of methyl formate and methyl acetate in the headspace over wheat and barley increased over a period of 6–8 h, relative to extraction in methanol without an acid catalyst. Extraction of

Table 3. Apparent residues of ethanol and ethyl formate in unfumigated commodities

Commodity	*Compound*[a]	*Solvent*	*Method*	*Extraction period* (*h*)	*n*	*Residue* ($mg\,kg^{-1}$)	
						Mean	*SE*
Wheat	EtF	MeOH	Headspace	24	6	0.08	0.07
Wheat	EtF	MeOH	Headspace	48	6	0.7	0.3
Wheat	EtF	MeOH	Liquid	24	6	0.5	0.2
Wheat	EtF	MeOH	Liquid	48	6	0.9	0.3
Wheat	EtF	PrOH	Liquid	24	6	0.006	0.0006
Wheat	EtF	PrOH	Liquid	48	6	0.006	0.0004
Wheat	EtF	EtOH	Headspace	48	6	<0.08	na[b]
Wheat	EtF	80% PrOH	Headspace	48	4	<0.08	na[b]
Wheat	Ethanol	PrOH	Liquid	24	6	0.006	0.009
Wheat	Ethanol	PrOH	Liquid	48	6	0.006	0.0004
Barley	EtF	water	Headspace	4	6	1.1	0.3
Barley	EtF	80% PrOH	Headspace	48	4	1.2[c]	0.2
Barley	EtF	MeOH	Headspace	24	9	1.7	0.6
Barley	EtF	MeOH	Headspace	48	9	2.7	0.8
Barley	EtF	MeOH	Liquid	24	9	2.4	0.1
Barley	EtF	MeOH	Liquid	48	9	3.6	0.5
Barley	Ethanol	PrOH	Liquid	24	4	<0.01	na[b]
Barley	Ethanol	PrOH	Liquid	48	4	<0.01	na[b]
Sultanas	EtF	80% PrOH	Headspace	48	4	<0.08	na[b]
Sultanas	EtF	water	Headspace	4	4	<0.08	na[b]
Sultanas	EtF	MeOH	Headspace	48	4	1.0	0.1
Sultanas	EtF	MeOH	Liquid	48	4	1.1	0.1
Sultanas	EtF	80% PrOH	Headspace	24	4	<0.08	na[b]
Sultanas	EtF	80% PrOH	Headspace	48	4	<0.08	na[b]
Sultanas	Ethanol	PrOH	Liquid	48	3	0.11	0.4

[a] EtF = ethyl formate;.
[b] na not applicable.
[c] The level was reduced to 0.13, standard error 0.025 $mg\,kg^{-1}$, after storing barley at 40 °C.

wheat in ethanol resulted in peaks that co-chromatographed with ethyl formate and ethyl acetate, but not with methyl formate.

Thus extraction of wheat, barley or sultanas with an alcohol results in some esterification of formic and acetic acids. Extraction with methanol results in methyl acetate, which interferes with the determination of ethyl formate.

The procedure of extracting commodity in alcohols, especially acidified alcohols, demonstrated the presence of formic acid and acetic acid in commodities, but we were unable to develop it into a quantitative method for estimation of these acids.

Natural levels of ethyl formate in barley were higher than in wheat or sultanas (Table 3). We postulated that the difference in levels between wheat and barley was due to higher natural levels of ethyl formate in the barley, which could be reduced by storage at higher temperatures. We therefore stored barley at 40 °C. After storage for 10 weeks, residues of ethyl formate and/or interfering substances, after 48 h extraction in propanol + water (80 + 20 by volume) had declined to 0.13 ($\pm$0.025) $mg\,kg^{-1}$. Thus the limit of detection of ethyl formate in wheat, barley and sultanas is low (approximately 0.1 $mg\,kg^{-1}$). However, for ethyl formate as for ethanol, there is evidence for a variation in natural levels, such that it would be difficult to describe low values of either compound as residues resulting from fumigation.

3.3 Discussion on analysis of ethyl formate and ethanol

Analysis of ethyl formate and ethanol in air was straightforward. Analysis of these compounds in commodities was also relatively straightforward, except where the level of interference in unfumigated commodities was significant relative to the residue to be determined.

The amount of biogenic ethanol and/or interfering compounds was low in wheat, barley and sultanas, for extraction with propanol. Such levels are unlikely to be of toxicological significance in most situations. Because ethanol has been found in all cereals tested,[6] levels of <10 $\mu g\,kg^{-1}$ (Table 3) are surprisingly low. However, the literature procedure used to measure ethanol[6] involved purging volatiles from a suspension of cereals in water, and it is possible that some fermentation occurred during this process.

'Background' levels of ethyl formate in wheat and sultanas were <0.1 $mg\,kg^{-1}$ but levels in barley were close to 1 $mg\,kg^{-1}$, except where barley had been stored at higher temperatures, when the background level was reduced to <0.2 $mg\,kg^{-1}$. This conclusion is based on the similarity of results from a variety of

extraction conditions (eg, from two solvents, and from at least two extraction periods). The apparent increase in background levels of ethyl formate resulting from methanol extraction are attributed to methyl acetate, formed from esterification of acetic acid with methanol.

Background levels of ethanol and ethyl formate, in all commodities, were low in comparison with the application rate of ethyl formate, which is in the range 322–564 mg kg^{-1} on raisins.[3] If ethyl formate is, as stated,[2] hydrolysed to formic acid and ethanol, background levels of ethanol and ethyl formate would be insignificant compared to residues arising from fumigation.

It is possible that natural levels of both ethyl formate and ethanol vary in individual commodities. The low background level of ethanol (and/or interference) in wheat, barley and sultanas and of ethyl formate (and/or interference) in wheat and sultanas establishes a low, but finite, level of interference arising from the method. We were unable to determine the composition of these interferences by GC/MS, because of lack of sensitivity.

Aqueous propanol is a suitable solvent for determination of low levels of ethyl formate in barley, wheat and sultanas, because of completeness of extraction and because of low levels of interferences. However, methanol has some advantages. First, it is cheaper than propanol. Second, with methanol as solvent, ethyl formate can be determined as part of a multi-residue screening procedure, as methanol has been widely tested as a solvent for extraction of many pesticides from foodstuffs.[15] It is also possible to determine ethyl formate residues from the difference in 'residues' from fumigated and unfumigated wheat, barley or sultanas, in a similar procedure to that used for determination of HCN residues in commodities.[10]

3.4 Natural levels of formic acid before fumigation

3.4.1 Amounts from GC analysis of formic acid without derivatisation

The peak area of underivatised formic acid in pure solvent was linear in the tested range of 0–2000 mg litre^{-1}, corresponding to 0–1000 mg kg^{-1} in commodity. However, the chromatogram of extracts of sultanas in either water or methanol had a low signal-to-noise ratio, such that the detection limit, with the thermal conductivity detector, was between 20 and 50 mg kg^{-1}, depending on conditions. The response of the flame ionisation detector was even less sensitive.

Extracts of unfumigated sultanas in methanol, water or propanol, contained a peak which had the same retention time as formic acid, and showed the same ratio of response to the two detectors, the flame ionisation and the thermal conductivity detector. The apparent level of formic acid in unfumigated sultanas, averaged from the response to two detectors, was determined as between 800 mg kg^{-1} and 1200 mg kg^{-1}.

It was concluded that analysis of underivatised formic acid was unsuitable for determination of low residues of formic acid, because of lack of sensitivity. The apparently high natural level of formic acid in unfumigated sultanas also required further confirmation.

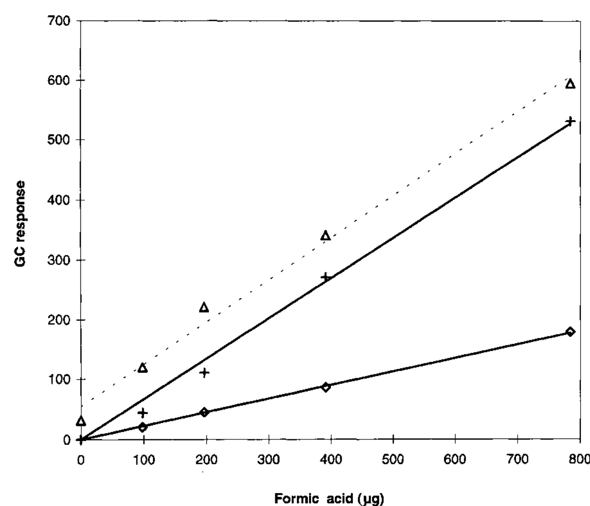

Figure 2. Response of the GC to headspace concentrations of butyl formate versus added formic acid, for three methods of esterification at 60 °C: (△) 48 h esterification with 2% nitric acid/butanol (v/v); esterification with 40 g litre^{-1} boric acid in butanol (◊) overnight and (+) for 96 h. The GC response is area/100 for boric acid catalysis and area/1000 for nitric acid catalysis.

3.4.2 Procedures for esterification of formic acid

Dose-response curves for esterification of formic acid in water, under three conditions, are shown in Fig 2. These conditions were esterification with butanol, using either nitric acid or boric acid (with two periods of esterification) as catalyst. In each case, the dose-response curve was linear. Esterification was more rapid with nitric acid than with boric acid (Fig 2), but the dose-response curve did not pass through the origin. In contrast, the dose-response curve with boric acid as catalyst passed through the origin, although more time was required for complete esterification.

In other experiments, it was shown that esterification with 2% strong acid (nitric, sulfuric or hydrochloric) was essentially complete after 4 h at 60 °C. At this time, the interference (ie apparent ester in solvent containing no added formic acid) was low (<1 µg ml^{-1}), but the interference increased if the esterification flasks were left for longer periods. In contrast, interference from esterification with boric acid remained below 1 µg ml^{-1} over a period of 96 h.

In experiments to determine optimal time for esterification of formic acid extracted from wheat with water, the amount of butyl formate formed was determined after esterification for 48 and 72 h. Residues were calculated from the ratio of butyl formate over commodity extract to that over standard. Results were averaged from 19 'complete' replicates, performed at different times with different standards. Residues determined after 48 h esterification with 40 g litre^{-1} boric acid averaged 99 (±17)% of those formed after 72 h. Thus the ratio of butyl formate formed in

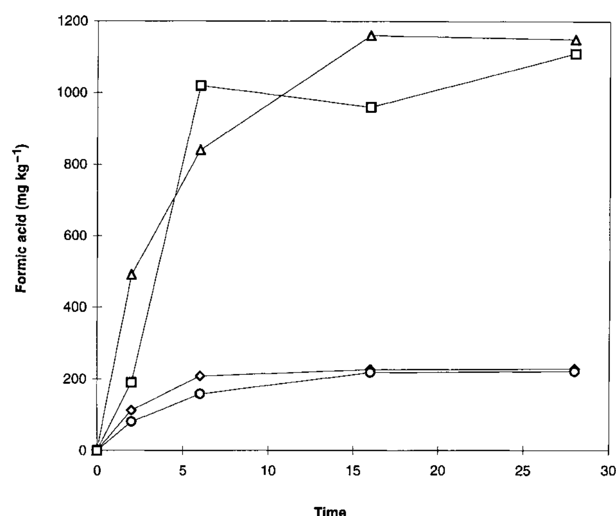

Figure 3. Time to extraction of formic acid from wheat and sultanas: (△) sultanas in water; (□) sultanas in MeOH; (◇) wheat in MeOH; (○) wheat in water. The unit of time is hours, for extraction in MeOH, and 0.25 h, for extraction in water.

commodity extracts to that formed in standards of formic acid was constant over the tested period of 24–48 h.

3.4.3 Completeness of extraction

The amount of formic acid extracted from sultanas and ground wheat into each of methanol and water is shown in Fig 3. In these studies, esterification was performed with boric acid and butanol. In each case, the amount of recovered formic acid reached a constant value which was not increased by extending the extraction period. Levels found after extraction with methanol were similar to those after extraction with water, though formic acid was extracted more quickly into water than into methanol.

3.4.4 Natural levels of formic acid in wheat, barley and sultanas

Levels of formic acid in unfumigated wheat, barley and sultanas are summarised in Table 4. There was no significant difference between results from the three different procedures used on wheat and sultanas. The coefficient of variation averaged 8.7% of the mean value for methods involving esterification. It is concluded that natural levels of formic acid in wheat and barley range between 200 mg kg^{-1} and 300 mg kg^{-1} and levels in sultanas are even higher. These natural levels are high, with respect to a food regulation that total formic acid should not exceed 250 parts per million,[4] but they are lower than those found in some other plant constituents, eg corn roots.[5]

3.4.5 Confirmation of results from esterification

Analysis using GC-MS of the sultana extracts after derivatisation with methanol showed a peak which, from its mass spectrum, was assigned as a mixture of methyl formate and formic acid. As formic acid does not itself elute on the column used, DB-624, and the peak displayed the same retention time as methyl formate, the acid must be formed by conversion of methyl formate in the ion trap. Esterification with propanol resulted in a peak which was assigned from its retention time and mass spectrum as propyl formate.

Attempts to quantify ethyl formate by GC/MS were abandoned because the limit of quantification exceeded 10 mg kg^{-1}. It is possible that the limit of quantification might be lower in other systems, eg those using chemical ionisation.

In experiments where commodity extracts of wheat, sorghum, barley, lupins and paddy rice were derivatised separately to four esters, and analysed by GC, the ratio of residues to the butyl ester taken as 1.0 was 0.89 for the methyl ester, 1.39 for the propyl ester and 1.07 for the octyl ester. These results confirm the identity of formic acid in commodities.

3.4.6 Semi-quantitative colorimetric test for formic acid

For formic acid in water, a violet colour was formed after reduction with nascent hydrogen and addition of chromotropic acid in 12 M sulfuric acid. Absorbance at 480 nm was proportional to concentration over the tested range of 10 to 500 µg of added formic acid. The method was unsuitable for sultanas, because the extract plus 12 M sulfuric acid, both in the presence and absence of nascent hydrogen, was intensely coloured, such that any violet colour formed from the reaction with chromotropic acid could not be observed. With wheat and barley, no colour was formed with acidified chromotropic acid in the absence of nascent hydrogen, indicating absence of formaldehyde and other interfering substances. However, a violet colour was formed after the reaction with nascent hydrogen, indicating the presence of formic acid in wheat and barley.

Table 4. Natural levels of formic acid in unfumigated wheat, barley and sultanas

Commodity	*Solvent*	*Extraction period (h)*	*Mean level (mg kg^{-1})*	*Standard deviation (mg kg^{-1})*	*Method of analysis*
Wheat	Water	4	197	22	Esterification with butanol/boric acid
Wheat	Water	4	243	28	Esterification with methanol/sulfuric acid
Wheat	MeOH	24	225	19	Esterification with butanol/boric acid
Barley	Water	6	237	48	Esterification with butanol/boric acid
Sultanas	PrOH	48	1050	208	Analysis of underivatised acid
Sultanas	MeOH	48	1243	92	Esterification with butanol/boric acid
Sultanas	Water	6	1180	102	Esterification with butanol/boric acid

3.5 Determination of residues of formic acid resulting from fumigation

3.5.1 Tests for interference from ethyl formate

In experiments where 200 µl of ethyl formate in methanol was subjected to the process used for derivatisation, no formic acid was detected as methyl formate, at a detection limit corresponding to 1 mg kg^{-1} in the commodity. Thus detectable levels of formic acid are not formed from hydrolysis of ethyl formate during the process of derivatisation. It is presumed that ethyl formate was removed by vaporisation during the work-up before it was hydrolysed.

3.5.2 Dose-response curves for added formic acid

Dose-response curves for wheat, barley and sultanas fortified with 0.5, 1, 1.5 and 2.0 times natural levels of formic acid were linear ($r^2 > 0.98$) for the tested procedure. This was 4-h extraction of ground commodity in water, esterification with butanol/boric acid, and taking the mean of triplicate determinations.

3.6 Discussion on measurement of formic acid

Analysis of formic acid in commodity extract was difficult, for three principal reasons. First, there is no GC detector that exhibits specific sensitivity to this compound, and the response of general detectors, such as the flame ionisation detector, to this compound is very low. Second, the mass spectra of formic acid and its esters are difficult to use to measure low levels of compounds, because of interference in quantification of the daughter ion, formic acid, caused by the presence of carbon dioxide. The third reason for the difficulty in analysis of formic acid in fumigated commodities is the presence of significant quantities of this compound in unfumigated sultanas, barley and wheat. The presence of formic acid was shown by co-chromatography of the acid, by the relative response of two detectors, by co-chromatography of four derivatised esters and from the mass spectra of methyl and propyl formate. The presence of formic acid in wheat was also confirmed by a semi-quantitative spot test, which relied on reduction with nascent hydrogen to formaldehyde, and a characteristic reaction of this compound. The methodology based on derivatisation proved suitable for the determination of formic acid in commodities, but the natural high level of formic acid makes the determination of any extra formic acid, resulting from fumigation, impractical where values resulting from fumigation are low, relative to natural levels.

4 CONCLUSIONS

The methodology for determining methods of analysis of residues was based on criteria[11,15] for validating methods of determining 'aged' residues. First, it was shown that recovery of fortified samples was adequate. It is necessary to obtain good recovery of fortified samples, but such good recovery of 'fresh' samples does not always result in good recovery of 'aged' samples. Second, it was shown that similar results from aged residues were obtained from different procedures.

For routine determination of residues of ethyl formate, and other formate esters, the polar FFAP columns are recommended, because the ester is detected before the more abundant alcohol peak.

The methods of analysis facilitate a proposed study on the fate of residues arising from fumigation with ethyl formate. The high natural levels of formic acid in wheat, barley and sultanas are relevant to the regulation[4] that 'total formic acid present, free and combined, in the finished product shall not exceed 250 parts per million'.

ACKNOWLEDGEMENTS

The Dried Fruit Research and Development Council and the partners to the Stored Grain Research Laboratory Agreement are thanked for financial assistance.

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