Final Report

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by

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Development of methods for the analysis of antioxidants in a range of foods

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EXECUTIVE SUMMARY

This project aimed to develop a method or methods for the reliable quantitation of a selection of synthetic antioxidants in the foods in which they are permitted. Antioxidants are added to foods in order to prevent deterioration of the foods through atmospheric oxidation. The antioxidants studied in this project were butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), octyl gallate (OG) and dodecyl gallate (also known as lauryl gallate, LG). These antioxidants are permitted in a limited range of foods, and at well-defined levels, shown in Table 1. There is a need for accurate methods for the measurement of these antioxidants in foods both for regulatory enforcement and for monitoring of the intake of additives by the population. Methods developed must be suitable for use in a general routine or commercial laboratory. Therefore methods must be robust, and ideally use non-specialist equipment.

Initial experimental work was carried out on commercially available samples containing antioxidants, as specified on their labels, but where the level was unknown. A range of different extraction procedures was used, and the antioxidants determined by High Performance Liquid Chromatography (HPLC). Levels of antioxidant found using different extraction methods were compared in order to gain an idea of the methods most suitable to carry forward for further development. Two types of extraction procedure were tested: (i) those that directly extract the antioxidant from the food and (ii) those that extract the fat from the food, followed by extraction of the antioxidant from the fat. It was found in general that the fat extraction methods were too harsh, and caused greater losses of antioxidants than the direct extraction methods. Of the direct extraction methods, one method was found to be suitable only for BHT, therefore was not selected for further development. A cold extraction method, using diethyl ether, was selected for further development.

Foods with known amounts of added antioxidant were prepared at Leatherhead Food International (LFI), in order to properly assess the recovery of antioxidants from foods. The cold extraction method was tested and optimised using these foods. Following this optimisation, ring trial samples were prepared and sent to external laboratories, with detailed protocols of the method to be used, in order to test the reproducibility of the method in other laboratories. Results from the laboratories were inconsistent, for a number of reasons, and therefore cannot be used as validation for the method to be put forward as a standard method. One laboratory had not used the method supplied to them, due to the length of time that would have been required, and had instead used an in-house method, therefore their results cannot be used to assess the method developed here. However, this is perhaps an indication that the method developed would not be attractive to a commercial laboratory or suitable for use as a routine method.

In addition to the use of traditional wet chemistry techniques for the extraction of antioxidants from foods, the project also aimed to develop molecular imprinted polymers against some of the antioxidants under consideration. Molecular imprinted polymers (MIPs) are polymers produced around a template molecule (in this case either BHA, BHT or propyl gallate). The template molecule is then removed by washing, leaving imprints in the polymer that will specifically retain an analyte that is structurally the same as the template molecule. Polymers were produced against

BHA, BHT and PG. A control polymer was also prepared, where the same ingredients were used as for the imprinted polymers, except that no template molecule was added. The effect of imprinting was assessed by packing each polymer into an HPLC column, and measuring the extent to which the polymer retained the analyte. This was compared to the retention of the same analyte by the control polymer. It was found that each of the MIPs retained BHA, BHT and PG to a greater extent than the control polymer. However, the MIPs did not show selective retention of their own imprint molecule. This demonstrated that, although imprinting had taken place, the polymers were not specific, and so could not be used for selective clean up of their corresponding template analyte.

For the MIP phase of the project, work was carried out in collaboration with Technical University Berlin (TU Berlin) who have significant experience of this technology. Polymers were produced there, and initial evaluations carried out before the technology was transferred to LFI. Work was carried out at both institutions to assess the possibility of using the polymers for solid phase extraction, but the MIPs were not found to be suitable for this use.

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INTRODUCTION

Antioxidants are added to foods to prevent deterioration of the food by atmospheric oxidation. This is particularly important in oils and fats, and in foods containing oils and fats, to prevent rancidity developing on storage. Rancidity is caused by oxidation of unsaturated fatty acids in the triglycerides which fats and oils consist of. There are a variety of antioxidants, many naturally occurring, and the antioxidants allowed as additives consist of both natural and synthetic antioxidants. According to the Food Regulations, an antioxidant is defined as any substance that is capable of delaying, retarding or preventing the development in food of rancidity or other flavour deterioration due to oxidation. The antioxidants of interest in this project were the synthetic antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), octyl gallate (OG) and dodecyl gallate (also known as lauryl gallate, LG). Structures are shown in Figure 1 to Figure 3. Octyl gallate and dodecyl gallate have structures analogous to propyl gallate, differing only in the length of the side chain.

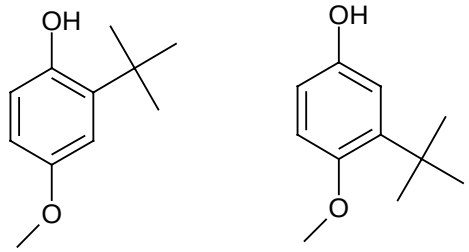

Figure 1 : Structure of BHA

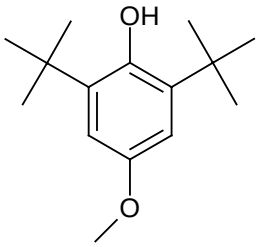

Figure 2: Structure of BHT

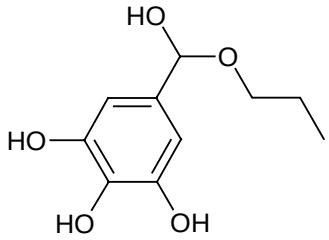

Figure 3: Structure of propyl gallate

There are reliable methods for the determination of antioxidants in edible oils and fats, but these are not immediately applicable to other foods. This project aimed to develop a method or methods for the reliable quantitation of a selection of synthetic antioxidants in the foods in which they are permitted. There is a need for accurate methods for the measurement of these antioxidants in foods both for regulatory enforcement and for monitoring of the intake of additives into the population. These antioxidants are permitted in a limited range of foods, and at well-defined levels, shown in Table 1 as laid down by the Miscellaneous Food Additive Regulations 1995 (as amended)

Extraction of the antioxidant or mixture of antioxidants from some foods is not straightforward as the antioxidant may be encapsulated or bound within the food

matrix. Furthermore, some methods of liberating antioxidants may actually lead to their destruction or loss. For these reasons, recovery of an antioxidant from the food matrix may not be 100%, and it is important to identify this and quantify any under recovery. The usual way to determine recovery of an analytical method is by adding a known amount of the analyte, generally in a solvent in which it is readily soluble, to the sample. This 'added' analyte then goes through the extraction procedure, is quantified and the recovered amount compared with the amount added in order to give % recovery. This is useful in determining losses due to extraction, especially in the case of analytes such as antioxidants which are likely to degrade easily due to light, heat, presence of oxygen or other pro-oxidants. However, this method does not assess the effectiveness of the extraction method in recovering the analyte from within the food matrix. Effectiveness of extraction can only be assessed by determining the recovery of the analyte after it has in some way been incorporated into the food matrix at a known level. It was proposed to liaise with manufacturing companies in Membership of Leatherhead Food International (LFI) to obtain specially manufactured samples of relevant foods with known levels of antioxidant addition. However, given that the majority of manufacturers contacted no longer use artificial antioxidants in their products, it was not possible to obtain such samples. Some food products with known amounts of added antioxidants were therefore produced at Leatherhead. In this way, the results of the analysis could be compared with the known levels of antioxidant added during processing. This would enable assessment of recovery of antioxidants that may have become bound into components of the food matrix during processing.

Table 1 : Permitted levels of antioxidants in foods

Food	Maximum level (mg/kg)
Cake mixes, cereal based snack foods, milk powder for vending machines, dehydrated soups and broths, sauces, dehydrated meat, processed nuts, seasonings and condiments, pre-cooked cereals	200 (gallates and BHA, individually or in combination, expressed on fat)
Dehydrated potatoes	25 (gallates and BHA, individually or in combination)
Chewing gum, dietary supplements	400 (gallates, BHT and BHA, individually or in combination)

Initial experimental work was carried out on commercially produced food products known to contain antioxidants, but where the level was unknown. This work was used to compare methods of extraction. In addition, a spiked oil sample was prepared, and analysed using various methods, to assess the effect of the extraction technique without the effect of the matrix having to be taken into account. These experiments allowed an initial assessment of the most suitable methods for extracting antioxidants from foods. The initial trials included methods that were intended to directly extract the antioxidants from the food and methods that first extracted fat from the foods and then extracted the antioxidants from the fat. Results of these first trials were used to narrow the range of methods under consideration. In the next stage, food samples with known amounts of antioxidant were prepared and analysed using a variety of techniques. The most suitable method, cold solvent extraction, was chosen for

optimisation and validation. Work at LFI focussed on using diethyl ether as the solvent, with some work carried out on the use of petroleum ether or hexane as alternatives, although these were not found to be suitable. Towards the end of the method development stage at LFI, Perrin and Meyer (2002) published work that evaluated two other solvent systems for the analysis of antioxidants in foods. Some analyses were carried out at LFI following the methods described, with mixed results, and so the LFI-developed method using diethyl ether was taken forward to the validation stage. After internal validation of the method, samples were sent out to three external laboratories for analysis by the developed method as a limited ring trial.

In addition to the use of traditional wet chemistry techniques for the extraction of antioxidants from foods, the project also aimed to develop molecular imprinted polymers (MIPs) against some of the antioxidants under consideration. Molecular imprinting has been shown to produce robust artificial receptors with recognition and even catalytic properties (Mosbach and Ramstrom, 1996, Wulff, 1995). Whilst the generation of a catalytic polymer is more complex, the generation of plastic recognition elements in general only requires that the molecule has at least one functional chemical group. Molecular imprinted polymers have been used for the separation of compounds of similar structure, for example β-lactam antibiotics (Skudar *et al*., 1999, Bruggemann *et al*., 2000), using the polymers as a stationary affinity phase for HPLC. MIPs have also been used in the analysis of complex matrices such as food (Ramstrom *et al*., 2001), and have been used in solid phase extraction applications for the purification of samples (Muldoon and Stanker, 1997, Andersson *et al*., 1997, Martin *et al*., 1997).

Molecular imprinted polymers are polymers produced around a template molecule (in this case either BHA, BHT or propyl gallate). The template molecule is then removed by washing, leaving imprints in the polymer that will specifically retain an analyte that is the same as that used for the template molecule. Initial assessments of whether such polymers do act to selectively retain the required analyte are carried out using the polymer packed into an HPLC column. The retention time of the analyte on the MIP is compared to the retention time of the analyte on a control polymer, produced without the addition of a template molecule. The aim of this project was to develop MIPs for use as a clean up technique, for example as a packing phase for solid phase extraction (SPE). This could be used for samples where there were a large number of co-extractives that might potentially interfere with the chromatographic analysis and hence with quantitation. Due to the nature of antioxidants, the longer the extraction procedure, the more likely the loss of antioxidant will be. Therefore, the more steps required to remove co-extractives, the less accurate the method. If a simple clean up procedure could be devised specific to the analyte, then this would improve the accuracy of the method by reducing the number of extraction steps and hence the length of time of the analysis. Therefore, after evaluating the MIPs packed into HPLC columns, they were then packed into cartridges and evaluated as SPE phases.

For the MIP phase of the project, work was carried out in collaboration with Technical University Berlin (TU Berlin) who have significant experience of this technology. Polymers were produced there, and initial evaluations carried out before the technology was transferred to LFI. Work was carried out at both institutions to assess the possibility of using the polymers for solid phase extraction.

MATERIALS AND METHODS

Unless specified, all solvents and reagents were purchased from Sigma, UK.

HPLC analysis

The initial HPLC set-up followed the method described in IUPAC 2.642 for the analysis of antioxidants in oils. Adjustments were made to improve the separation, described below in the results, leading to the following conditions used in the remainder of the project:

Mobile phase A: 5% (V/V) acetic acid (glacial, analytical grade) in distilled, deionised, filtered water
Mobile phase B: Methanol (HPLC grade) : Acetonitrile (HPLC grade) :acetic acid in the ratio 50:47.5:2.5
Temp: 25 ºC Injection volume: 10 µl Flow rate: 2.0 ml/min
Detection at 280 nm
Column: Suplelcosil (Supelco, UK) LC-18 15cm x 4.6mm

Table 2 : Gradient method for HPLC analysis

Time (min)	%A	%B
0	70	30
11	0	100
16	0	100
17	70	30
20	70	30

Under these conditions, the standards were found to elute in the following order:
propyl gallate, TBHQ, BHA, octyl gallate, BHT, lauryl gallate

The SOP for HPLC analysis is contained in Appendix 1.

TLC analysis

TLC plates (Pre-coated SIL G25 0.25 mm, Macherey-Nagel, and G1500 silica gel, Schleicher and Schull) were conditioned for 1 hour at 120 ºC and cooled in a dessicator before use. Standards of each antioxidant were prepared in 1:1 2-propanol:acetonitrile. Individual spots of 5 µl of each solution were loaded onto the plate. A mixed standard was also run, by spotting 2µl of each standard onto the same spot on the baseline. The plate was developed with 1:4 glacial acetic acid: petroleum ether 40-60. Visualisation of the spots was carried out using Gibbs reagent and $K_3Fe(CN)_6/FeCl_3$.

Gibbs reagent: 2,6 Dichloro-p-benzoquinone-4-chlorimine 0.5% w/v in ethanol. After spraying, the plate was developed at 120ºC for five minutes to reveal spots.

$K_3Fe(CN)_6/FeCl_3$ was prepared by taking 1.5 ml of each of 5% w/v $K_3Fe(CN)_6$ (aq) and 5% $FeCl_3$ (aq) and making to 20 ml with 80% aqueous ethanol. Spots were revealed on spraying with this reagent.

Variations were made to the acetic acid: petroleum ether ratio to optimise the separation of the standards. The SOP for the developed method is contained in Appendix 2.

Fat extraction methods

Several fat extraction methods were used, described below. Antioxidants were extracted from the fat using the extraction procedure described in the standard IUPAC method 2.642, and analysed by HPLC, using the conditions described above.

Bolton extraction

A weighed amount of sample was placed into an extraction thimble and continuously extracted with hot petroleum ether. The solvent was removed by evaporation at elevated temperature.

Werner Schmidt

A suitable amount of sample (approximately 5 g) was weighed into a boiling tube. Water (5 ml) and conc. HCl (5 ml) were added, and the mixture heated on a boiling water bath for 5 min with shaking. After cooling, 95% ethanol (10 ml) was added, mixed, then diethyl ether (15 ml) was added. The tube was stoppered, shaken for 1 min, then petroleum ether 40-60 (15 ml) was added. The tube was again stoppered and shaken for 1 minute. The upper solvent layer was removed into a conical flask using a siphon. 1:1 diethyl ether: petroleum ether 40-60 (mixed solvent, 10 ml) was used to wash the siphon tube, collecting in the boiling tube. This upper layer was again siphoned off. A further portion of mixed solvent (30 ml) was added to the tube, shaken, allowed to separate, and the upper layer siphoned off. The tube was again washed with mixed solvent (10 ml), which was collected and siphoned off. A final portion of mixed solvent (30 ml) was added to the tube, shaken, allowed to separate, and siphoned off. All extracts were pooled, dried over sodium sulphate, filtered and rotary evaporated to dryness. Any remaining solvent was removed under a stream of nitrogen and the extracted fat stored at +4 ºC until required for analysis, or stored at –20 ºC if the period of storage exceeded 24 hours.

Rose Gottlieb

A suitable amount of sample (5-10 g) was weighed into a boiling tube. Sodium chloride solution (8 ml, 0.5% w/v) and ammonia solution (1 ml, 0.91 SG) were added, followed by mixing on a vortex mixer. The tube was stoppered and allowed to stand overnight at room temperature. 95% ethanol (10 ml) was added, mixed, then diethyl ether (15 ml) was added. The tube was stoppered, shaken for 1 min, then petroleum ether 40-60 (15 ml) was added. The tube was again stoppered and shaken for 1 minute. The upper solvent layer was removed into a conical flask using a siphon. 1:1 diethyl ether: petroleum ether 40-60 (10 ml, mixed solvent) was used to wash the siphon tube, collecting in the boiling tube. This upper layer was again siphoned off. A

further portion of mixed solvent (30 ml) was added to the tube, shaken, allowed to separate, and the upper layer siphoned off. The tube was again washed with mixed solvent (10 ml), which was collected and siphoned off. A final portion of mixed solvent (30 ml) was added to the tube, shaken, allowed to separate, and siphoned off. All extracts were pooled, dried over sodium sulphate, filtered and rotary evaporated to dryness. Any remaining solvent was removed under a stream of nitrogen and the extracted fat stored at +4 ºC until required for analysis, or stored at –20 ºC if the period of storage exceeded 24 hours.

Modified Bligh and Dyer

A suitable amount of sample (10-20 g) was weighed into a beaker. Samples with low water content (e.g. cake mix, extruded snack) were mixed with 10 ml water. 80 ml methanol was added, mixed and allowed to stand. 40 ml chloroform was added, and homogenised for 4 min. A further 20 ml chloroform was added, and homogenised for 1 min. The sample was filtered under gravity, the residue washed with 20 ml chloroform, and the filtrate transferred to a 500 ml separating funnel. 40 ml water were added, shaken gently, and the layers allowed to separate. The chloroform layer was collected, dried over sodium sulphate, filtered and the filtrate rotary evaporated to dryness. Any residual solvent was removed under nitrogen, and the extracted fat stored at +4 ºC until required for analysis, or stored at –20 ºC if storage exceeded 24 hours.

Direct extraction methods

A number of direct extraction methods were used, described below. Extracts were analysed by HPLC (see above) for quantification of the antioxidants.

Cold direct diethyl ether extraction
Approximately 10 g (9.5-10.5g) of sample was weighed into a conical flask. Diethyl ether (100 ml) was added, the flask stoppered and shaken for 30 s. The sample was left to extract for one hour with occasional shaking. The ether solution was filtered, and the residue re-extracted with ether (2 x 50 ml), allowed to stand for 30 minutes with occasional shaking followed by filtration. Extracts were combined in a round-bottom flask and rotary evaporated to dryness. The extract was redissolved in hexane saturated with acetonitrile (approximately* 5 ml) and transferred to a 10 ml volumetric flask. The round bottom flask was rinsed with propan-2-ol and the rinsings transferred to the volumetric flask, to make to volume.
*Sufficient hexane was used to dissolve all of the fat, with gentle warming if necessary.

Hot direct diethyl ether extraction
A suitable amount of sample was taken (enough to fill an extraction thimble e.g. 5 g of extruded snack, 25 g cake mix). The sample was placed in a soxhlet extractor and extracted for 1 hour with hot diethyl ether. The solvent was evaporated to dryness, with the final traces of solvent being removed under nitrogen. The extract was redissolved in hexane saturated with acetonitrile (approximately* 5 ml) and transferred to a 10 ml volumetric flask. The round bottom flask was rinsed with propan-2-ol and the rinsings transferred to the volumetric flask, to make to volume.

*Sufficient hexane was used to dissolve all of the fat, with gentle warming if necessary.

Clavenger extraction
Sample (10g) was weighed into a round bottom flask. Saturated sodium chloride solution (150 ml) was added, with ascorbic acid (0.5 g) and 3-4 drops of an antifoaming agent. The Clavenger distillation apparatus was attached to the flask and the side arm filled with water. Ethyl acetate (2 ml) was pipetted into the side arm. The flask was heated using a heating mantle, and distillation was carried out for a total of two hours. After this time, the water in the side arm was removed, and the ethyl acetate collected into a 2 ml volumetric flask. The side arm was rinsed with further small portions of ethyl acetate until a final total volume of 2 ml had been collected. This solution was used for analysis.

Cold methanol extraction
(Method used by Perrin and Meyer, 2002)
Sample (5g) was weighed into a centrifuge tube, methanol (25 ml) was added, and shaken vigorously for 10 minutes. The extract was then centrifuged at 2500 rpm for 5 minutes, and the supernatant collected. The extraction and centrifugation were repeated twice, the collected supernatant extracts were then pooled in a round bottom flask, and rotary evaporated to dryness at less than 40 ºC. The residue was dissolved in methanol (10 ml) and analysed.

Cold hexane / propan-2-ol extraction
(Method used by Perrin and Meyer, 2002)
Sample (5g) was weighed into a centrifuge tube, hexane / propan-2-ol 1:1 (25 ml) was added, and shaken vigorously for 10 minutes. The extract was then centrifuged at 2500 rpm for 5 minutes, and the supernatant collected. The extraction and centrifugation were repeated twice, the collected supernatant extracts were then pooled in a round bottom flask, and rotary evaporated to dryness at less than 40 ºC. The residue was dissolved in methanol (10 ml) and analysed.

Preparation of food products with added antioxidant

Cake mix, extruded corn snack and mayonnaise were prepared at LFI with a known amount of antioxidant added into the oils used. Full recipes are given in Appendix 3.

Preparation of spiked oil

A blank oil (analysed by IUPAC method 2.642 to verify the absence of antioxidants) was spiked with the following concentrations of antioxidants:

BHA	201 mg/kg
BHT	239 mg/kg
propyl gallate	205 mg/kg
octyl gallate	228 mg/kg
TBHQ	228 mg/kg

Antioxidants were dissolved in the oil with stirring and warming to ensure homogeneity.

Preparation of ring trial samples

Preparation of fat containing antioxidant
Shortening (Brand name 'Sweetex', high ratio, all vegetable, Rowallan Creamery) was liquefied by gentle warming. Weighed antioxidant (BHA and BHT) was added to the melted fat under nitrogen, whilst stirring using a magnetic stirrer. Calculated concentrations of BHA and BHT in the oil samples are given in Table 3. Two samples of fat were prepared with added antioxidant at different levels. One was used to prepare samples A and B (blind duplicates) and the other to prepare sample C. One portion of fat was kept as the blank, used to prepare sample D.

Table 3: Calculated concentrations of BHA and BHT in fat used to prepare ring trial samples

Sample	BHT mg/kg	BHA mg/kg
A and B	137.4	140.2
C	103.1	105.2
D	None	None

Preparation of cake mixes

Samples A/B and D were prepared as follows: The prepared fat was added in liquid state to the dry ingredients given in Table 4. Mixing was carried out in a domestic-type food processor, and was continued for three hours to ensure homogeneity.

Sample C was prepared by mixing samples A and D in the ratio 3:1.

Subsamples were taken and stored frozen until required.

Table 4: Recipe for ring trial cake mix samples

Ingredient	Weight (g)
Flour	594.6
Granulated sugar	485.4
Icing sugar	206.2
Shortening	121.4
Starch	38.9
Sodium bicarbonate	16.9
Salt	14.1
SALP	7.9
SAPP	6.2
MCP	5.7
Xanthan	2.7

Preparation of molecular imprinted polymers (MIPs) (TU Berlin)

In all cases, the recipe in Table 5 was used for the preparation of the polymers.

Table 5: Recipe for preparation of MIPs

Compound	Function	Molar ratio	Mass/g	Volume/ml
MAA	Funct. monomer	4	1.148	
EGDMA	Crosslinker	12	7.929	
AIBN	Initiator	0.17	0.080	
AcN	Porogen			5

MAA: methacrylic acid; EGDMA: ethylene glycol dimethacrylate
AIBN: azo(bis-iso-butyronitrile); AcN: acetonitrile

One polymer was prepared for each antioxidant. Antioxidants were added in the following amounts to act as the template for the corresponding MIP:

BHA: 0.6g
BHT: 0.733 g
Propyl gallate: 0.707g

A control polymer was prepared in the same way, but without the addition of a template molecule.

Polymers were prepared in the following way: The porogen (acetonitrile) was filled into a glass beaker. This was followed by the addition of the liquid functional monomer, the liquid crosslinker and the initiator using for all three components a balance for determining the exact weight. For the MIP, the relevant template was weighed and added to the mixtures. After homogenization, the imprinting mixtures were flushed with gaseous nitrogen for 3 min at room temperature in order to remove the polymerization inhibitor oxygen.

The mixtures were placed in glass ampoules (20 ml) and polymerization was carried out overnight at 70°C in an oven. The resulting bulk polymers were ground using a ball mill (Retsch, type S 100, 20 ml steel beaker, 15 steel balls), wet sieved (mesh 25 µm) with acetone, until all material had passed the sieve, i.e., three grinding procedures had to be performed (each 300 rpm for 45 min). This was followed by sedimentation and the removal of the supernatant of remaining suspended fine particles. The polymer was cleaned with methanol and dried at 50 °C in an oven overnight. The resulting powder was weighed and 3 g of each sample were resuspended in acetone, packed with an Alltech slurry packer (model 1666) into HPLC columns (4.6 mm x 250 mm), extracted with methanol:acetic acid 7:1 v/v at 2 ml/min, to remove the template molecule, and washed with methanol to remove the acid. Elution of the template was monitored online via UV-detection at 220, 260, 300 and 330 nm until a stable baseline was observed. At this stage, it was concluded that all of the template had been removed.

Column packing (LFI)

Polymer (2g) was weighed into a vial, acetone added (5 ml) and the mixture vortexed to obtain a slurry. Two empty 15 cm HPLC columns were coupled together, and the slurry pipetted into the columns. The columns were then attached to an HPLC pump, and acetone was pumped through (2.0 ml/min) for 30 minutes. At the end of this time, the top (empty) column was removed, and more slurry added to fill the bottom column. Again the column was attached to the pump, and acetone was pumped through. This procedure was repeated until the column appeared to be packed. The column was then washed with methanol:acetic acid (7:1) to remove the template, and the solvent changed to 1:1 propan-2-ol:water, and left pumping at 0.3 ml/min overnight. At the end of this time, the column packing was re-checked, to ensure that no further settling had occurred, and further packing added as necessary.

HPLC evaluation of MIPs (TU Berlin)

Experiments were carried out to determine the optimal mobile phase for retention of the antioxidant on its respective polymer. Table 6 shows the mobile phase conditions investigated:

Table 6: Mobile phase compositions tested for optimal retention of analytes on MIPs

Mobile phase[a]	Composition (v/v)	Flow rate (ml/min)
AcN/AcOH	99:1	2
AcN/AcOH	99:1	1
MeOH	100 %	1
AcN/H_2O	1:1	1
AcN/H_2O	1:1	0.5
$CHCl_3$	100 %	1
AcOAc	100 %	2
Hexane	100 %	1
MeOH:AcN	1:1	1
MeOH:H_2O	1:1	1
MeOH:H_2O	7:3	1
i-propanol:H_2O	1.1	1

[a]AcN: acetonitrile; AcOH: acetic acid; MeOH: methanol; AcOAc: ethyl acetate

Batch extraction of antioxidants from standard solutions (TU Berlin)

For the first extraction experiments, the fine particles derived from the sedimentation procedure from the supernatants were used as solid phase to allow an efficient migration or diffusion of the analyte molecules into the pores, to avoid any hindrance by pore diffusion phenomena. Prior to their use the particles were washed twice with methanol/acetic acid 7:1 (v/v) in batch mode, followed by two methanol washes. In order to re-suspend the fine particles after filtration they were ultra-sonicated after adding the solvents.

For the extraction of BHA, BHT and PG from organic solvents, stock solutions were generated by dissolving 1 g of the individual antioxidants in 100 ml methanol. These solutions of 10 mg/ml were diluted to 1, 0.5, 0.1, 0.05 and 0.01 mg/ml with methanol,

and were used for HPLC calibration. 20 ml of a 0.1 mg/ml solution was added to 500 mg MIP or CP, respectively, and shaken on a rocking desk. After a defined period of time, 1 ml samples were taken from these batches, centrifuged to separate fine particles from the supernatant, followed by the chromatographic determination of the concentration of the antioxidants remaining in the supernatants.

Experiments were also carried out using acetonitrile as solvent for the extraction procedure, in place of methanol. Since acetonitrile was the selected porogen in the imprinting process, it was expected to allow the template molecules an unhindered access to the imprints in the MIP due to similar swelling effects. The concentration of the individual antioxidant solutions were chosen with a value of 0.0125 mg/ml, but exactly determined via HPLC.

Breakthrough curves of antioxidant solutions on SPE columns (TU Berlin)

The LC columns packed with CP and the PG-MIP already used for the determination of the affinity of the polymers were applied as SPE modules in the Dionex LC apparatus. The columns were thoroughly flushed with methanol/acetic acid 7:1 (v/v) until a stable baseline was observed at 280 nm. Then the columns were washed with methanol in order to remove the acid. After again reaching a stable baseline, the column was removed from the LC system, and the tube was flushed from the reservoir to the inlet of the column with a solution of the antioxidant (0.105 mg/ml PG in methanol). The column was again connected to the pump, and by starting the pump (flow rate 1 ml/min) and simultaneously recording the signal at 280 nm, the breakthrough of the antioxidant was determined.

Solid phase extraction (SPE) of apple juice spiked with antioxidants (TU Berlin)

Apple juice mixed with 0.105 mg/ml PG in methanol (1:1) was pumped through the column. After a period of 1 h, the MIP column was removed and replaced by an HPLC C18 reversed phase column with particle sizes of 12 µm (Supelcosil). After equilibrating the LC system with MeOH/H_2O 1:1 (v/v), the PG-MIP column was placed in front of the HPLC column and MeOH/H_2O 1:1 (v/v) as mobile phase was pumped through the two connected columns. The experiment was repeated with a 1:1 mixture of apple juice:methanol, with no added PG, as a control.

Evaluation of MIPs for solid phase extraction (LFI)

Polymer (0.5g) was packed into an empty cartridge with an integral frit at the bottom, and a second frit was placed on top of the polymer. Standard solutions of each antioxidant, prepared in a variety of solvents were passed through the polymer under positive pressure provided by a hand-held syringe. The non-retained portion was collected. A further portion of the same solvent was passed through the polymer, and again collected. A different solvent or series of solvents was then passed through the polymer and collected. All of the collected fractions were analysed by HPLC to determine if any of the antioxidant had been retained by the polymer.

RESULTS AND DISCUSSION

Development of HPLC analysis

Initial analysis of a mixture of propyl gallate, TBHQ, BHA, octyl gallate, BHT and lauryl gallate by the method used in IUPAC method 2.642 gave good separation for propyl gallate, TBHQ, BHA and octyl gallate, but BHT and lauryl gallate coeluted. A reduction in the flow rate to 1.5 ml/min resulted in slight separation of these compounds, as did a reduction in the slope of the gradient, but neither resulted in baseline separation of the peaks.

Eluent B was changed from (acetonitrile : acetic acid) (95:5) to (methanol : acetonitrile : acetic acid) in the ratio (50:47.5:2.5), keeping the same gradient and flow rate conditions as before. This resulted in separation of all standards. Appendix 1 contains the SOP for the modified HPLC method.

Development of TLC analysis

It was necessary to establish a TLC method for the confirmation of peaks found in the HPLC chromatogram, where the identity of these might be disputed e.g. where an antioxidant might be apparently above a permitted level, or appear to be present without having been stated on the label.

Initial analysis using SIL G25 plates (Macherey Nagel) or G1500 silica gel plates (Schleicher and Schull), developing with 1:4 glacial acetic acid: petroleum ether 40-60 resulted in separation of all compounds except TBHQ and lauryl gallate. Spraying with $K_3Fe(CN)_6/FeCl_3$ showed all of the antioxidants as blue spots. Spraying with Gibbs reagent showed the antioxidants as spots with varying shades of red/brown. TBHQ and lauryl gallate gave slightly different colour spots, but this was not considered to be sufficient to identify an unknown considering their similar retention factors.

Changes were made to the developing solvent; developing with 1:2 acetic acid:petroleum ether resolved TBHQ from lauryl gallate, but brought it to the same position as octyl gallate. Changing to 1.5:4 acetic acid: petroleum ether also resolved TBHQ from lauryl gallate, but again moved the TBHQ spot towards octyl gallate. Adjusting the ratio to 5:16 acetic acid:petroleum ether separated TBHQ from both octyl gallate and lauryl gallate. Table 7 shows results obtained for standards run under these conditions. Appendix 3 contains the SOP for the TLC method.

Table 7 : TLC results for antioxidant standards

Antioxidant	Rf value	Visualised with $K_3Fe(CN)_6/FeCl_3$	Visualised with Gibbs reagent
BHA	0.48	blue spot	orange red
BHT	0.95	blue spot	red
Propyl gallate	0.07	blue spot	red brown
Octyl gallate	0.15	blue spot	red brown
Lauryl gallate	0.25	blue spot	red brown
TBHQ	0.2	blue spot	dark red

Analyses of spiked oil

Table 8 shows the data for recovery of antioxidants from a spiked oil using six different methods of analysis. It can be seen that the IUPAC method 2.642 for determination of antioxidants in oils gives good recovery for all of the antioxidants with the exception of TBHQ.

Clavenger distillation only gave good recovery values for BHT, and even here the results were variable for repeat analyses. This steam distillation method was not expected to give good recoveries for the gallates, but the low recovery of BHA and the variable recovery of BHT suggest that other methods might be more suitable for all of the antioxidants being considered.

Table 8 : Recovery of antioxidant from a spiked oil, using different methods of analysis

Method	% Recovery				
	propyl gallate	BHT	octyl gallate	BHA	TBHQ
IUPAC 2.642	93-96	88-91	103-106	103-105	71-79
Clavenger	0	58-99	15-19	26-54	3-4
Bligh and Dyer then IUPAC 2.642	8-10	77-78	31-36	69	9-20
Rose-Gottlieb then IUPAC 2.642	0	79-90	0	90-99	0
Werner-Schmidt then IUPAC 2.642	41-43	102-104	93-97	97-101	41-69
Cold diethyl ether direct extraction	84	*	*	88	*

*not determined

Bligh and Dyer extraction resulted in fairly good recoveries of BHA and BHT, but low recoveries of the gallates and TBHQ. Similarly, the Rose-Gottlieb and Werner Schmidt methods resulted in good recoveries of BHA and BHT. However, no gallate or TBHQ were recovered from the oil treated by the Rose Gottlieb method, although better recoveries were seen using the Werner-Schmidt method. Cold ether extraction gave good recoveries for propyl gallate and BHA. Of the methods evaluated, this is the only one which has shown good recovery of propyl gallate.

Analysis of commercial samples

Cereal/chocolate snack bar

Table 9 shows the levels of BHA determined in a commercial snack bar using four different extraction methods. The fat content stated on the label was 10%, and E320 (BHA) was listed as an ingredient. Values for BHA recovery in Table 9 only give an indication of the usefulness of the method in recovering the antioxidant, since the absolute concentrations of antioxidants were not known for these samples. It appears

that the Bligh and Dyer method is most useful for this sample. However, the results from the spiked oil showed that this method could not be used for the recovery of gallates, a factor which must be considered in the development of a method for the analysis of a range of antioxidants.

Table 9 : Extraction of BHA from chocolate/cereal bar

Extraction method	Recovered BHA mg/kg expressed on fat
Werner Schmid	not detected
Rose Gottlieb	110
Bolton	35
Bligh and Dyer	166

Table 10 shows the results of analysis of BHA in chewing gum. Two different samples were analysed, both listing E320 (BHA) in the ingredients. Since chewing gum does not contain fat, the methods used were direct extraction only. Again, since the absolute levels of antioxidant were not known, these values can only be used to evaluate the relative usefulness of the methods. Both the Clavenger and diethyl ether methods gave similar levels for BHA in the sample.

Table 10: Extraction of BHA from chewing gum

Extraction method	BHA mg/kg	
	Sample 1	Sample 2
Clavenger	95	115
Diethyl ether	90	105

Two samples of mashed potato were analysed, again by direct extraction only, due to the low levels of fat present. Both samples were labelled as containing BHA. Sample 1 had a fat content of 0.2%, sample 2 had a fat content of 1.7%. Table 11 shows the values of BHA recovered from the samples using Clavenger and diethyl ether methods. Greater levels of BHA were obtained for the Clavenger extracted samples.

Table 11: Extraction of BHA from mashed potato

Extraction method	Recovered BHA mg/kg	
	Sample 1	Sample 2
Clavenger	1	3.4
Diethyl ether	0.2	0.1

Analysis of samples prepared at Leatherhead

Extruded snack
The extruded snack was prepared with oil containing antioxidants at the following concentrations:

BHA: 63 mg/kg
Propyl gallate: 64 mg/kg
Lauryl gallate: 51 mg/kg

Due to the way in which extruded snacks are produced, it was not possible to pre-determine the concentration of fat in the final product, and so the fat content was measured, by a standard method, (Weibull Bernthrop) without needing to conserve the antioxidant. Fat content in the snack without added antioxidant was 4.1%, and in the snack with added antioxidant was 4.2%. The antioxidant recoveries are expressed on this measured value, but have also been calculated on the amount of fat extracted during the extraction of antioxidants, where applicable. The fat extraction methods used in this project are not necessarily those that might have been chosen for the measurement of fat content, but have been selected for their potential ability to extract antioxidants intact. Therefore, the methods may not completely extract both the fat and the antioxidant.

- Bligh and Dyer

This method recovered all three of the antioxidants, although at low levels when expressed on the total fat (propyl gallate 9-23%, BHA 11-27%, lauryl gallate 9-25%). Recoveries expressed on the actual amount of fat extracted give higher values (propyl gallate 24-102%, BHA 48-122%, lauryl gallate 29-94%). However, it can be seen that this method did not give consistent recoveries of the antioxidants. In one particular extraction, only BHA was recovered, and so this method does not appear to be suitable for further development.

- Hot ether

No antioxidants were recovered by this method

- Cold ether

This method again gave variable recoveries. Initial extractions did not recover any of the antioxidants. However, changes to the method to include more extractions and larger solvent volumes resulted in recovery of BHA and lauryl gallate, although at low levels (12-18% and 21% respectively), and no recovery of propyl gallate. This method does not measure the amount of fat extracted, and so no comparison can be made with the Bligh and Dyer extraction in this respect.

The recovery of antioxidants from the extruded snack was low in all cases. It is not clear whether the harsh processing conditions cause high losses of antioxidants during the processing, in which case the low recoveries are not due to difficulties in extraction but to the absence of intact antioxidants. It may equally be true that the processing causes binding of the fat inside the matrix, and so the fat, and antioxidants, are particularly difficult to extract. Harsher extraction conditions may be required, but this may then cause losses of antioxidant.

Cold ether extractions were made on a blank extruded snack (made without added antioxidants), with external spikes added. Recoveries of BHA and BHT were 60-78% and 79-91% respectively. However, recoveries of the gallates were low (propyl gallate 4-41%, octyl gallate 6-51%, lauryl gallate 17-66%), suggesting that something in the extruded snack is binding these antioxidants, even when they have been added in solution, and were not cooked into the matrix.

Mayonnaise

A high fat dressing was prepared using oil with the following concentrations of antioxidants:

BHT:	76 mg/kg
BHA:	57 mg/kg
lauryl gallate	55 mg/kg
propyl gallate	53 mg/kg

Recoveries of antioxidant have been expressed on the known concentrations added, on the known fat content. For the Bligh and Dyer method, recoveries were also calculated on the fat recovered during the extraction of antioxidants.

- Bligh and Dyer extraction

BHA was recovered at 68%, but no propyl gallate was detected, and the recovery of lauryl gallate was low (26%)

- Cold ether extraction

Recoveries of both BHA and lauryl gallate were high (89 and 83% respectively). Propyl gallate appeared to be massively over-recovered. This was due to a co-extractive eluting at the same time as propyl gallate in the HPLC chromatogram. Analysis of each ingredient separately showed this compound to be potassium sorbate.

- Cold hexane / propan-2-ol extraction

Determination of BHA and propyl gallate was not possible due to co-extractives interfering in the HPLC chromatogram. Recoveries for BHT were 18 and 47%, and for lauryl gallate were 30 and 17% in duplicate determinations.

Cake mix

The cake mix prepared at LFI contained propyl gallate and BHA each at 100 mg/kg fat. Since both the fat content and the antioxidant concentration in the fat was known, recoveries have been expressed on the known values, unless indicated.

- Clavenger extraction

Propyl gallate was not recovered at all using Clavenger extraction, as expected from previous analyses. Recoveries of BHA were variable, ranging from 5 to 132%. Possible explanations for the variation include the complexity of the glassware and the small amounts of solvent used. LFI has two pieces of Clavenger distillation glassware, each with a different side-arm volume, and each giving different results. Rinsing of the glassware to ensure all antioxidant is collected is difficult, and there are also difficulties associated with sufficiently extracting solid dry samples. Samples are placed in a round bottom flask with water, and heated with a heating mantle. Therefore dry samples must be mixed thoroughly in the water to ensure that the entire sample is wetted, and no lumps remain, before heating begins.

- Bligh and Dyer extraction

Recovery of propyl gallate was low (2-8%). Recovery of BHA was also poor (40-50%) but expression of the antioxidant concentration on the weight of fat recovered, rather than the known amount added gave better recoveries (102-103%). This suggests that although not all of the fat was extracted from the cake mix by this method, the antioxidant was extracted in the correct ratio to the fat.

- Hot ether extraction

This method gave better recoveries for propyl gallate (35-40%) than the Clavenger or Bligh and Dyer methods, although recoveries were still low. Recovery of BHA was also low, with values of 48-51%.

- Cold diethyl ether extraction

From preliminary results on spiked oil, the cold ether method appeared to be most suitable for the analysis of both BHA and propyl gallate. In the cake mix, this method gave the highest recoveries for BHA (51-79%), and propyl gallate (21-96%), although the results were widely variable. Recoveries of external spikes were generally good (propyl gallate 67-80%, lauryl gallate 74-93%, BHA 81-104%, octyl gallate 54-63%), indicating that losses of antioxidant were minimal during the extraction. The variation in recoveries from the oil in the cake mix was likely to be due to insufficient extraction, or to inhomogeneity of the sample. Results from the chewing gum also suggested that this method might be suitable for further development. The lack of heating or harsh conditions in this extraction reduces losses of antioxidants. This method was chosen for further development.

Trials were carried out on the most suitable solvent for use in a cold extraction method. Cake mix was analysed in duplicate using three solvent systems: diethyl ether, methanol and hexane / propan-2-ol (1:1). Table 12 shows the results obtained. The methanol extract gave the worst results, with good recoveries for only BHT and BHA. Hexane / propan-2-ol gave the best recoveries for all of the antioxidants tested. Further analyses were carried out using the hexane / propan-2-ol solvent system, on a series of samples prepared with a mixture of antioxidant-containing and blank cake mix, in order to test the recoveries over a range of concentrations. Results are shown in Table 13. This solvent system compares well with the diethyl ether method already being used. It gives higher recoveries for propyl gallate and lauryl gallate than the ether method, although in some cases propyl gallate is over-recovered significantly. However, the recoveries for BHT are lower, and the linearity is not as good as the ether method (see below).

Table 12: Recoveries from cake mix using different cold solvent extraction systems

Antioxidant	**% recovery Diethyl ether extract**		**% recovery Methanol extract**		**% recovery Hexane / propan-2-ol extract**	
propyl gallate	44	53	60	30	70	67
BHT	115	105	89	103	88	98
octyl gallate	58	66	15	0	70	71
BHA	84	80	80	85	91	87
lauryl gallate	82	91	30	10	106	105

Table 13: Experiments carried out using hexane:propan-2-ol for extraction of cake mix

Antioxidant	**10% cake mix with antiox**		**30% cake mix with antiox**		**60% cake mix with antiox**		**100% cake mix with antiox**	
	Actual mg/kg	% recovery	Actual mg/kg	% recovery	Actual mg/kg	% recovery	Actual mg/kg	% recovery
propyl gallate	8	**140**	22	**75**	45	**109**	74	**73**
BHT	13	**76**	38	**74**	77	**78**	128	**86**
octyl gallate	9	**89**	26	**67**	53	**65**	87	**70**
BHA	10	**86**	30	**86**	60	**76**	99	**88**
lauryl gallate	11	**115**	33	**84**	66	**72**	110	**70**

Development of cold ether extraction

A fresh sample of cake mix was prepared, containing all of the antioxidants of interest (BHA, BHT, propyl gallate, octyl gallate, lauryl gallate). The possibility of the variability discussed above being due to inhomogeneity of the sample was tested by measuring the fat in each subsample analysed. Previously, the concentration of antioxidant in the sample had been expressed on the amount of fat in the sample, assuming homogeneity of the bulk sample. The cake mix sample was prepared in 1 kg batches. Antioxidant was dissolved in the oil, and this was added to the dry ingredients, then mixed thoroughly using a domestic mixer. However, it was possible that the oil was not distributed evenly throughout the sample. In order to check this possibility, it was decided to test the oil content of the actual sample extracted. In this way, the antioxidant content could be expressed on the true amount of oil from which the antioxidant had been extracted. Samples were extracted with ether, and the fat content of the remaining solids was determined by the Weibull-Berntrop method. In addition, the fat removed in the ether extract was also weighed after removal of the ether. These two values were added, and the antioxidant concentration in the sample expressed on this value. However, fat weights determined in this way were not found to be very different from the fat weights that would be used by assuming a homogeneous sample. The addition of extra steps to determine the fat content on each sample would add significantly to the work required for each analysis, with little or no benefit, and so these extra steps were not added to the method.

Recoveries of antioxidants from cake mix have so far been expressed on the concentrations of antioxidants in oil based on the weights of antioxidant added to a known amount of oil. An investigation of possible losses during the addition of the antioxidants to the oil was carried out. IUPAC method 2.642 was used to determine the concentration of antioxidants in the oil, before addition to the cake mix. The oil was then added to the cake mix and the concentration of antioxidants was determined using the diethyl ether extraction. Results are shown in Table 14. It can be seen that for propyl gallate and octyl gallate, a significant amount of antioxidant is lost in the preparation of the oil. Therefore recovery data expressed on the amount of antioxidant added into the oil is inaccurate for these two antioxidants. Subsequent values have been expressed on the measured values for concentration of antioxidant in the oil, in order to overcome this.

Table 14 : Losses of antioxidant on dissolving in oil

Antioxidant	mg/kg added	mg/kg measured in oil (% recovery)	mg/kg measured in cake mix (% recovery*)
Propyl gallate	126	74 (59%)	42 (57%)
BHT	131	128 (98%)	120 (94%)
BHA	107	99 (93%)	84 (85%)
Octyl gallate	119	87 (73%)	66 (76%)
Lauryl gallate	110	110 (100%)	69 (62%)

* Expressed on the measured concentration of antioxidant in the oil.

Improvements to the extraction were made by lengthening the time of extraction, and experiments were carried out on the length of time, and number of extractions required to maximise the recovery of antioxidant. Extractions were carried out with constant stirring. To minimise oxidation, extractions were carried out in an ice bath, and flasks were flushed with nitrogen. The final developed method is attached as Appendix 4.

Validation data for the developed method

Repeatability data

Six replicate analyses were carried out on cake mix containing oil with known amounts of antioxidant. Expected values: BHT: 128 mg/kg fat, BHA: 99 mg/kg fat, propyl gallate: 74 mg/kg fat, octyl gallate: 87 mg/kg fat, lauryl gallate 110 mg/kg fat. Table 15 shows the repeatability data for BHA and BHT.

Table 15 : Repeatability data for extraction of BHA and BHT from cake mix

	BHT		BHA	
Replicate	**Analytical value**	**% Recovery**	**Analytical value**	**% Recovery**
1	114	89	82	83
2	116	91	84	85
3	134	105	91	92
4	118	92	75	76
5	120	94	85	86
6	118	92	84	85
Mean	120		84	
Std dev	7.16		5.17	
% Coefficient variation	5.97		6.15	

Repeatability was also determined for the gallates. Recovery of propyl gallate was low and variable (38-72%). Recovery of lauryl gallate was more consistent, but still low (52-67%). Of the gallates, recovery of octyl gallate was highest. Table 16 shows the repeatability data:

Table 16: Repeatability data for recovery of octyl gallate from cake mix

Replicate	Analytical value mg/kg	% Recovery
1	69	79
2	68	78
3	67	77
4	54	62
5	68	78
6	70	81
Mean	66	
Std dev	5.97	
% Coefficient variation	9.04	

Reproducibility data

Data from twelve separate analyses of cake mix carried out on three different days gave coefficients of variation of 8.6 for BHT and 8 for BHA.

Linearity of the method

Linearity was determined by measuring the antioxidant concentrations of mixtures of cake mix with added antioxidant and 'blank' cake mix, in different proportions. Figure 4 shows results obtained for BHA. The dashed line demonstrates the ideal slope, if recovery had been 100% at all levels. Similarly, **Figure 5** shows the linearity data for analysis of BHT in cake mix. It can be seen that the linearity of the method for these two analytes is good. Table 17 shows the % recovery for all of the antioxidants. It can be seen that for the gallates, recoveries are good at low levels of antioxidant, but are reduced at higher levels of antioxidant. Recoveries for BHA and BHT are good at all levels.

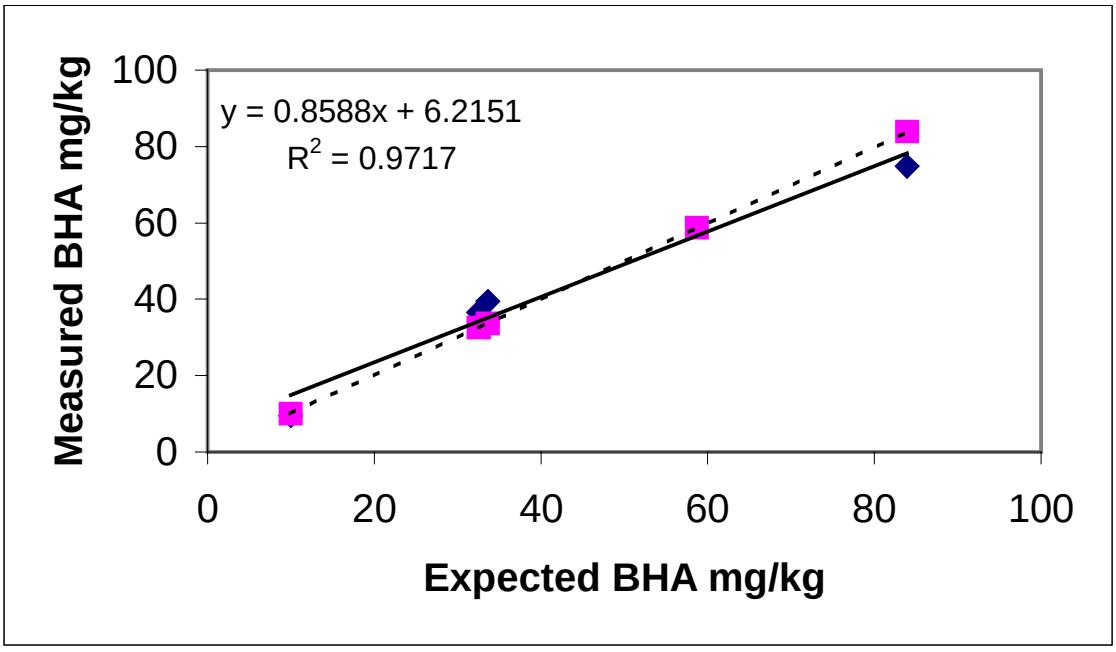

Figure 4: Linearity of recovery of BHA from cake mix (dashed line indicates ideal slope for 100% recovery at all levels)

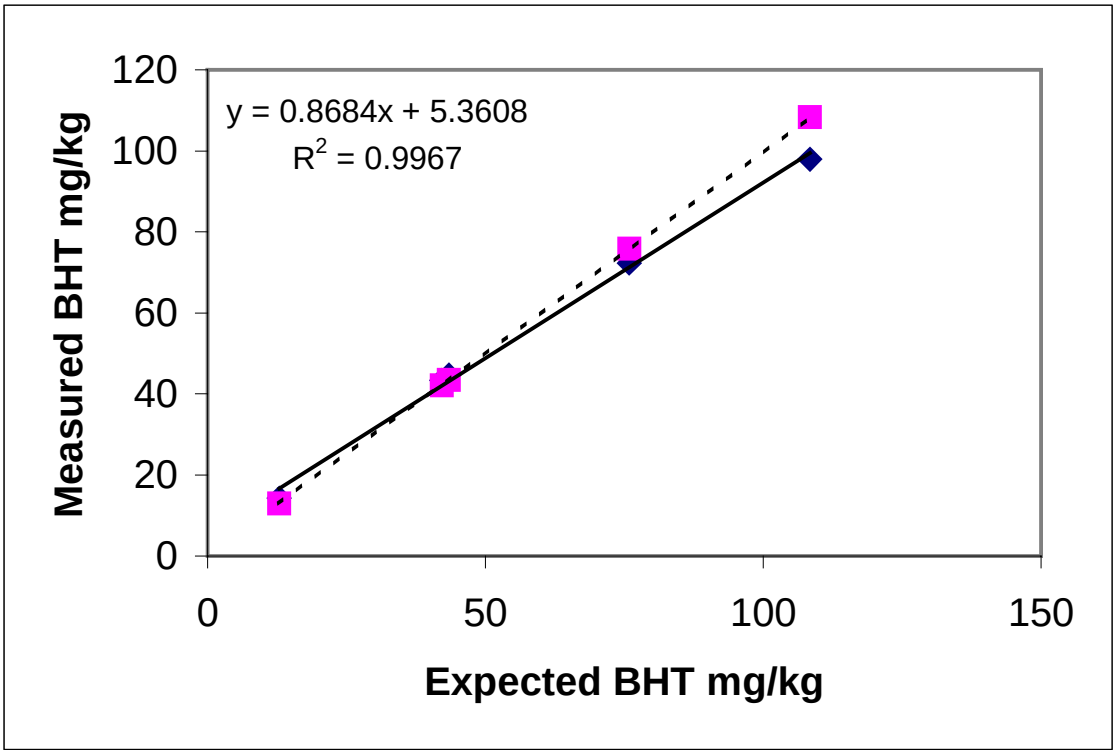

Figure 5: Linearity of recovery of BHT from cake mix (dashed line indicates ideal slope for 100% recovery at all levels)

Table 17 : Linearity data for recovery of antioxidants from cake mix

% cake mix with antioxidant	% cake mix with no antioxidant	% Recovery				
		BHT	BHA	Propyl gallate	Octyl gallate	Lauryl gallate
10	90	110	95	82	80	97
33	67	103	112	60	78	77
34	66	103	117	40	43	47
59	41	95	100	47	63	60
85	15	90	89	38	56	53

Linearity trials were also carried out on the mayonnaise sample prepared at Leatherhead. In the same way as for the cake mix, samples were prepared by adding mayonnaise containing antioxidant to a blank mayonnaise. As before, the peak co-eluting with propyl gallate meant that this antioxidant could not be quantified. In addition, a large number of co-extractives made quantification of BHA and octyl gallate difficult, in that it was not possible to identify the correct peaks. Recoveries for BHT were low (53-63%), but consistent across the range of samples, from 10% mayonnaise-containing-antioxidant to 100% mayonnaise-containing-antioxidant. Recoveries for lauryl gallate were also low, and decreased with increasing

concentration of antioxidant (from 54% recovery for a 10% mayonnaise + antioxidant mix, to 9% for a 100% mayonnaise + antioxidant mix).

Results from the ring trial

Homogeneity test on prepared cake mix samples

To check that the cake mix samples were fully homogenous, the fat content was determined by acid hydrolysis followed by solvent extraction on a soxhlet system on six randomly selected samples from each of A and B, C and D. This extraction method was chosen as the most suitable for accurate determination of fat on this matrix. Preservation of the antioxidants did not need to be considered here.

Table 18 : % fat in ring trial samples for homogeneity check

	Sample A and B	**Sample C**	**Sample D**
	8.58	8.55	8.54
	8.55	8.65	8.49
	8.54	8.58	8.45
	8.58	8.56	8.45
	8.64	8.68	8.52
	8.52	8.60	8.46
Mean	8.57	8.60	8.49
% Coefficient deviation	0.50	0.60	0.45

The above results show that each sample is homogenous. The variation obtained for each sample type is within the limits of repeatability for the fat determination method.

Analysis of antioxidants

Antioxidant levels in the oil used for preparation of the cake mix were determined by IUPAC 2.642, as described previously. Results are given in Table 19. These values give an indication of the antioxidant levels expected in the cake mix samples. However, there may have been some losses of antioxidant during incorporation of the oil into the cake mix. Table 20 shows the determined levels of antioxidants in the cake mix, carried out at LFI using the developed cold ether extraction.

Table 19: Antioxidant levels in the oil used for ring trial samples, determined by IUPAC 2.642

Sample	**mg of BHT per kg fat**	**mg of BHA per kg fat**
A and B	145	112
C	109	84
D	nd	nd

nd = not detected

Table 20: Antioxidant levels in ring trial samples, determined by cold ether extraction

Sample	BHT (mg/kg fat)	% Recovery of BHT	BHA (mg/ kg fat)	% Recovery of BHA
A and B	105 / 123	73 / 85	96 / 96	86
C	81	74	73	87
D	nd	---	nd	---

nd = not detected

Recovery data was calculated by comparison with BHT and BHA levels in the oil, reported in Table 19.

Ring trial results

Table 21 and Table 22 show the results of the ring trial, obtained from two external laboratories, and with the LFI values for comparison. Laboratory 1 did not use the protocol provided with the samples, but used a method currently in use in their laboratory (based on a heptane extraction followed by analysis by GC). Therefore these data cannot be used for assessment of the method developed at LFI. Laboratory 3 did not return any results. Therefore only one set of data was obtained from external laboratories in this trial.

The results from laboratory 2 were very variable. Samples A and B were duplicates, but the results from laboratory 2 are very different for both BHA and BHT. Sample C should have contained 75% of the levels found in samples A and B. However, the result for C is at a similar level to sample A. Therefore results from laboratory 2 are not showing a systematic under-recovery. Results from laboratory 1, which used a different method of analysis, are closer to the antioxidant levels found in the oil (Table 19) for both BHA and BHT.

Table 21: Ring trial results for BHT

	BHT mg/kg fat			
	LFI	**Laboratory 1**	**Laboratory 2**	**Laboratory 3**
A	105	146 125	50	-
B	123	120 115	18	-
C	81	104 98	53	-
D	0	0 0	< 5	-

Table 22: Ring trial results for BHA

	BHA mg/kg fat			
	LFI	**Laboratory 1**	**Laboratory 2**	**Laboratory 3**
A	96	113 110	54	-
B	96	106 108	19	-
C	73	89 90	56	-
D	0	0 0	< 5	-

Retention characteristics of MIPs

Retention characteristics - TU, Berlin

Solubility of the templates was tested in a number of solvents. All templates were soluble in acetonitrile, which was also selected due to its versatility as an HPLC mobile phase component. Methacrylic acid (MAA) and ethyleneglycol dimethacrylate (EGDMA) were chosen as reliable functional monomer and cross linker, respectively. It was expected that MAA would interact non-covalently with the three template molecules via hydrogen bonds. The ratio of template to MAA to EGDMA was kept constant (Table 5).

Preliminary experiments were carried out using propyl gallate and the corresponding PG-MIP. It was found that mobile phases containing acetonitrile did not lead to any retention of propyl gallate on the polymer. When an analyte interacts with a solid phase imprinted with the same compound, the analyte will be retarded, and the peak shape will show tailing due to the heterogeneity of the imprints. Retardation of an analyte is evaluated by comparing its retention time on the MIP with that of the same analyte on a control polymer under the same conditions. The control polymer has not been imprinted with a template molecule, and so any retention of the analyte will be due to non-specific interactions. Extra retention of the analyte on its corresponding MIP, in addition to any non-specific interactions, is expressed as the separation factor, α. If a MIP retains the analyte to a greater extent than the control polymer, then α will be greater than 1, and higher α values indicate greater specific retention.

A mobile phase of methanol/water at a ratio of 1:1 showed interaction of propyl gallate with the PG-MIP, demonstrated by clear retardation, and tailing of the peak. Attempts to use this mobile phase for BHA and BHT were halted by solubility problems, and the water content of the mobile phase was reduced to a ratio of methanol:water 7:3. The analytes were dissolved in methanol for analysis. A mixed sample of propyl gallate, BHA and BHT was analysed on the PG-MIP, and all of the components were separated, although propyl gallate was eluted first. This was not as expected, since in theory propyl gallate would be retained in preference to compounds which were not used as the template molecule. In addition, retention times were long for the later eluting compounds (13.6 min for BHA and 27.8 min for BHT)

In order to shorten the run times, the mobile phase was changed to i-propanol/H_2O 1:1. The mixture of antioxidants was analysed on the PG-MIP column and on the control polymer. The order of elution was not changed by the change in mobile phase, but the retention times were reduced. Analytes eluted faster from the control polymer, suggesting that imprinting of the polymer was resulting in retardation of all of the analytes. Repeating this experiment with the BHA and BHT-MIPs also resulted in retardation of all of the analytes when compared with the control polymer. Table 23 shows the retention times of the antioxidants on each MIP and on the control polymer. Although this showed that the MIPs produced did not specifically retain one analyte, they did appear to retain the antioxidants and so showed some potential for use in sample clean up.

Table 23: Retention times for antioxidants on MIPs and control polymer

Polymer	Retention time (min)		
	PG	**BHA**	**BHT**
Control	4.79	8.72	12.18
PG-MIP	5.40	10.26	15.74
BHA-MIP	5.14	9.93	15.39
BHT-MIP	5.26	*	15.14

* Peak not clearly resolved

For quantification of the relative retention of the analytes on the MIPs and control polymer, capacity factors and separation factors were determined. Acetone was added to the sample solutions, as an unretained marker (void marker), and used to calculate the capacity factor:

Capacity factor, $k' = (t - t_0) / t_0$

where t = retention time in min of analyte, t_0 = retention time in min of the void marker .

Capacity factors for an analyte on the control polymer (k'_{CP}) and on the MIP corresponding to the analyte (k'_{MIP}) were used to determine the separation factor.

Separation factor, $\alpha = k'_{MIP}/k'_{CP}$

The separation factor for propyl gallate on the PG-MIP was 1.15 for the preliminary analysis. Three repeat analyses gave values of 3.44, 3.41 and 3.63 (ave = 3.49) for k'_{MIP}, and 2.86, 2.82 and 2.76 (ave = 2.81) for k'_{CP}, giving an α of 1.24. The repeat analyses were carried out on a new batch of polymer, and the tailing effects were more prominent with this batch of polymer, which may be explained by different packings or particle sizes. It should be noted that these analyses were carried out using MeOH/H_2O 1:1 as the mobile phase.

Replicate analyses were also carried out for the BHA and BHT MIPs with their respective analytes. These analyses were carried out using i-propanol/H_2O as the mobile phase, and so the capacity factors are lower than for the values given above for propyl gallate. Capacity factors and separation factors are given in Table 24 and Table 25.

Table 24: Capacity factors and separation factor for BHA analysed with BHA-MIP

	k'_1	k'_2	k'_3	k'average	α
MIP	1.61	1.57	1.56	1.58	1.12
CP	1.42	1.40	1.42	1.42	

Table 25: Capacity factors and separation factor for BHT analysed with BHT-MIP

	k'_1	k'_2	k'_3	k'average	α
MIP	3.62	3.68	3.57	3.62	1.47
CP	2.45	2.46	2.48	2.46	

The highest α-value of 1.47 was found for BHT, containing only one hydroxy-function, but in the direct neighbourhood of two t-butyl-groups. BHA which was used as a mixture of two isomers (Figure 1) as template as well as analyte, only resulted in an α-value of 1.12. When evaluating the PG-MIP a separation factor of 1.24 was obtained, lower than in the case of BHT, although PG consists of four free hydroxy-groups (Figure 3). Example chromatograms for PG are shown in Figure 6 and Figure 7.

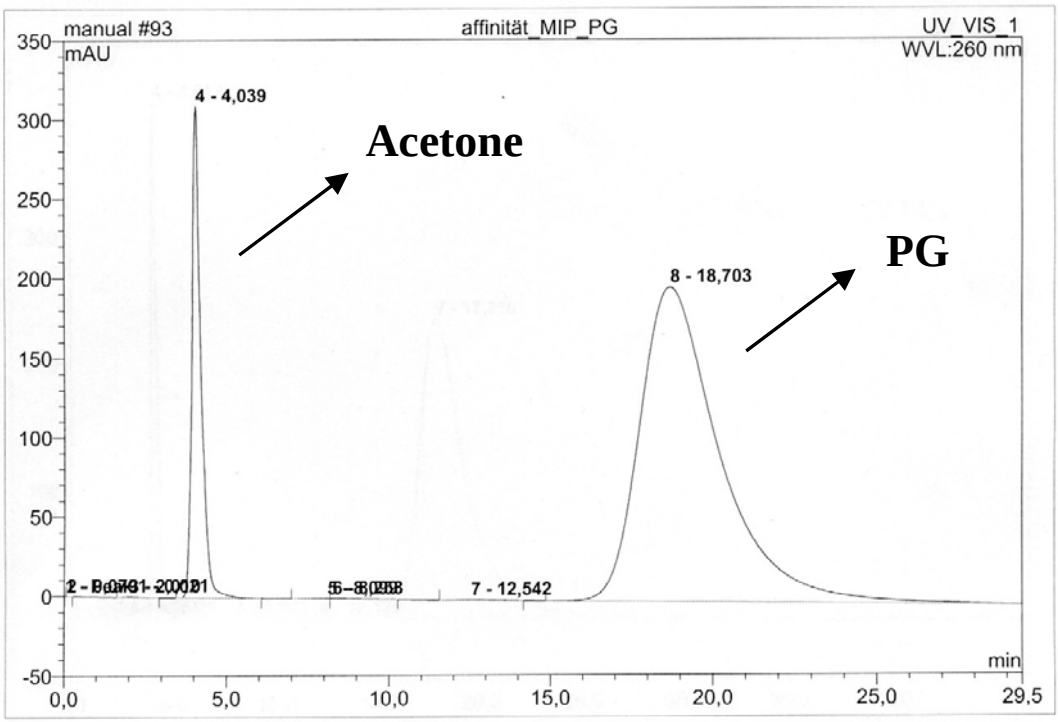

Figure 6: PG on the PG MIP

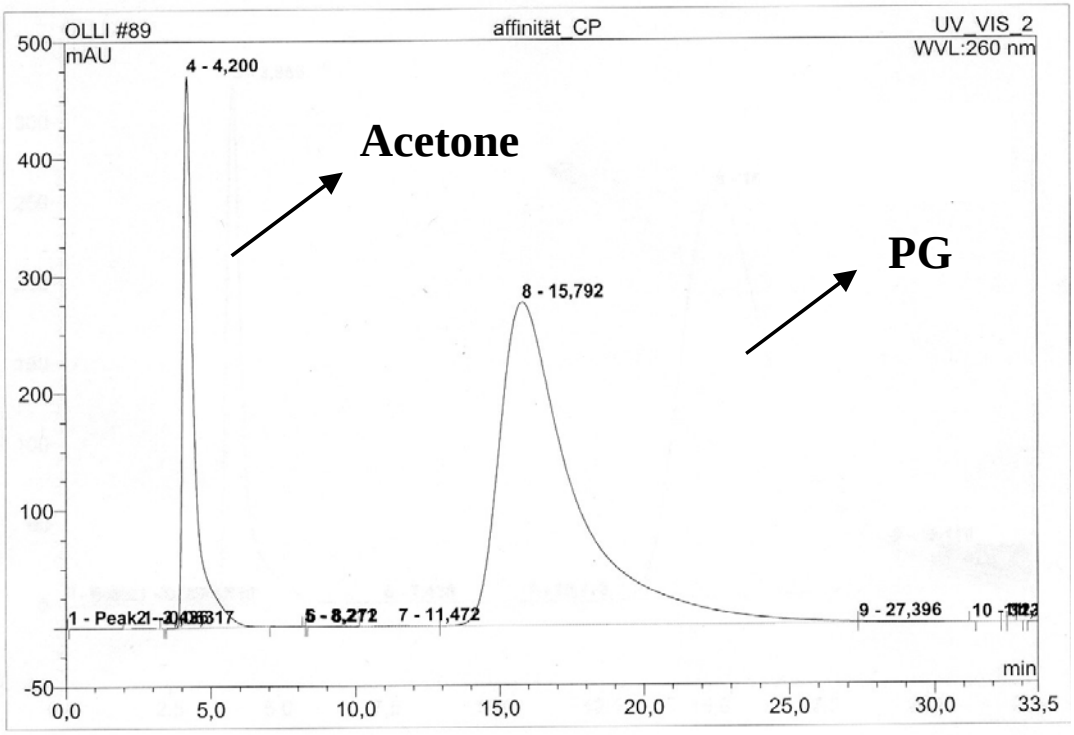

Figure 7: PG on the control polymer

MIPs for solid phase extraction – TU Berlin

In these experiments, MIP and CP were evaluated with respect to their applicability in batch extraction approaches and SPE columns. Reverse phase HPLC was used to determine the concentrations of the antioxidants in solution.

When using the different MIP for the batch extraction of their individual antioxidants from standard solutions in MeOH, both the PG-MIP and BHT-MIP showed unspecific behavior in comparison with the CP, i.e., the CP adsorbed both PG and BHT to a higher extent than the MIP. In some cases the supernatants showed higher concentrations of the antioxidants than the original solution added to the polymer. For the MIP this could have been explained by bleeding of template from the imprints, but it was also observed for the CP which had not been in contact with the template/analyte prior to the extraction. Most probably this is due to a lack of sensitivity of the analytical method, for example there may have been bleeding of one of the components of the polymer, leading to increased UV absorption.

Only one combination led to an expected specific adsorption of an antioxidant on its respective MIP. When using a solution of BHA in methanol the BHA-MIP was able to extract more BHA from that solution than the CP, and in both supernatants the concentration was lower than in the original solution. This effect was observed after an extraction period of 42.5 h, and became much more obvious after an extraction time of 233.5 h. Table 26 shows the results for the extractions from methanol solutions.

Table 26 : Results for the batch extractions of antioxidants from methanol solutions using the respective MIP or CP fine particles.

BHA			
Extraction time	**Original solution**	**CP supernatant**	**BHA-MIP supernatant**
42.5 h	98.6 µg/ml	98 µg/ml	96.5 µg/ml
233.5 h	96.6 µg/ml	81.6 µg/ml	7.6 µg/ml
BHT			
Extraction time	**Original solution**	**CP supernatant**	**BHT-MIP supernatant**
186.5 h	99.5 µg/ml	97.9 µg/ml	99.5 µg/ml
PG			
Extraction time	**Original solution**	**CP supernatant**	**PG-MIP supernatant**
21 h	78.1 µg/ml	86.1 µg/ml	77.9 µg/ml

Using acetonitrile as solvent for the extraction procedures, similar results were observed for all three different MIP and the CP when extracting their respective antioxidants. Again, PG-MIP and BHT-MIP showed lower affinity for PG and BHT, respectively, than the CP. For the supernatants of these MIP higher concentrations of the antioxidants were determined than for the supernatants of the CP. However, in the same way as for the methanol-based extractions, the BHA-MIP adsorbed more BHA from an acetonitrile solution than the CP, although the difference was not so pronounced. Whereas after the first 24 h MIP and CP showed a higher BHA concentration than the original acetonitrile solution, after a period of 64.3 h both supernatants contained less BHA than the original solution, and the MIP showed a higher affinity. Table 27 gives an overview of the acetonitrile based extraction experiments.

Table 27: Results for the batch extractions of antioxidants from acetonitrile solutions using the respective MIP or CP fine particles.

BHA			
Extraction time	**Original solution**	**CP supernatant**	**BHA-MIP supernatant**
23.2 h	2.7 µg/ml	3 µg/ml	2.9 µg/ml
64.3 h	3.3 µg/ml	3.2 µg/ml	2.7 µg/ml
BHT			
Extraction time	**Original solution**	**CP supernatant**	**BHT-MIP supernatant**
24.8 h	17.3 µg/ml	16.8 µg/ml	17.3 µg/ml
65.9 h	17.4 µg/ml	17.1 µg/ml	17.7 µg/ml
PG			
Extraction time	**Original solution**	**CP supernatant**	**PG-MIP supernatant**
24 h	7.5 µg/ml	5.4 µg/ml	6.2 µg/ml
68.5 h	9 µg/ml	9.1 µg/ml	9.9 µg/ml

Comparing these first extraction experiments with the findings of the chromatographic evaluations of the MIP and the CP, contrary results were found. The BHA-MIP showed the lowest α-value in LC, but the highest affinity in the batch extraction procedure, compared to the PG-MIP or the BHT-MIP.

Breakthrough curves of antioxidant solutions on SPE columns – TU Berlin

When the PG MIP and the CP were applied as solid phases packed in HPLC columns for selective extraction, the higher affinity of the PG-MIP compared to its CP was again apparent. Figure 8, top, shows for the PG-MIP a breakthrough curve for a solution of 0.105 mg/ml PG in methanol at 1 ml/min, with t_b = 3.35 min, whereas the use of the CP column leads to a breakthrough time of t_b = 3.2 min (Figure 8, bottom). Considering the dead volume between the column and the detector which was determined to be 0.15 ml, the determined breakthrough volumes of 3.2 ml for the MIP and 3.05 ml for the CP mean a relative affinity effect of 1.05 for the MIP compared to the CP.

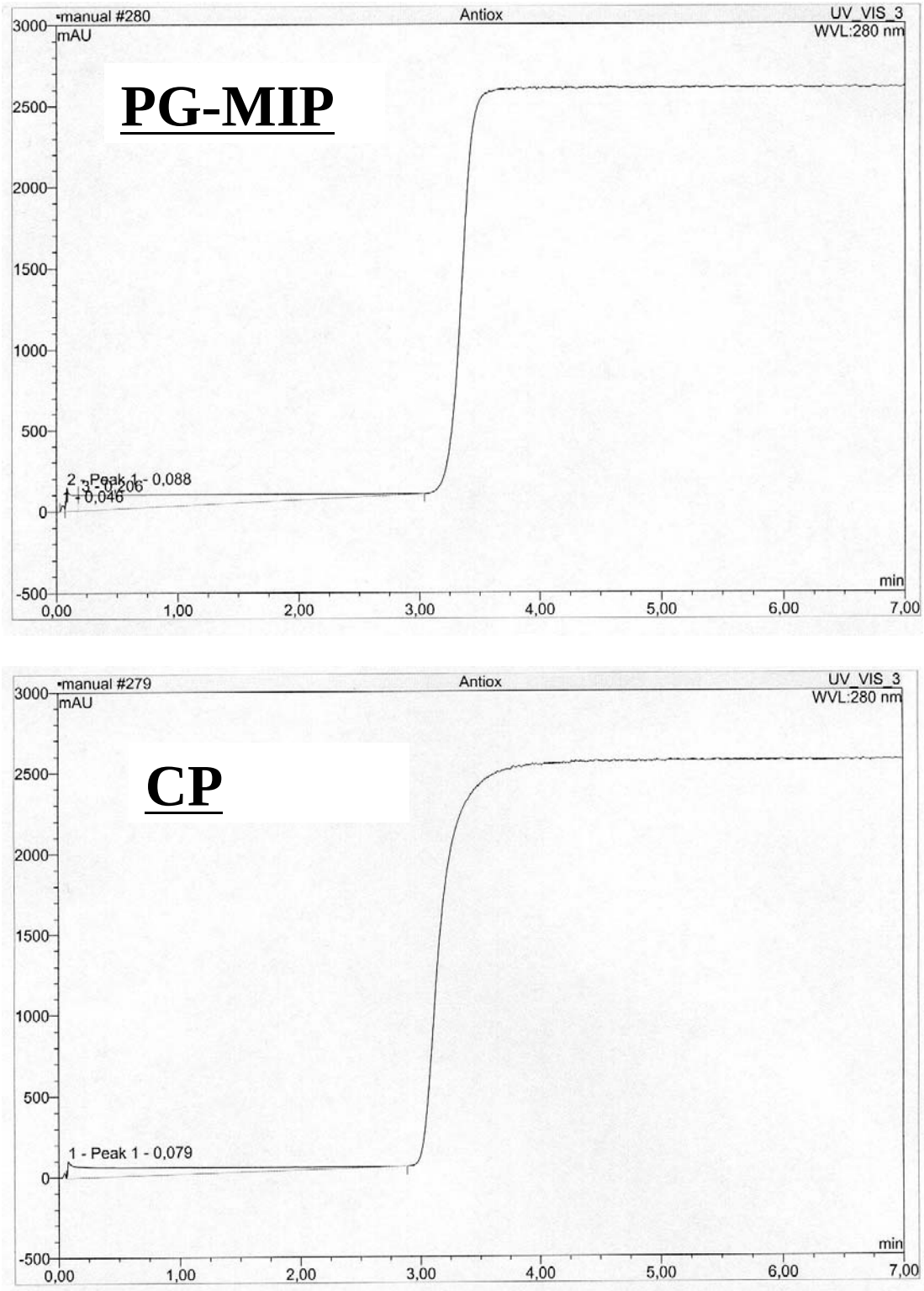

Figure 8: Breakthrough curves of 0.105 mg/ml PG in methanol on polymer solid phase columns at a flow rate of 1 ml/min; detection at 280 nm; top: PG-MIP, bottom: CP as solid phases

SPE of apple juice spiked with antioxidants – TU Berlin

First attempts to extract PG from apple juice spiked with PG followed by analysis on the HPLC 18 column did not lead to PG signals clearly distinguishable from the strong background noise (not shown). The PG-MIP obviously requires more thorough washing directly after loading with the apple juice / PG mixture, in order to remove interfering analytes. Therefore, further optimization of this SPE-approach is required

The above results were obtained at the Technical University, Berlin. Polymers produced there were then used at LFI, in the first instance to attempt to reproduce the work carried out on packed HPLC columns, and then for further development of uses for the polymers.

HPLC results obtained at Leatherhead Food International

Table 28 shows data obtained for a column packed with BHT-MIP. Standards were prepared in 1:1 isopropanol:acetonitrile, with 5% acetone added, and the mobile phase was 1:1 isopropanol:water. Each standard was run individually to determine the retention time and k' factor. k' factors for BHT compare well with those obtained at TU Berlin.

Table 28: Initial results obtained at LFI for BHT-MIP

Analyte	**Acetone retention time (min)**	**Antioxidant retention time (min)**	**k'**
acetone	1.95		
BHT	2.2 / 2.2 / 2.2	10.42 / 10.42 / 10.27	3.74 / 3.74 / 3.67
BHA	2.2	6.6	2
propyl gallate	2.2	3.22	0.46

Table 29 shows the retention times and k' factors for the antioxidants analysed individually on a column packed with a control polymer. Again the standards were prepared in 1:1 isopropanol:acetonitrile, and the mobile phase was 1:1 isopropanol:water. Average k' factors were: propyl gallate 0.5, BHT 3.52, BHA 2.06. These were quite different from the values obtained at TU Berlin. This may have been due to the difference in the solvent used to prepare the standards, and so standards were prepared in isopropanol, with 5% acetone to act as the void marker. Table 30 shows the data obtained. The k' factors were lower, showing that the solvent used for preparation of the analyte must also be considered during future method development and evaluation of the polymers. The separation factor was calculated for BHT on the BHT-MIP, using the values obtained for the analyte prepared in isopropanol:acetonitrile, giving a value of 1.06. This shows some retention of BHT on the BHT-MIP.

Table 29: Retention times and k' factors for antioxidants on the control polymer

Analyte	Acetone retention time (min)	Antioxidant retention time (min)	k’
acetone	1.82		
BHT	1.95 / 1.93 / 1.93	8.63 / 8.77 / 8.85	3.43 / 3.54 / 3.59
BHA	1.95 / 1.93 / 1.93	5.87 / 5.93 / 5.97	2.01 / 2.07 / 2.09
propyl gallate	1.97 / 1.95 / 1.93 / 1.95	2.9 / 2.92 / 2.92 / 2.95	0.47 / 0.50 / 0.51 / 0.51

Table 30: Retention times and k' factors for antioxidants prepared in isopropanol on the control polymer

Analyte	Acetone retention time (min)	Antioxidant retention time (min)	k’
acetone	2.2		
BHT	2.22 / 2.22 / 2.22	9.32 / 9.31 / 9.31	3.20 / 3.19 / 3.19
BHA	2.22 / 2.20 / 2.22	6.22 / 6.30 / 6.40	1.80 / 1.86 / 1.88
propyl gallate	2.23 / 2.23 / 2.23 / 2.23	3.1 / 3.13 / 3.13 / 3.18	0.39 / 0.40 / 0.40 / 0.43

Evaluation of MIPs for use in sample clean up

Standard solutions

Antioxidants were tested on their respective MIPs, adding a solution of the antioxidant to the MIP and collecting eluted fractions. Table 31 contains a summary of the experiments carried out on the BHT-MIP. Where there was no retention of the analyte, HPLC analysis of the fractions showed that the majority of the antioxidant either passed straight through the polymer, or was eluted from the cartridge during a wash with the same solvent. This suggests that the antioxidant was not bound to the polymer, but had simply remained in the cartridge with residual solvent. Wash solvents are listed in the table, but are irrelevant for all but the system in which the solvent was loaded in hexane. In this system, some antioxidant was retained. However, a repeat of the analysis using the control polymer showed similar results, showing that it is some general property of the polymer and not a specific imprinting effect that has retained the analyte. Table 32 and Table 33 show the results using the PG-MIP and BHA-MIP. These results are similar to those for the BHT-MIP, with again the apparent retention of the antioxidant loaded in hexane. Tests on the cartridges with no polymer loaded showed no retention of the antioxidants, demonstrating that it is the polymer retaining the antioxidants and not the frits.

Table 31 : Results using BHT-MIP for solid phase extraction

Solvent used in standard soln.	Wash solvent	Retention of analyte by polymer?
Acetonitrile / water	Hexane	No
Acetonitrile	Hexane	No
Acetonitrile	Diethyl ether	No
Hexane	Acetonitrile	Some
Methanol / water	Hexane	No

Table 32: Results using PG-MIP for solid phase extraction

Solvent used in standard soln.	Wash solvent	Retention of analyte by polymer?
Acetonitrile / water	Hexane	No
Acetonitrile / water	Diethyl ether	No
Propan-2-ol	-	No
Acetonitrile	Hexane	No
Acetonitrile	Diethyl ether	No
Acetonitrile	Methanol	No
Acetonitrile	Propan-2-ol	No
Hexane	Acetonitrile	Some

Table 33: Results using BHA-MIP for solid phase extraction

Solvent used in standard soln.	Wash solvent	Retention of analyte by polymer?
Acetonitrile	Hexane	No
Acetonitrile	Diethyl ether	No
Hexane	Acetonitrile	Some
Acetonitrile / water	Hexane	No
Methanol / water	Hexane	No

Sample extracts

Cake mix was extracted using hexane. The extract was passed though the polymer, the polymer washed with hexane, followed by acetonitrile, and all fractions were collected and analysed by HPLC. Results from all polymers, including the control polymer, showed some retention of the antioxidants. Therefore, as for the standard solutions, the polymers appear to retain the antioxidants under these solvent conditions, but the retention is non-specific, occurring for both the imprinted and non-imprinted polymers.

CONCLUSIONS

The aim of this project was to develop and validate a method for the accurate quantification of selected synthetic antioxidants in food matrices. Two approaches were taken. The first evaluated traditional wet chemistry methods for the extraction of antioxidants. The second used new technology with the aim of cleaning up and extracting samples much more selectively than could be achieved using traditional methods.

A range of fat extraction methods was evaluated in the initial stages, but in the main these were found to be too harsh for the intact extraction of the antioxidants being considered. In addition, extraction of the fat followed by extraction of the antioxidants was time consuming, and generally led to a two-day extraction procedure, which is not ideal either from a practical point of view or from the point of view of losses of antioxidants.

Clavenger extraction was not time consuming, but the number of samples extracted per day was limited by lack of apparatus. LFI has two pieces of Clavenger extraction glassware, but it was considered that other laboratories might not have any suitable glassware, making it an expensive analysis to set up. It was also noted that of the apparatus at LFI, each piece had different dimensions, which led to differences in recovery. Although this might have been resolved by further development work, it is likely to have been difficult to produce a method which would give reproducible results in any laboratory on any piece of glassware. The fact that this extraction only recovers BHT adds to the limitation of the method, and so it was not pursued.

Cold extraction methods were found to give the best recoveries for the range of antioxidants considered. Development work at LFI found diethyl ether extraction to be suitable, and work was carried out to determine optimum conditions. Recoveries from the extruded snack were low, probably due to losses of the antioxidants from the oil during production of the snack, but also possibly due to binding effects. This was shown by low recoveries of antioxidants from the snack even when added as external spikes and not cooked into the matrix. Analysis was also carried out on mayonnaise and cake mix, with better results. Best recoveries were obtained for BHA and BHT and lauryl gallate. Lower recoveries were achieved for propyl and octyl gallates. Coefficients of variation for repeatability and reproducibility were less than 10% for BHA, BHT and lauryl gallate. However, results from the ring trial suggest that the developed method is not easily transferable to other laboratories. Lack of time for training was the reason given by one laboratory for not using the LFI-developed method. This laboratory did return results obtained using their own method. These results gave higher recoveries than even the LFI results using the diethyl ether extraction. This might therefore be a useful lead for carrying out further work. Results from the laboratory that did use the LFI developed method were very variable. In all cases the recovery was low, and the results for blind duplicates were very far apart.

Extraction of the mayonnaise sample using diethyl ether resulted in an interfering peak in the HPLC chromatogram at the same retention time as propyl gallate. The possibility of co-extractives interfering in the HPLC analysis was an issue that it was hoped could be resolved with the use of MIPs as a solid phase extraction clean-up

step. The MIPs produced did not show selective retention of their template molecules, although each MIP retained all of the antioxidants to a greater extent than the control polymer. This showed that some imprinting had occurred, and the retention was not simply due to non-specific interactions of the analytes with the polymer. All of the template molecules used here for the production of MIPs were phenolic antioxidants, and it is likely that the interaction was via a phenolic hydroxy group, common to all of the structures. Since the retention was not specific to a particular molecule, then it is likely that solubility effects were also a major factor to be considered. The first-eluting compound was propyl gallate, regardless of which MIP was being considered, and this is probably due to the greater number of hydroxy groups making propyl gallate more soluble in the polar mobile phases used. BHT is insoluble in water, BHA is practically insoluble in water whilst propyl gallate has a solubility of 0.35 g/100ml water at 25 ºC (Merck Index). This is also seen in the order in which these compounds elute from the reverse phase HPLC column (propyl gallate before BHA before BHT), which is the same order in which the compounds elute from the MIP-packed columns.

Attempts to use the MIPs as a solid phase clean-up, however, did not show selective retention of the analytes. Only analytes dissolved in hexane were found to be retained on the packing material. However, this effect was also seen in the control polymer, and therefore was not due to an imprinting effect of the template on the polymer. As suggested above, the polarity of the analytes due to the hydroxy groups might also explain the retention of the compounds on the MIPs when hexane was used as the loading solvent.

In summary, a method was developed to extract and quantitate some of the antioxidants from a food matrix. Validation data produced at LFI showed that the method could be used reliably in our own laboratory. Transfer of the method to other laboratories was not successful, but it should be noted that only one laboratory actually returned results using this method. Whilst this allows the possibility that in other laboratories, or with sufficient training, the method might be shown to be reliable, it also suggests that a simpler method would be preferred for routine use. The development of selective MIPs might have been one way of simplifying the extraction procedure, but work carried out in this project has not succeeded in producing polymers that can be used for solid phase extraction.

ACKNOWLEDGMENTS

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APPENDICES

Appendix 1: SOP for HPLC analysis

HPLC METHOD FOR ANALYSIS OF ANTIOXIDANTS

Method developed from IUPAC method 2.642.

1 Aim

To separate and quantify the antioxidants BHA, BHT, propylgallate, dodecylgallate and TBHQ.

2 Principle

The antioxidants in a mixture are separated by HPLC. Quantification is carried out by the preparation of a calibration curve using standards.

3 Reagents

3.1 Acetonitrile, HPLC grade

3.2 Methanol, HPLC grade

3.3 2-propanol, HPLC grade

3.4 Glacial acetic acid, analytical grade

3.5 Standard solvent
1:1 (V/V) 2-propanol (3.3)/acetonitrile (3.1)

3.6 Antioxidant standards: BHA, BHT, propyl gallate, dodecyl gallate, TBHQ

3.6.1 Antioxidant stock solutions
Weigh accurately about 25 mg of each antioxidant into separate 25 ml volumetric flasks. Make to volume with standard solvent (3.5).

3.6.2 Antioxidant standard solution 1 (approx. 20µg/ml)
Pipette 1 ml of each stock solution into a 50 ml flask. Make to volume with standard solvent (3.5).

3.6.3 Antioxidant standard solution 2 (approx. 10µg/ml)
Pipette 0.5 ml of each stock solution into a 50 ml flask. Make to volume with standard solvent (3.5).

3.6.4 Antioxidant standard solution 3 (approx. 5µg/ml)
Pipette 0.5 ml of each stock solution into a 100 ml flask. Make to volume with standard solvent (3.5).

3.7 Mobile phase A
5% (V/V) acetic acid in distilled, deionised, filtered water

3.8 Mobile phase B
Methanol (3.2): Acetonitrile (3.1) :acetic acid (3.4) in the ratio 50:47.5:2.5

4 Apparatus

Usual laboratory equipment, and in particular:

4.1 High performance liquid chromatograph consisting of an HPLC pump capable of producing a gradient, and injector capable of 10 µl injections.

4.2 UV detector system to measure absorbance at 280 nm.

4.3 C18 HPLC column 15 cm x 4.6 mm with guard column.

5 Procedure

HPLC conditions are as follows:

Temp: 25 ºC Injection volume: 10 µl Flow rate 2.0 ml/min

Detection at 280 nm

Gradient programme:

Time	%A	%B
0	70	30
11	0	100
16	0	100
17	70	30
20	70	30

Notes
Under these conditions, the standards were found to elute in the following order:

propyl gallate
TBHQ
BHA
BHT
lauryl gallate

Appendix 2: SOP for TLC analysis

TLC method for analysis of antioxidants

Method developed from the method described in Endean, M.E. (1976). "The detection and determination of food antioxidants - a literature review". Leatherhead Food RA. Scientific and Technical Surveys No. 91.

1 Aim

To separate the antioxidants BHA, BHT, propylgallate, dodecylgallate and TBHQ.

2 Principle

Separation of antioxidants by thin layer chromatography.

3 Reagents

3.1 Glacial acetic acid A.R.

3.2 Petroleum ether 40-60

3.3 Ferric chloride A.R.

3.3.1 5% aqueous ferric chloride
Weigh 5g $FeCl_3$ into a 100 ml flask. Make to volume with water.

3.4 Potassium ferricyanide A.R.

3.4.1 5% aqueous potassium ferricyanide
Weigh 5g $K_3Fe(CN)_6$ into a 100 ml flask. Make to volume with water.

3.5 2,6 Dichloro-p-benzoquinone-4-chlorimine

3.5.1 Gibbs' spray reagent
Weigh 0.1g 2,6 dichloro-p-benzoquinone-4-chlorimine. Dissolve in 20 ml absolute ethanol. This reagent should be freshly prepared immediately before use.

3.6 Methanol A.R.

3.7 Absolute ethanol

3.7.1 80% ethanol
Mix 4 parts absolute ethanol with 1 part distilled, deionised water

3.8 Developing solvent
Mix 5 parts acetic acid (3.1) with 16 parts petroleum ether 40-60 (3.2)

3.9 $K_3Fe(CN)_6/FeCl_3$ spray reagent

Take 1.5 ml each of 5% aqueous ferric chloride (3.9) and 5% aqueous potassium ferricyanide (3.10). Make up to 20 ml with 80 % ethanol (3.7.1). This reagent should be freshly prepared immediately before use.

3.10 Standard antioxidants: BHA, BHT, propyl gallate, octyl gallate, dodecylgallate, TBHQ

> **3.10.1** Solutions of standard antioxidants
> Prepare solutions containing 0.1% of each antioxidant in methanol, and also a mixed standard.

4 Apparatus

Usual laboratory equipment, and in particular:

4.1 Pre coated silica gel TLC plates e.g. Machery Nagel SIL-G25, 0.25mm layer

4.2 Developing tank for thin layer chromatography, fitted with a ground glass lid.

4.3 Drying oven capable of heating to 130 ºC

4.4 Syringe, capacity 20µl

4.5 Dessicator

4.6 Hot air dryer

4.7 Spray apparatus

5 Procedure

Line the chromatography tank with paper and add developing solvent to a depth of approximately 1cm. Place the lid on the tank and allow to equilibrate for 3 hours.

Activate two thin layer plates in the oven at 130 ºC for 1 hour. Remove from the oven and place in the dessicator to cool.

Score a line 17 cm from the base of the plates. Inject 5 µl of each standard antioxidant solution 2 cm from the base of the plate at 2cm intervals, producing the same spotting pattern on each plate. Rinse the syringe thoroughly with methanol between injections. Dry the spots using a hot-air dryer then cool the plates in a dessicator.

Develop the plates in the tank until the solvent has reached the line scored 17 cm from the base of the plate. Remove the plates from the tank and air dry in a fume cupboard. Spray one plate with the $K_3Fe(CN)_6/FeCl_3$ reagent. The antioxidants are revealed as

blue spots on a yellow-green background. The background will eventually also turn blue (after about three hours) so that the spots can no longer be seen. Spray the other plate with Gibbs' reagent and place in an oven at 103 ºC for 10 minutes. The antioxidants will show up as characteristically coloured spots on a white background.

Antioxidants extracted from foods can be identified by comparison of Rf values and colour with those of the standards.

Notes
The following describes the results seen at LFRA:

Antioxidant moving furthest from origin:	BHT
	BHA
	Dodecyl gallate
	TBHQ
	Octyl gallate
Antioxidant moving least from the origin:	Propyl gallate

All antioxidants gave blue spots with $K_3Fe(CN)_6/FeCl_3$ reagent. With the Gibbs reagent, the gallates all gave red/brown spots, BHT gave a red spot, BHA gave a red/orange spot and TBHQ gave a dark red spot.

Appendix 3*:* Recipes used in the production of foods with known concentrations of antioxidants

Cake mix:

Ingredient	**Weight (g)**
Flour	277.48
Fat / shortening	56.63
Granulated sugar	226.52
Icing sugar	96.25
Starch	18.13
Sodium bicarbonate	7.91
Salt	6.58
SAPP	2.87
SALP	3.71
MCP	2.66
Xanthan	1.26

Propyl gallate:	5.69 mg (equivalent to 100 mg/kg fat)
BHA:	5.65 mg (equivalent to 100 mg/kg fat)

Mayonnaise:

Ingredient	**%**	**Ingredient**	**%**
Water	39.75	Modified starch	4.50
Vinegar	3.20	Salt	1.80
Sugar	3.50	Potassium sorbate	0.10
Mustard powder	0.15	Egg yolk	7.00
Oil*	40.00		

* Oil without antioxidant was used to prepare a control sample. For preparation of a sample with antioxidant, oil with the following antioxidant concentrations was used:

BHT:	76 mg/kg	BHA:	57 mg/kg
lauryl gallate	55 mg/kg	propyl gallate	53 mg/kg

Extruded snacks:

Ingredient	**%**
Maize grits	75.0
Wheat flour	20.0
Sucrose	5.0

Oil and water were added to this powder as it passed through the extruder. Due to the process employed, the fat content of the final product had to be measured analytically, and could not be determined from the amount of oil used.

Oil without antioxidant was used to prepare a control sample. For preparation of a sample with antioxidant, oil with the following antioxidant concentrations was used:

BHA:	63 mg/kg	Lauryl gallate:	51 mg/kg
Propyl gallate:	64 mg/kg		

Appendix 4: SOP for extraction of antioxidants from foods

DETERMINATION OF THE ANTIOXIDANTS BUTYLATED HYDROXYTOLUENE (BHT) AND BUTYLATED HYDROXYANISOLE (BHA) BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reagents

1. Diethyl ether (purchased as stabilised with ethanol)
2. Anhydrous sodium sulphate (AR)
3. Nitrogen
4. Acetonitrile (HPLC grade or glass distilled).
5. Hexane (HPLC grade or glass distilled).
6. Hexane saturated with acetonitrile.
   Shake an equal volume of hexane and acetonitrile in a separating funnel, allow to separate, and keep the upper level.
7. 2-Propanol (HPLC grade or glass distilled).
8. Standard solvent 1:1 (v/v) mixture of 2-propanol and acetonitrile.
9. Butylated Hydroxytoluene (BHT) (Sigma)
10. Butylated Hydroxyanisole (BHA) (Sigma)

Equipment

1. Magnetic stirrers.
2. Rotary evaporator.
3. Analytical balance capable of weighing to 4 decimal places.
4. 20^0C water bath.
5. Hand held calibrated pipette or dispenser.
6. Glass syringe (Preferably 50 ml).
7. Ice making facility.
8. Filter papers: Whatman GF/A (glass fibre), size 150mm, Whatman 541 (Hardened), size 125 mm.
9. NESCOFILM (Nippon Shoji Kaisha Ltd, Osaka, Japan, available from general laboratory suppliers).
10. Syringe filters: Whatman 13mm disposable, 0.45um pore size (or equivalent).
11. A normal supply of laboratory glassware etc including: conical flasks (Quickfit), round bottom flasks (Quickfit), separating funnel, beakers, funnels, measuring cylinders, disposable plastic syringes etc.

Procedure

1. Weigh between 9.5000g and 10.5000g of sample into a 250 ml Quickfit conical flask.

2. Add 100ml of diethyl ether, a magnetic flea and place in a small plastic reservoir full of ice standing on a magnetic stirrer.

3. Stir for 1 hour, occasionally swirling to remove sediment stuck to the side of the flask, and replacing the ice as necessary to keep the sample cold.

4. Remove from the magnetic stirrer, still keeping cold, and allow to settle, (usually about 15 minutes).

5. Carefully remove the stopper, and with the aid of a glass syringe, remove the liquid, disturbing the sediment as little as possible, and filter through an GF/A filter paper, loaded with about 5g of anhydrous sodium sulphate, and stood inside a 541 filter paper for strength. Collect the filtrate in a 250 ml round bottom flask kept cool with ice. Wash the filter paper and contents with about 5 ml of diethyl ether.

6. Place 45 ml of diethyl ether into a beaker and use it to wash the syringe and the residue from the filter paper back into the conical flask containing the sample residue.

7. Repeat stages 3 to 6, but stirring for only 30 minutes,

8. Repeat stages 3 to 5, again with only 30 minutes stirring. Finally wash the syringe into the filter paper.

9. Wash the filter paper with 2 x 10 ml portions of diethyl ether.

10. Rotary evaporate at less than 40^0C, to dryness. Ice can be used to cool the distillate to shorten the time of drying. Try to ensure the drying takes no longer than 10 – 15 minutes.

11. Remove excess solvents with a stream of nitrogen.

12. Gently warm the flask, and wash the fat into a 10 ml volumetric flask with two 2.5ml portions of hexane, saturated with acetonitrile.

13. Wash out the round bottom flask into the 10 ml volumetric flask, with small portions of 2-propanol and make up to volume at 20^0C with the propan-2-ol.

14. Syringe filter into vials ready for the HPLC determination.

15. Refer to the HPLC method sheets for the HPLC conditions.

Precautions / Notes

1. During each of the extraction stages, both the conical and round bottom flasks had nitrogen added and the stopper sealed in place with Nescofilm. (Caution: When the nitrogen is added the stopper may be ejected with considerable force, and must be held in place until the Nescofilm is added).

2. Both the extraction and the filtrate flasks were cooled with ice for as much time as was practically possible between the stages of weighing out the sample, up until placing the samples in vials ready for the HPLC.

3. Additional anhydrous sodium sulphate was added during each filtering stage.

4. For all three extractions the filtrate was collected in the same round bottom flask. If the combined volume is to too large to be rotary evaporated, it may be necessary to rotary evaporate between extractions.

5. The extracted solutions must be run on the HPLC the day of extraction.