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REPORT FD 04/38

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FINAL REPORT

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SUMMARY

1. This report details the findings of Food Standards Agency Project A01036: Development of a validated method for sucrose esters (E473) and sucroglycerides (E474) in permitted foods. These additives are complex mixtures and have a high molecular weight. These factors prevent their direct analysis in complex food samples using readily-available techniques.

2. The analytical strategy adopted therefore was to take advantage of the fact that the additives are soluble in organic solvents. The additives were extracted from foods using a mixture of tetrahydrofuran and ethyl acetate, and the extract freed of any co-extracted free sugars by washing with saturated sodium sulfate. The extract was then hydrolysed, thus liberating free sucrose from any E473 or E474 present. The sucrose was then hydrolysed and assayed by silylation and GC-MS determination of the glucose and fructose derivatives formed.

3. Since the specifications and descriptions of additives E473 and E474 cover a wide range of degrees of esterification and esterified fatty acid types, this presented a challenge for calibration and quantitation in food analysis. Without prior knowledge of the exact composition of any E473 or E474 added to a food sample, the factor used to convert the liberated sucrose content into sucrose ester content was based on the sucrose ester with the lowest sucrose content (i.e. the higher degree of esterification with fatty acids). This approach was adopted because it is conservative and is suitable for making upper-bound estimates of potential additive intake.

4. The new method was applied to the analysis of a range of 8 different food types (bakery wares, sugar confectionery, dairy product, margarine, meat pies and a sauce) spiked with 0.5 to 1% of a mixture of 3 sucrose esters that spanned the hydrophilic/lipophilic balance (HLB) range. The limit of quantification was around 50 mg/kg, which is more than adequate for these additives. The analytical recovery was in the range 73 to 106% with an average of 91%. The precision of the method (rsd) was in the range 6 to 18% (n= 3 to 20 for each food type) with an average rsd of 10.8%.

5. In conclusion, the method determines the content of total sucrose esters in a food sample but it is not rapid and it cannot identify and discriminate between individual sucrose esters when present as a mixture. SuEs are not available as purified standards in the normal sense hence direct quantitative analysis was difficult, requiring the use of single conversion factor. The uncertainty associated with this approach was considered to be large compared to the likely measurement uncertainty. For these reasons, it was agreed that there was no need for an independent laboratory to test the method provisionally, prior to in-house validation of the SOP.

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GLOSSARY

ADI	Acceptable Daily Intake
APCI	Atmospheric pressure chemical ionisation
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
n-BuOH	n-Butanol
CHX	Cyclohexane
CI	Chemical ionization
DMSO	Dimethyl sulfoxide
DCM	Dichloromethane
EFSA	European Food Safety Authority
EtOAc	Ethyl acetate
G6P	Glucose-6-phosphate
GC	Gas chromatography
GP	Gel permeation
FID	Flame ionisation detection
HLB	Hydrophilic/lipophilic balance (value)
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
IHVM	In-house validation matrix
IQC	Internal quality control
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
PAD	Pulsed amperometric detection
RI	Refractive index
RP	Reverse phase
RSD (%)	Relative standard deviation (percent)
SAIB	Sucrose acetate isobutyrate
SFC	Supercritical fluid chromatography
SPE	Solid phase extraction
SOP	Standard Operating Procedure
STDEV	Standard deviation
SuE	Sucrose ester
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TMCS	Trimethylchlorosilane
TMS	Tetramethylsilane
TMSI	Trimethylsilylimidazole
UV	Ultra violet

1. INTRODUCTION

1.1. Sucrose esters of fatty acids (E473) and sucroglycerides (E474) are additives controlled by European parliament and Council Directive 95/2/EC (as amended) [1]. These additives may be used as emulsifiers in a wide range of foods, including bakery products e .g. cakes, beverage whiteners, desserts, , confectionery, sauces and dairy-based drinks. Each Member State has a legal requirement to monitor consumption and usage of food additives (Council Directives 94/35/EC, 94/36/EC and 95/2/EC refer). To fulfil this obligation, the European Commission published a report, which provided an overview of food additive intake in the European Union [2].

1.2. This report highlighted several additives or groups of additives, including E473, needing further review by Member States due to: (i) the theoretical estimated additive intake being close to, or exceeding, the acceptable daily intake (ADI) limit for adults and/or children; (ii) limited existing data or (iii) limited available data for additives permitted at *quantum satis*. The report recommended intake estimates for these additives should be carried out using actual occurrence and consumption data. Therefore a method was needed to measure levels of E473 in foods. Reliable methods of analysis are also needed to assist enforcement. A review of published methods for measurement of E473 in foods indicated that only very limited methods of analysis are available. Therefore this work has developed a method for the extraction and quantitative determination of these additives in foodstuffs.

1.3. Sucrose esters (sucrose esters of fatty acids, sugar esters, SuE) are defined in the regulations as the mono-, di- and tri-esters of sucrose with fatty acids occurring in food fats and oils [3]. They may be prepared from sucrose and the methyl and ethyl esters of food fatty acids or by extraction from sucroglycerides. Sucroglycerides are produced by reacting sucrose with edible fat or oil to produce a similar mixture of SuE with fatty acids together with residual mono-, di- and tri-glycerides from fat or oil (Figure 1).

1.4. These two groups of emulsifiers comprise a range of products from stiff gels, soft solids to white or slightly greyish-white powders. They are sparingly soluble in water but soluble in ethanol and other organic solvents, mineral oil and vegetable oils. SuE and sucroglycerides are permitted in a wide range of foodstuffs under Annex IV of the EU regulations. SuE are also permitted under Annex V as a carrier for food colours and fat-soluble antioxidants, and under Annex VI Parts 1 and 2.

1.5. During 2004, in the light of new studies on short- and long-term toxicity in experimental animals as well as toxicokinetic studies in animals and humans, the European Food Safety Authority (EFSA) Scientific Panel on Food Additives, Flavourings, processing Aids and materials in Contact With Food, evaluated E473 and E474 [4] and Group ADI of 0-30 mg/kg b.w. was established. These additives have been considered several times by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and, in its most recent evaluation was given an ADI of 0-30 mg/kg bw, expressed as sucrose ester content [5].

1.6. Naturally-occurring and synthetic surface-active agents are used in food processing when a decrease in surface tension is required e.g. in the production and stabilisation of disperse systems. Dispersions include emulsions, foams, aerosols and suspensions. A characteristic of most emulsifiers is that they are not single compounds but rather mixtures of several well-defined substances in quantities, which vary according to their origin and manufacture. Thus there is a multiplicity of possible components within this group of emulsifiers depending upon the degree of esterification, the fatty acid chain length and the presence of monounsaturates ($C_{18:1}$). Moreover, they are closely related physicochemically to other groups of permitted emulsifiers (e.g. salts of fatty acid esters (E470a,b; mono- and di-glycerides of fatty acids (E471); sorbitan tristearate (E92),) from which they must be discriminated analytically. Since the listed emulsifiers are not single substances, it is necessary to chemically characterise appropriate reference materials and a range of additive formulations.

1.7. *Review of published methodology*

The quantitative analysis of emulsifiers in food is difficult, primarily because: (i) the multiple components are similar in structure, (ii) most commercial sources of emulsifiers are quite heterogeneous and (iii) emulsifiers can be difficult to extract from foods that contain significant amounts of lipid, starch and/or protein. Several different analytical strategies have been employed for the analysis of SuE as such, but there are very few literature references to their determination in foods.

1.8. Since SuE are liposoluble their quantitative measurement in foods requires differentiation of SuE from other lipids/liposoluble materials. The JECFA specification for SuE contains a method of assay which prescribes solubilization in tetrahydrofuran followed by gel permeation chromatography with refractive index (RI) detection [6]. This method however is only applicable to relatively pure SuE additive formulations. SuE can be determined by high-performance liquid chromatography (HPLC) using reverse-phase (RP) columns and either a methanol/water or methanol/isopropanol solvent system [7]. However, difficulties with obtaining good peak separation were reported when fatty acid esters of glycerol were present. Sucrose polyesters are a related class of emulsifiers that are synthesized by esterifying 6 to 8 of the hydroxyl groups of sucrose with fatty acids and they exhibit similar physicochemical properties to SuE. Methods of analysis for sucrose polyesters based on the direct analysis of the liposoluble fraction have been developed using supercritical fluid chromatography [8] or high performance gel permeation chromatography [9, 10]. While thin layer chromatography (TLC) with dual wavelength scanning has been used [11], RP-HPLC is reported to be superior to TLC for the separation and determination of 18 different SuE and sucrose polyesters, unreacted sugar and other fatty acid esters, using aqueous methanol and UV detection [12]. Moh *et al.* used an aqueous methanol gradient RP-HPLC system for the separation of SuE isomers with light-scattering detection and atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) for confirmation [13].

1.9. Other workers have used an indirect approach following hydrolysis [14,15]. Drake *et al.* extracted the liposoluble fraction from foodstuffs with petroleum ether,

which was then hydrolysed by saponification in methanolic KOH [15]. The free sucrose from the hydrolysate was extracted with water and quantified colorimetrically using a phenol-sulfuric acid test. Koh *et al.* [16] determined the composition of commercial SuEs using TLC and gas chromatography (GC) and subsequently developed an indirect method for their analysis in foods (crackers, Vienna sausage and ice cream) using ion-exchange HPLC with pulsed amperometric detection (PAD) and RI detection of free sucrose following alkaline hydrolysis. The liposoluble fraction of the foods was extracted with a selective mixed solvent comprising ethyl acetate (EtOAc) and tetrahydrofuran (THF) (7:3 v/v), and further purified using diol solid phase extraction (SPE) cartridges prior to hydrolysis. The reported recoveries of SuE ranged from 86-99% and the limit of detection for sucrose using PAD was 4 mg/kg. Such methods however did not discriminate between the different mono-, di- and tri-esters nor did any allow for identification of the fatty acid residues associated with the SuE.

1.10. Specific methods for the analysis of mono- and di-SuE have been developed. Murakami *et al.* determined mono- and di-SuE in food by aqueous acetonitrile gradient reverse-phase (C_8) HPLC with UV detection (230nm) [17]. Samples were extracted into THF, the extracts purified by liquid chromatography (LC) on silica gel and derivatised with dinitrobenzoyl chloride. SuE were reported to be selectively determined without interference. Recoveries of SuE added to various foods was 75-80% and the detection limit was 10 mg/kg for each ester. High temperature (400°C) GC-FID has been used to analyse SuE as trimethylsilyl derivatives [18]. The method performance was checked by

supercritical fluid chromatography (SFC) and direct chemical ionisation MS of standard compounds, and allowed high molecular weight carbohydrate derivatives (1500 Da) to be determined whilst maintaining high separation efficiency.

1.11. More recently, Uematsu *et al.* have developed a GC/ GC-MS method for the determination of sucrose monoesters of fatty acids and sucrose acetate isobutyrate (SAIB) in food additive premixes and some foods [19]. A reverse-phase octylsilane (C_8) SPE column was used to extract mono-SuE and SAIB from aqueous samples. A silica-gel SPE column effectively removed diglycerides from mono-SuE in THF extracts of solid / fatty samples. Mono-SuEs based on fatty acid residues of C_{12}, C_{14}, C_{16}, C_{18} and $C_{18:1}$ were acetylated and determined using wide-bore capillary GC with splitless injection and flame-ionization detection. Peak identities were confirmed using GC-MS where the mono-SuEs were characterised using 6 fragment ions. The analyte peaks were clearly separated from acetylated derivatives of sucrose, tocopherols, diglycerides and triglycerides.

1.12. The key aspects of available methods were taken forward for development whilst ensuring that the developed method would be readily accessible to a wide base of users. The analytical strategy was based on selective solvent extraction of SuE from food followed by a clean up procedure to remove unwanted co-extractives using solvent partition and/or SPE technologies. End determination methods considered included colorimetry and gas chromatography, as these offered a lower degree of complexity and were considered as relatively easy to

roll out to other laboratories. Technologies such as size exclusion and LC-MS were therefore excluded. It was recognised that analysis of intact SuE would be complicated by the wide range sucrose esters that may be present, so hydrolysis of SuE and measurement of the released sucrose was investigated, with derivatisation and gas chromatography considered the upper limit of desired complexity for this aspect.

2. PROJECT OBJECTIVES

The objectives were to:

2.1. Obtain, purify (if necessary) and chemically characterise E473-4 emulsifiers for use as reference materials, identify suitable analytical marker(s) and discriminate between the target analytes and other emulsifier types or related compounds (especially triglycerides) that may be present in emulsifier formulations and foods.

2.2. Develop an analytical method for the determination of the E473-4 in foods as defined in 95/2/EC (as amended).

2.3. Validate the developed method internally. Ensure that the method is sufficiently accurate, precise and robust for the determination of E473-4 in the full range of foodstuffs listed in EU Directives. The target level of determination will be one tenth of the lowest maximum permissible level i.e. 200 mg/kg.

2.4. Identify and prepare a homogeneous sample for use as in-house validation matrix for internal quality control (IQC) purposes.

2.5. Prepare an in-house standard operating procedure (SOP) sufficient in scope to cover all E473-4/food commodity combinations as laid down in legislation.

2.6. Analyse an agreed number of generic 'test' food samples in order to prove the method

2.7. Validate the SOP using a suitable independent laboratory in order to prove that the method is technologically transferable and repeatable.

2.8. Produce a final report describing the method development, the final SOP and the performance characteristics of the method.

3. METHODOLOGY

The methods are described briefly below. The full standard operating procedure is given in Annex 1.

3.1. For the purposes of this study, published methods were improved in general terms by reducing the analysis time and minimising exposure of extracts to aggressive reagents. The steps taken to achieve this involved minimising the number of times that extracts were transferred between different sets of glassware, and reducing evaporation times and temperatures when removing solvents and during derivatization of sugars for GC-MS analysis. The reagents used were all of recognised analytical grade except for water, which was HPLC grade.

4. ANALYSIS OF SAMPLES

4.1. *Sampling regime*

Since E473 and E474 do have widespread use in the UK, retail outlets in the York area were scouted for likely commodities, based on those permitted to contain these additives [1].

4.2. *Sample preparation and extraction*

Briefly, 0.25-1.0 g foodstuff homogenate was weighed accurately into a 40 ml glass vial and 10 ml of THF: EtOAc (7:3 v/v) added. Samples containing significant amounts of starch and/or gelatine were dispersed with 2 ml of water before adding the solvent. The sample/solvent mixture was shaken thoroughly or if necessary, blended with an Ulta-Turrax probe for 30 seconds. The vial was placed on a shaker for 30 minutes then centrifuged at 700-1000 x *g* at 20°C for 5 minutes and the supernatant carefully transfered to a 40 ml vial. To remove free sucrose and other sugars, 10 ml of saturated sodium sulfate solution was added, the vial placed on a shaker for 20 minutes, centrifuged at 700-1000 x *g* at 20°C for 5 minutes and the upper (organic) phase transferred to a 12 ml vial. The solvent was removed using a nitrogen blow-dry apparatus at 70°C and 2 ml of n-butanol (n-BuOH):cyclohexane (CHX) (1:1) added. After shaking to dissolve, 2 ml of 4M sodium hydroxide was added and the mixture placed on a shaker for 40 minutes to hydrolyse the sucrose esters. The lower aqueous layer was transferred to a 4ml vial and a 100 µl aliquot taken and placed in a separate 4 ml vial. To this was added 100 µl of internal standard solution (0.1 mg/l fucose) and 125 µl of 6M hydrochloric acid to hydrolyse the sucrose to glucose and fructose. The solvent was removed using a nitrogen blow-dry apparatus at 50°C until the residue was dry, whereupon 250 µl of silylating reagent (Tri-Sil® Z in pyridine) was added and the mixture heated at 70°C for 30 minutes. After cooling, the mixture was transferred to a 300 µl vial for GC-MS analysis.

4.3. *GC-MS conditions*

GC-MS was carried out using a model HP 5890 capillary GC with split/splitless injector (Agilent UK, Bracknell), model HP 5971 mass selective detector and HP ChemStation data processing facility (Agilent UK, Bracknell). The following conditions were used:

Column	HP-5 ((5%) phenyl-methylpolysiloxane) MS fused silica column, 30m x 0.25mm x 0.25µm (Agilent UK, Bracknell)
Oven program	80°C initial temp, hold for 1 min. then 5°C/min to 200°C, hold for 3 mins.
Injection volume	1µl
Split mode	Splitless
Inlet temp	280°C
Carrier gas	Helium at 1ml/min
Tranfer line temp.	250°C
MS detection	Selected ion impact mode, ions monitored (m/z) 204 and 217

5. QUALITY CONTROL

5.1. *Method validation*

Whilst low limits of determination were not a primary consideration of this project, the method was developed with sufficient scope to allow for the determination of *ca.* 200 mg/kg sucrose ester.

5.2. *Sucrose ester reference materials*

Fourteen commercially available sucrose ester materials were obtained from European (4) and Japanese (10) manufacturers. These comprised samples with hydrophilic-lipophilic balance (HLB) values ranging from 1 to 16 (Table 1).

5.3. *Standards and GC-MS calibration*

Selected sucrose esters were used to determine the efficacy of the extraction and hydrolysis at the appropriate stages of method development. GC-MS measurement was calibrated using sucrose solutions containing a fixed amount of fucose internal standard, which were hydrolysed and derivatised in the same way as sample extracts. Full details are given in the SOP (Annex 1).

5.4. Each calibration set was run at the beginning or end of each analytical batch. A calibration graph was constructed by plotting the ratio of the standard summed peak areas for glucose and fructose to that of the peak area for the internal standard, against concentration and the regression equation calculated. The linear regression coefficients obtained from batch calibration plots were consistently 0.990 or greater. The preparation and testing of the in-house validation matrix is given in 6.24.

6. RESULTS AND DISCUSSION

General points

6.1. All but one of the initial objectives agreed for this project have been achieved. It was agreed that independent laboratory evaluation of the method would not be undertaken (see below). Extensive development/adaptation was undertaken on

the method for the extraction of SuEs, which span a broad range of application in water- and oil-soluble or dispersible food commodities, for which published methods were not available.

6.2. One IHVM was prepared which was spiked with 3 SuEs covering the HLB range and was suitable for the foodstuffs examined (6.24). Due to the broad range hydrophyllic/lipophyllic properties of the SuEs included in the scope of this project, development of the extraction regime was not straightforward and its application to the analysis of permitted foodstuffs was analytically challenging. Retail food commodities containing E473 or E474 proved difficult to locate (only one sample was obtained) hence quality assurance data were obtained by the less desirable route of spiking experiments. Moreover, the method is not rapid and the sucrose esters themselves are not well-defined analytes available as 'pure' standards in the normal sense. Rather, they are mixtures manufactured to varying degrees of esterification to meet a particular technological need (i.e. HLB number). This made direct quantification difficult hence a single conversion factor was used to calculate sucrose ester content from measured glucose and fructose. Since the uncertainty that is explicit in this assumption is greater than the likely measurement uncertainty, it was agreed that there was no need for an independent laboratory to test the method provisionally, prior to in-house validation of the SOP.

6.3. *Method development*

The range of SuEs available for this work (Table 1) are not all freely soluble in any one solvent recommended in literature methods. Since SuEs are used in

food at high concentrations, a solvent system was required that could dissolve high concentrations of SuEs to allow extracts to be transferred. A value of 100 mg/ml was chosen as the required minimal solvating power. SuE are reported to dissolve in chloroform, THF, water/methanol (1:1), diethylether/EtOAc (3:7) and dimethyl sulfoxide (DMSO), while being insoluble in hexane. SuE can reportedly be extracted from aqueous organic solutions with n-BuOH /CHX (1:1) and washed with water [23]. Manufacturers solubility tables suggested that most SuE solutions require heating to 75°C to aid solubility; propylene glycol was the only solvent reported to achieve dissolution of all listed SuEs at this temperature [27].

6.4. As expected, the more hydrophobic SuEs were soluble in dichloromethane, while the more hydrophilic SuEs were soluble in acetone. A 1:1 mixture of DCM/acetone was ineffective at increasing the solvating range. Most SuEs dissolved in DMSO (100 mg/ml), but many formed froth while shaking to dissolve (Table 3). This froth later sets as a gel; hence DMSO was clearly an unsuitable solvent for most SuEs. Similarly methanol/water (1:1) failed to dissolve SuEs at ambient temperature. Certain solvent mixtures were considered to be suitable for specific roles in the method work-up other than for stock solution preparation. Two solvent mixtures which dissolved all of the SuE standards at high concentrations (100 mg/ml) were n-BuOH/CHX (1:1) and THF/acetone (1:1) (Table 3). Concentrated stock solutions required warming to 50°C, or sonication to ensure complete dissolution (especially S-170).

6.5. *Detection of intact SuEs*

The recommended industry method for assessing the presence of SuE in ester mixes is gel permeation (GP) HPLC (JECFA, 1997). This method resolves the SuE into separate mono- di- and tri-ester peaks and uses RI detection. This method was not assessed in this project on the grounds of lack of applicability in a wide range of laboratories. Reverse phase HPLC with or without MS, RI and evaporative light scattering detection has been attempted [19,30]. Due to the large number of peaks in each SuE, these approaches would appear to work best on standards rather than food extracts, so they were not considered further.

6.6. Food extracts spiked with SuE were evaluated by TLC but this technique required relatively clean extracts in order to visualise the SuE spot. Sample extract concentrates or standards spotted onto silica gel coated glass plates (5 x 5 cm) and developed by elution in chloroform/methanol/water/acetic acid (70:26:2:2), showed SuE elution with high R_f values (0:68-0.79). Except for free sucrose (0.14), certain detergents, SAIB, sucrose acetate and sucrose benzoate, all eluted at the solvent front. Mixing 2 portions of chloroform with 1 portion of this mix (diluting to 8% methanol) slowed the elution of SuEs, with SAIB, sucrose acetate and sucrose benzoate again eluting at the solvent front. The standard SuEs all gave Rf values of between 0.25-0.28. A second series of fainter lines at RF(0.54) suggested the presence of di-esters.

6.7. TLC spots were visualized by immersion in vanillin: H_2SO_4: water (5:5:90) after heating to 100^0C on a hot block. Purple spots were the first to develop, followed by grey-coloured SuE spots, which often eluted as dumbell-shaped

double spots. Since this was a non-specific detection system, 2-5 µl of the SuE standard solutions also spotted onto the TLC plate at 50 mg/ml concentration for identification purposes.

6.8. *Carbohydrate assays*

Although various test kits and reagents are readily available for detecting sugars, mainly as glucose, chemical reaction was favoured over enzymatic test kits for ease of use and availability, without the limitation of a fixed shelf life. The phenol-sulfuric acid and anthrone carbohydrate assays were examined and the latter selected as the most suitable for this work. Details of the experimental findings are given in Annex 2.

6.9. *Removal of free sucrose*

To check the removal of free sucrose from SuE standards in various solvent mixtures, SuE Mono-P (10 mg, 100 µl) in organic phase was diluted to 5 ml with 1:1 n-BuOH/CHX. Separate aliquots were mixed with an equal volume of water, saturated sodium chloride or sodium sulfate solutions.

6.10. The water/solvent system did not readily separate into distinct phases, even after the solvents were mutually presaturated before addition of SuE. The aqueous phases from each experiment were removed and tested with the anthrone reagent (Annex 2). The first extract from water gave an intense reaction (blue colour), while the two salt solutions produced a light blue colour reaction. Secondary extracts gave a negative reaction by the anthrone test. As the amount of free sucrose was fixed, the pure water extract was examined by TLC where it

was observed that the anthrone reactivity was due to the SuE Mono-P and not to free sucrose. It was concluded that water is not suitable without addition of a salt for washing sucrose out of organic phase while retaining SuEs in the organic phase. Moreover, since the sodium chloride solution reacted with sulfuric acid to produce chlorine (fumes), and the pure water resulted in loss of SuE, saturated sodium sulfate was taken forward as the most suitable washing solvent. This partitioning experiment was repeated with EtOAc/THF (1:1), but this solvent system was found to be inefficient.

6.11. *Hydrolysis*

A number of reports suggest simple base-alcohol mixtures may be used to affect hydrolysis of SuE to sucrose [15,16,30]. While this method may work for incurred SuE where triglcyerides and hexane assist transport of the SuE into the reagents, it was not successful for pure (50 mg) SuE standards as described by Tsuda [30]. Sucrose esters Mono-P, L-595, S-170, S-1170 and PS750 (50 mg) were placed in volumetric flasks with 1M NaOH (50 ml). After sonication and standing overnight, the SuE were not dissolved or hydrolysed.

6.12. Hydrolysis could therefore realistically be achieved only when the SuEs were in solution. Heating under reflux as described by Koh *et al.* did not achieve dissolution in this work [16]. Even when the SuEs (Mono-P, L-595, S-170, S-1170, PS750 were added as dissolved solutions (THF/acetone 1:1) into the hydrolysis reagent, a precipitate quickly formed while being heated under reflux. There was a second issue of retaining the hydrolysed sucrose in solution, since any observed solid could be either undissolved SuE or sucrose. Moreover,

when short-chain SuEs (L-1695, L-595, SuE C12:0) were pre-dissolved in THF/acetone and dried as a thin film (100 mg) then heated (100°C 1h) in ethanol/water (5 ml, 1:1 with 0.5 M NaOH), they dissolved (as did sucrose) and hydrolysed readily. When neutralised with *ca* 3 ml HCl (1 M), sucrose solutions remained clear, while SuE solutions produced a fine colloid. When volumes were reduced by *c.a.* half under a nitrogen stream and acidified (250 µl conc HCl), a layer of fatty acid was formed on top of the aqueous mixture. The free fatty acid readily dissolved in and was extracted into ether (5 ml). It was concluded that while this hydrolysis method worked to an extent, it was not robust enough to hydrolyse SuEs of more limited solubility.

6.13. It was clear that a solvent system that could dissolve all reactants and products was required. Sucrose did not dissolve in ether, ethanol, ACN/water, n-BuOH/CHX or in THF/acetone, but did dissolve in water/ethanol (0.5 M NaOH) at 20 mg/ml.

6.14. n-BuOH/ CHX /water has been used as a purification step in the synthesis of SuEs (Crues, 2001), combing this with a salt wash, SPE and the anthrone reagent test, a much simplified and more robust hydrolysis method was produced. Briefly, *ca.* 1 mg each of longer chain SuEs, S-1170 (C_{18}) and (L-1695, L-595, SuE $C_{12:0}$) were dissolved in n-BuOH/c-Hexane (2 ml). Each SuE solution was washed with saturated sodium sulfate solution (2 ml) to remove free sucrose. Aqueous sodium hydroxide (1M, 2 ml) was added to the remaining organic phase and the mixture placed on a vial roller. Aliquots were removed at specific time points and allow to settle out into two phases (*ca* 1 min). The

lower aqueous phase was removed and passed through an Oasis SPE cartridge (dry). One ml of hydrolysate was added to anthrone reagent (2 ml), allowed to stand for 10 min and pipetted into disposable UV cuvette. It was not necessary to neutralise the sucrose in dilute aqueous hydroxide as the anthrone reagent provided necessary buffering. Hydrolysis was rapid (Figure 2), hence there was no reason to change from the suggested 40 min reaction time. Hydrolysis released a different ratio of fatty acids for a specific SuE, which were observed as characteristic peaks in the GC chromatograms of crude hydrolysis mixes that had not undergone cleanup.

6.15. *Derivatisation for GC analysis*

The well-established technique of silylation was used to derivatize SuE and free sugars prior to GC analysis, details of which are given in Annex 2. TMSI (Trimethylsilylimidazole) as *Tri-Sil® Z* proprietary reagent was selected as the most suitable silylation reagent.

6.16. *Solid phase extraction (SPE)*

Due to the limited number of solvents that could be used as carriers, development of an SPE method for the available range of SuEs was predictably difficult. Experimental details are given in Annex 2. Application of this technique to the cleanup of food extracts is discussed in 6.19.

Method refinement and application to foodstuffs

6.17. *SuE extraction*

The SOP established through experimentation on SuE standards was used as the basis for carrying out further method development on foodstuffs, especially where particular problems had been experienced. Samples of sausage roll and pork pie containing E473 as a declared ingredient were used as test samples along with similar samples containing no E473 or E474 for use as extraction 'blanks'. Experiments were designed to determine whether the clean up stages were effective at removing free sucrose and lipid, whilst at the same time allowing the SuE present to be isolated, purified, hydrolysed and analysed by GC.

6.18. TLC analysis of extracts showed that free sucrose was successfully removed from the sample and that the SuE was retained. Analysis of extracts from samples spiked with a small range of standard SuEs (S170, S1570 and E473 stearate) were also showed successful extraction.

6.19. *SPE cleanup and hydrolysis*

To affect an acceptable removal of extraneous lipid and other interfering components, the residue was taken up in dichloromethane and loaded on to a solid phase (diol) extraction cartridge. Lipid was removed by washing with hexane: ether (1:1), the sucrose esters eluted with tetrahydrofuran and the eluate blown dry under nitrogen. Free sucrose and other potential interfering components were then removed by partition using n-BuOH: CHX and saturated sodium sulfate. However, consistently low recovery rates were found for certain

SuE standard materials taken through various stages of the SOP. The SuE hydrolysis and sucrose hydrolysis procedures of the SOP were considered to be the most likely stages to incur analyte losses, so a set of experiments was undertaken on three SuE standards of low, medium and high HLB values: S170 (HLB=1), S570 (HLB=5) and L1695 (HLB=16) to determine efficacy. The sucrose hydrolysis stage was not found to be the main cause of low recoveries, although the acid concentration was increased from 1M to 6M to increase hydrolysis efficiency.

6.20. Modifications to the base-mediated biphasic ester hydrolysis stage improved recovery of all 3 SuEs, especially those of medium and high HLB value. The low HLB value SuE showed a recovery rate of 62% through this stage. Re-examination of the SPE cleanup procedures afforded little improvement, even when solvents such as hexane, ethylacetate:THF (7:3) and n-BuOH: CHX (1:1) were used to try and improve the recovery of SuEs from the SPE cartridge. Mixtures of THF:methanol used to elute the SPE cartridges showed that the an 80:20 mixture was better for the low and high value HLB SuEs, whereas a 95:5 mixture was superior for elution of the medium HLB value SuE (Table 5). Clearly, several factors affected retention of SuEs on the SPE phase, not just solvent polarity, which served to compound the problem. At the risk of obtaining less pure extracts (i.e. contaminated with lipid) to improve recoveries, the SPE cleanup stage was removed.

6.21. *GC-MS analysis*

Since the aim of this project was to develop a method that would be readily accessible to a wide base of users, technologies such as size exclusion and LC-MS were excluded as being too complex to roll out easily to other laboratories. Derivatization and gas chromatography was considered the upper limit of desired complexity. In order to facilitate simple GC analysis, the sucrose esters were hydrolysed with sodium hydroxide to liberate free sucrose, which was then acid hydrolysed to glucose and fructose, and analysed by GC-MS following silylation. Fucose (6-deoxy-D-galactose) was used as an internal standard prior to derivatization. However, preliminary GC-MS analysis of standards showed that the hydrolysis conditions produced variable levels of sucrose and its hydrolysis products; glucose and fructose. The aqueous alkaline SuE hydrolysate was therefore treated with excess acid (6M HCl, 125µl) prior to water removal in order to hydrolyse the sucrose fully to glucose and fructose. As a consequence, the GC-MS analysis of standards revealed several anomer peaks for both glucose and fructose. These were well separated from one another, as well as from those due to intact sucrose and the fucose internal standard. Thus it was possible to calibrate the GC using sucrose standards by summing the total number of peaks due to glucose and fructose. Fructose peaks (2) at *ca.* 21.6 and 21.8 mins were monitored at m/z 217, glucose peaks (3) at *ca.* 23.5, 23.8 and 25.4 mins were monitored at m/z 204, and fucose internal standard peaks (2) at *ca.* 18.5 and 19.5 mins were monitored at m/z 204. Calibration was achieved by dividing the total analyte peak areas for sucrose standards by the internal standard peak areas to obtain the peak area ratios and plotting the peak area ratio values against the concentration of sucrose in µg/ml.

6.22. *Conversion factor for sucrose to SuE*

Preliminary analysis of 3 sucrose ester standards using this method gave recovery values for liberated sucrose (i.e. theroretical/observed * 100) of 76-103%. However, the theoretical values are based on mono-, di-, tri- and poly-ester contents of SuEs, which are crude estimates taken from the manufacturers specifications. Therefore, before the quantitative aspects of the SOP could be finalised, the most appropriate way to express the results i.e. in terms of SuE content based on sucrose measured, needed to be ascertained. From compositional data supplied by industry, factors for calculating the original SuE content of a foodstuff based on the measured sucrose (as glucose + fructose) were used to calculate recoveries of SuE standards analysed using the SOP. However, lower than expected recoveries were observed, especially for SuEs with lower HLB values (i.e. highly non-polar types).

6.23. The compositional data appeared to be in conflict with that provided on the manufacturers website. For example, according to the original data, sample S170 (HLB 1) should comprise 100% sucrose distearate, which should give a theoretical sucrose contribution of 39.1% by weight. However, the website graph from the same manufacturer (Figure 3) clearly shows that a SuE with HLB of 1 should comprise 2% monostearate, 2% distearate, 8% tristearate, 14% tetrastearate, 26% pentastearate, 22% hexastearate, 20% heptastearate and 6% octastearate. Based on these values, the theoretical sucrose contribution is 20.9% i.e. almost half of that calculated using the original data. Clearly, such a difference will have a significant effect on calculated recoveries of SuEs, especially those of lower HLB value. Thus, using the values shown in Figure 3,

the theoretical sucrose contributions for the remaining HLB values were calculated and plotted on a graph (Figure 4). The resultant curve was logarithmic ($R^2 = 0.9874$) and followed the equation:

$$y = 10.81 \times \text{Ln}(x) + 22.259$$

Using this formula, a table of HLB value versus theoretical sucrose contribution was constructed (Table 4). These data show that the conversion factor for a SuE of unknown composition extracted from a foodstuff could lie between 22.3 and 52.2%. Thus in an extreme hypothetical case, the use of the wrong conversion factor would incur an error of *ca.* 2.3 times (or reciprocal thereof), which is clearly not acceptable. The calculated arithmetic mean conversion factor conveniently falls around the centre of the HLB scale and could therefore be used as an 'average' conversion factor, the incurred error notwithstanding. As an example, Table 5 shows the recoveries of the three SuEs discussed above through SPE cleanup using mixtures of THF:methanol using the original, improved and 'average' conversion factors. However, the use of a conversion factor for higher esters (low HLB) would seem appropriate because it would incur an overestimation of the SuE content. Thus a factor of 4.5 is recommended to convert from sucrose content to SuE content (i.e. approximately 100/22.3). This approach was adopted because it is conservative and is suitable for making upper-bound estimates of potential additive intake.

6.24. *Preparation and homogeneity testing of IHVM*

A pack of (6) mini Cornish pasties were finely chopped in a food processor. To 500 g was added 200 mL of water to facilitate homogenisation. A 250 g portion of the homogenate was spiked with a mixture of 3 different commercial sucrose esters dissolved in THF:EtOAc (7:3 v/v). The spiking details are given in Table 2. The total SuE content was 6060 mg/kg. The IHVM was stored at –20°C.

6.25. Sucrose was spiked into blank IHVM at 1 and 20 mg/g and analysed using the SOP to determine the efficacy of free sucrose removal. No sucrose was detected above the limit of quantitation (see 6.29) in either sample and no peaks due to sucrose were observed in the chromatogram from a reagent blank. An example chromatogram from blank IHVM spiked with 20 mg/g sucrose is shown in Figure 5. Two batches of ten replicate sub-samples of the IHVM were extracted using the SOP on two separate days. The results are given in Table 6.

6.26. Statistical analysis of the mean recovery figures from each batch showed that they were not significantly different (t-test at p=0.05). A control chart was established over the +/- 2 s.d. range based on a mean recovery of 81.1 % (Figure 6) for use in subsequent batch analyses of test samples. An example of a chromatogram obtained from the IHVM is shown in Figure 7.

6.27. Analysis of the blank IHVM (before spiking) showed that the saturated sodium sulfate / solvent partition was effective at removing free sucrose present at high level and that co-extracted lipid from the reasonably high fat product used to prepare the IHVM (Cornish pasty) did not interfere significantly with the

subsequent hydrolysis, derivatization and GC-MS stages. The results from the repeat analyses show that the IHVM was homogeneous and that the analysis was repeatable.

6.28. *Analysis of food samples*

The application range for sucrose esters may be categorized into 5 main groups, from which it was agreed that at least one commodity would be selected to cover the range of applications stated by the major manufacturer. Since SuE usage in the UK is very sparse, commodities were spiked at levels in line with those appropriate to the maximum permitted. Samples were purchased locally from outlets in the York area and analysed using the developed SOP. The results are given in Table 7. Recoveries of SuE spiked into foodstuffs at a concentration of between 0.5 to 1.0 % were in the range 80% (sausage roll) to 106% (ice cream), except for soft mint sweets which had an average recovery of 73%. The mean recovery for all commodities was 94%, with the mean RSD of 10.8%, though initially some problems were experienced with certain commodities (see 6.42).

6.29. *Quality assurance*

Samples were analysed on a batch basis with at least one IHVM sample. All recovery values for the IHVM were within the boundaries of the analytical control chart (Figure 6). The limit of quantitation (LOQ) was 12 mg/kg for equivalent to 54 mg/kg sucrose ester, calculated from an analyte peak with a signal to noise ratio of 10. The limit of detection (LOD) was 6 mg/kg for sucrose, equivalent to 27 mg/kg sucrose ester, calculated from an analyte peak with a signal to noise ratio of 5.

Apart from the presence of multiple analyte peaks, the detection system was essentially free from interference. The internal standard peak was found to be consistently free from any co-eluting material. Where extracts exhibited poor recovery for the internal standard, this was in most instances due to insufficient drying of the acidic hydrolysate prior to derivatization.

6.30. The sample matrices that presented the most difficulties were marshmallow biscuit and margarine. The presence of gelatine and starch in the marshmallow biscuit product caused the sample to congeal during the initial solvent extraction, thereby compromising SuE extraction. This was ameliorated by the addition of 2 mL of water to the 1 g sample and homogenising prior to solvent extraction. Although the mean recovery of 97% for this commodity was very good, the RSD value obtained (18%) mirrored the difficulty with analysis of this matrix.

6.31. The margarine comprised almost 100% fat, which effectively neutralised the sodium hydroxide during attempted hydrolysis of the SuE (to release free sucrose) i.e. through saponification. No GC-MS peaks for sucrose or fructose were detected in chromatograms from extracts of margarine samples, hence it was not possible to calculate meaningful recoveries (i.e. they were effectively zero). Since samples containing moderate levels of fat were analysed successfully (e.g. sausage roll and biscuit), the sample weight of the margarine was reduced to *ca.* 0.25g so as not to overload the base hydrolysis step. The recoveries obtained from analysing the smaller sample size were very good (mean 101%).

7. Conclusions

7.1. The results from this study suggest that E473 and E474 have very limited use in the UK, borne out by the difficulty in obtaining samples. Nevertheless, the developed method provides a means by which the total sucrose ester contents of a limited range of food commodities may be estimated at levels well below the maximum levels specified in the Council Directive [1]. This is particularly relevant to the generation of intake data for E473/474. Further development of extraction regimen for food commodities containing significant amounts of starch such as biscuits and fine bakery wares, and high-fat commodities may be necessary.

7.2. The factor for converting the free sucrose content to SuE content was based on the SuE with the lowest sucrose content (i.e. higher degree of esterification) and was adopted on the grounds that its use effectively gives an over estimation of lower esters but does err on the side of caution with respect to public protection. The alternative was to express the results as a range (based on factors derived from the highest and lowest theoretical sucrose contents across the HLB range). This was considered to be less appropriate for monitoring SuE intake.

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TABLES

Table 1. Sucrose ester reference materials

CSL Code	SuE standard	% Composition	Fatty acid	Source
3L0713	Monoester P	95%	monopalmitate	Japan
		5%	dipalmitate	
3LO711	Monoester L-595	29-33	monolaurate	Japan
		38-42	dilaurate	
		25-29	trilaurate	
3L0712	Sugar Ester L-1695	78-81	monolaurate	Japan
		14-15	dilaurate	
		<3	trilaurate	
3L0710	Sugar Ester S-170	-	hexastearate	Japan
		-	pentastearate	
		-	heptastearate	
		-	tetrastearate	
3L0708	Sugar Ester S-570	18-20	monostearate	Japan
		8-9	dipalmitate	
		23-25	distearate	
		15-17	tristearate	
3L0709	Sugar Ester S-1170	37-39	monostearate	Japan
		20-22	distearate	
		14-15	monopalmitate	
		7-8	dipalmitate	
		13-19	alkylate	
3L0707	Sugar Ester S-1570	46-49	monostearate	Japan
		14-15	monopalmitate	
		14-16	distearate	
		5-6	dipalmitate	
		7-16	alkylate	
3L0705	Sugar Ester S-1670	-		Japan
3L0704	Sugar Ester P-1670	-		Japan
3L0706	Sugar Ester P-1570	-		Japan
3L0716	Sisterna PS750	-	monopalmitate	Europe
		-	monosteatate	
3L0717	Sisterna SP70	-	monosteatate	Europe
		-	monopalmitate	
3LO715	Sisterna SP50	-	monosteatate	Europe
		-	monopalmitate	
3L0714	Sisterna SP30	-	disteatate	Europe
		-	monopalmitate	

[The first letter code L, S or P specifies the fatty acid (lauric, palmitic, or stearic), the first number codes (where four are given) are the HLB value 1-16 and the last two numbers are the percentage of SuE in the formulation].

Table 2. IHVM spiking details

Sucrose ester	HLB value	Amount added (mg)	Spike level in IHVM (mg/kg)
L1695	16	504	2016
S570	5	502	2008
S170	1	509	2036

[*By weight, based on manufacturers data]

Table 3. Sucrose ester solubilities

CSL Code	SuE-Standard	Acetone	DCM	DMSO	BuOH/ cycloexane (1:1)	THF/ Acetone (1:1)
3L0713	Monoester P	✓	x	✓	✓	✓
3LO711	Monoester L-595	✓	✓	✓	✓	✓
3L0712	Sugar Ester L-1695	✓	x	✓	✓	✓
3L0710	Sugar Ester S-170	x	x	Not wettable	✓	✓
3L0708	Sugar Ester S-570	✓	✓	Gel	✓	✓
3L0709	Sugar Ester S-1170	✓	x		✓	✓
3L0707	Sugar Ester S-1570	✓	x	Gel	✓	✓
3L0706	Sugar Ester P-1570		x		✓	✓
3L0705	Sugar Ester S-1670		x		✓	✓
3L0704	Sugar Ester P-1670		x		✓	✓
			x		✓	✓
3L0716	Sisterna PS750		x	Gel	✓	✓
3L0717	Sisterna SP70		x	Gel	✓	✓
3L0715	Sisterna SP50		✓		✓	✓
3L0714	Sisterna SP30		✓		✓	✓
	Sucrose acetate		✓		✓	x
	SAIB		✓		x	✓

[key: ✓=soluble, x=insoluble]

Table 4. HLB value vs. theoretical % sucrose contribution by weight for standard SuEs.

HLB value	% Sucrose contribution	HLB value	% Sucrose contribution
1	22.3	9	46.0
2	29.8	10	47.1
3	34.1	11	48.2
4	37.2	12	49.1
5	39.7	13	50.0
6	41.6	14	50.8
7	43.3	15	51.5
8	44.7	16	52.2

Table 5. Recoveries of SuEs through SPE cleanup using mixtures of THF:methanol (%).

	Original sucrose factor		Improved sucrose factor		Average sucrose factor*	
SuE	80:20	95:5	80:20	95:5	80:20	95:5
L1695 (16)	12	5	14	6	17	7
S570 (5)	24	31	27	34	25	31
S170 (1)	46	7	80	13	42	7

[*Arithmetic mean of improved factors across all HLB values]

Table 6. Homogeneity and recovery of SuE in IHVM (n=10)

Batch No.	Analysis date	Mean recovery (%)*	Standard deviation	RSD (%)
1	19/01/05	88.8	5.8	5.9
2	25/01/05	73.4	5.8	5.8

[*Based on a mean theoretical sucrose content of 45%]

Table 7. Recoveries of SuE from spiked food commodities.

Category	Sample	SuE Spike level (%)	Mean recovery (%)	RSD (%)	n
1. Fine bakery wares	(1) Fruit scone	0.5	88	5.7	6
	(2) Marshmallow biscuit (i)	0.75	97	18.0	3
	Marshmallow biscuit (ii)	0.75	93	15.3	6
2. Sugar confectionery	Soft mint sweets	0.5	73	12.0	6
3. Dairy products	Ice-cream	0.75	106	7.7	3
4. Processed fats & oils	Margarine *	1.0	101	11.5	6
5. Other	Sausage roll	1.0	80	10.2	5
	Cornish pastie**	0.6	81	5.9	20
	Stir-fry sauce	0.5	96	11.3	6

[* Sample size 0.25g. **IHVM]

FIGURES

Figure 1. Chemical structure of sucrose esters. R_1-R_3 are usually comprised mixed residues of $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and/or $C_{18:1}$ fatty acids.

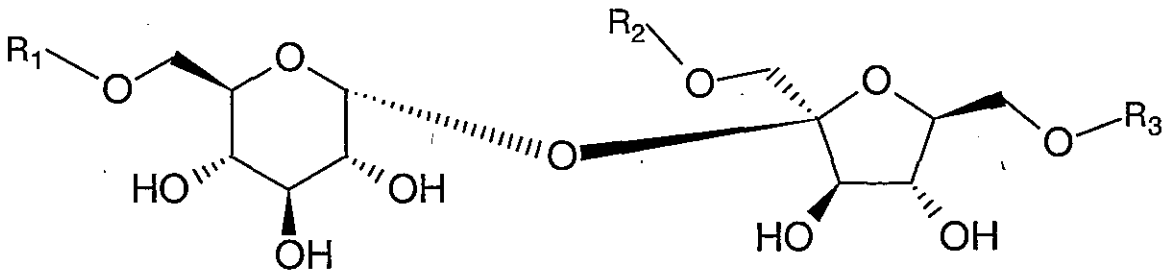


Figure 2. Hydrolysis profile of sucrose ester S1170.

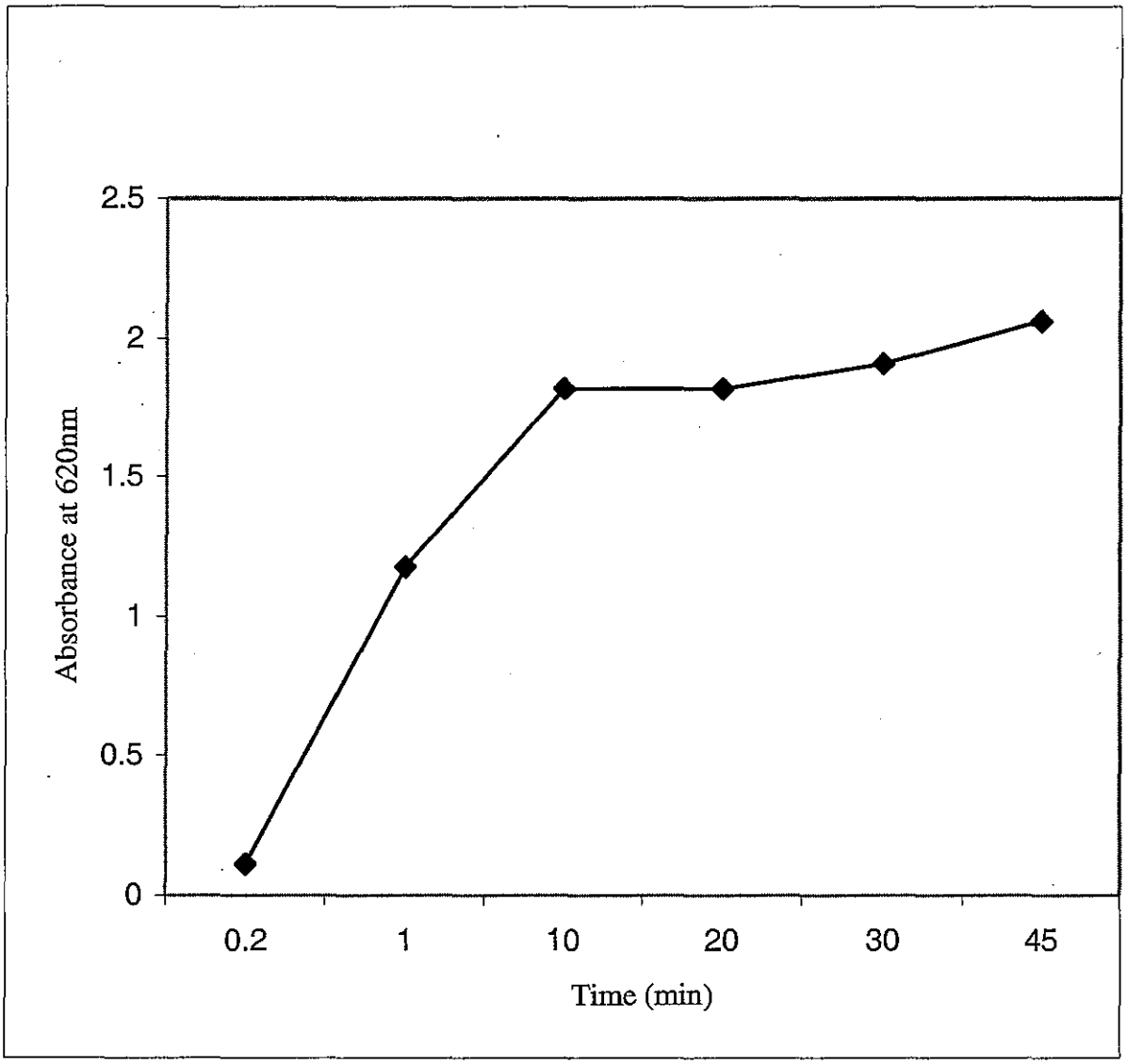


Figure 3. Sucrose ester composition by HLB value [27].

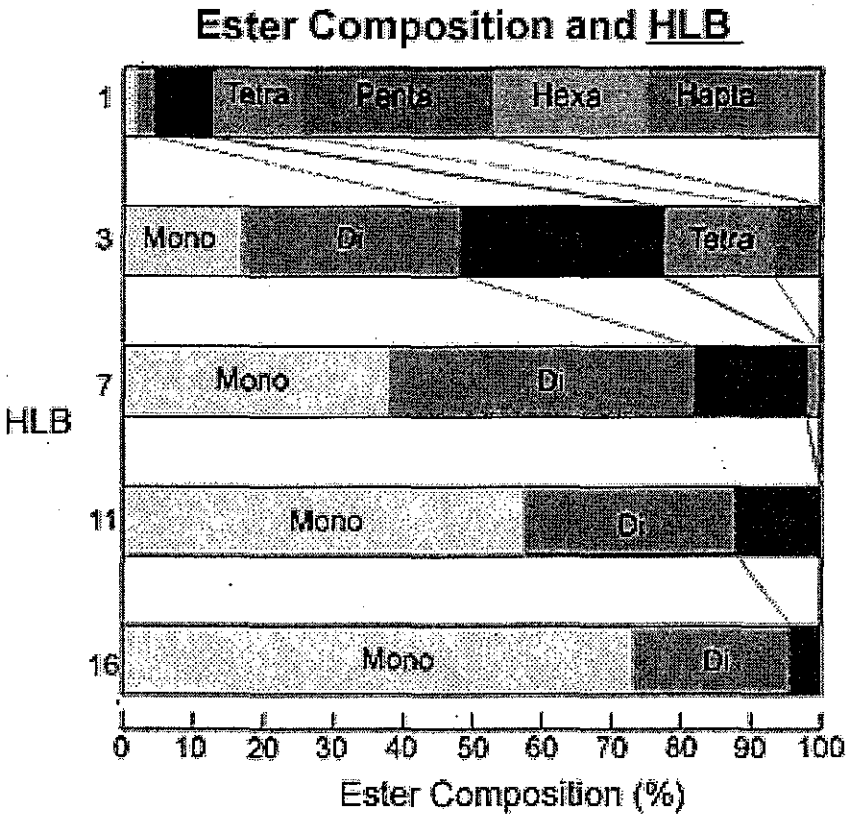


Figure 4. Plot of HLB value vs. theoretical sucrose contribution .

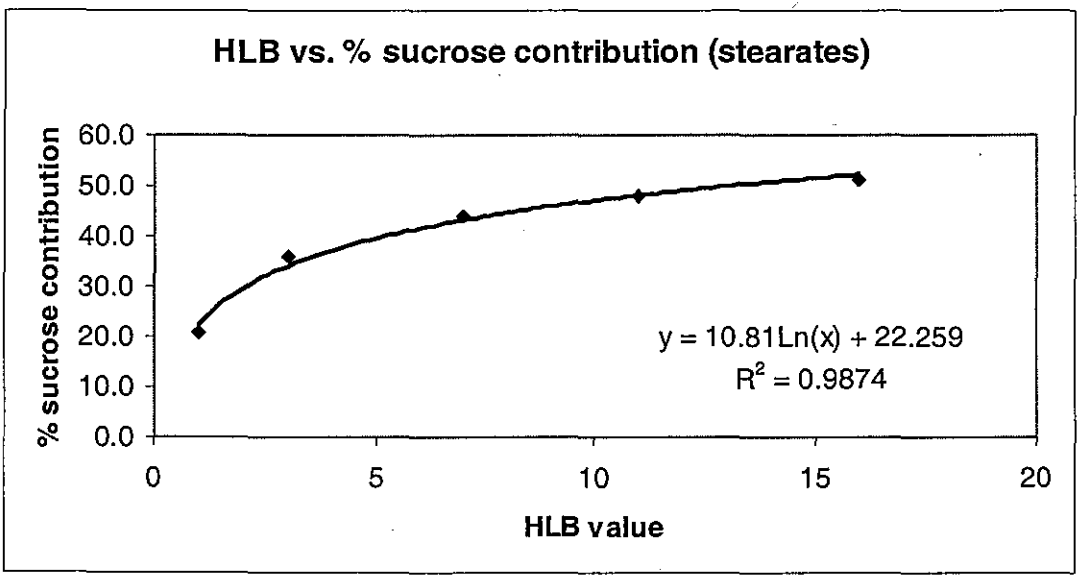
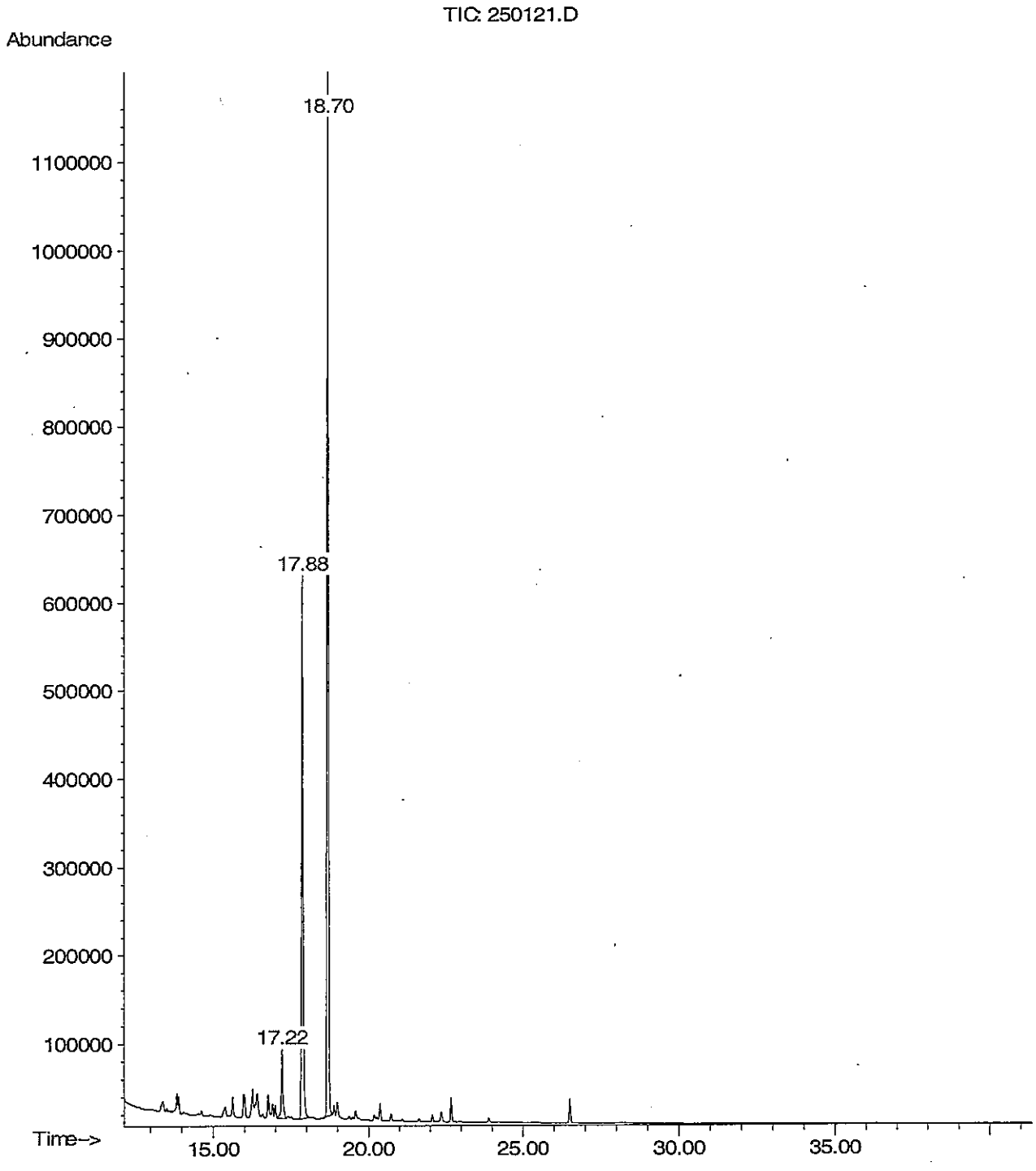


Figure 5. GC-MS analysis of blank IHVM spiked with sucrose at 20 mg/g.



[Peaks between 17 and 19 minutes are fucose internal standard]

Figure 6. Control chart for batch analysis of Sucrose esters based on the IHVM.

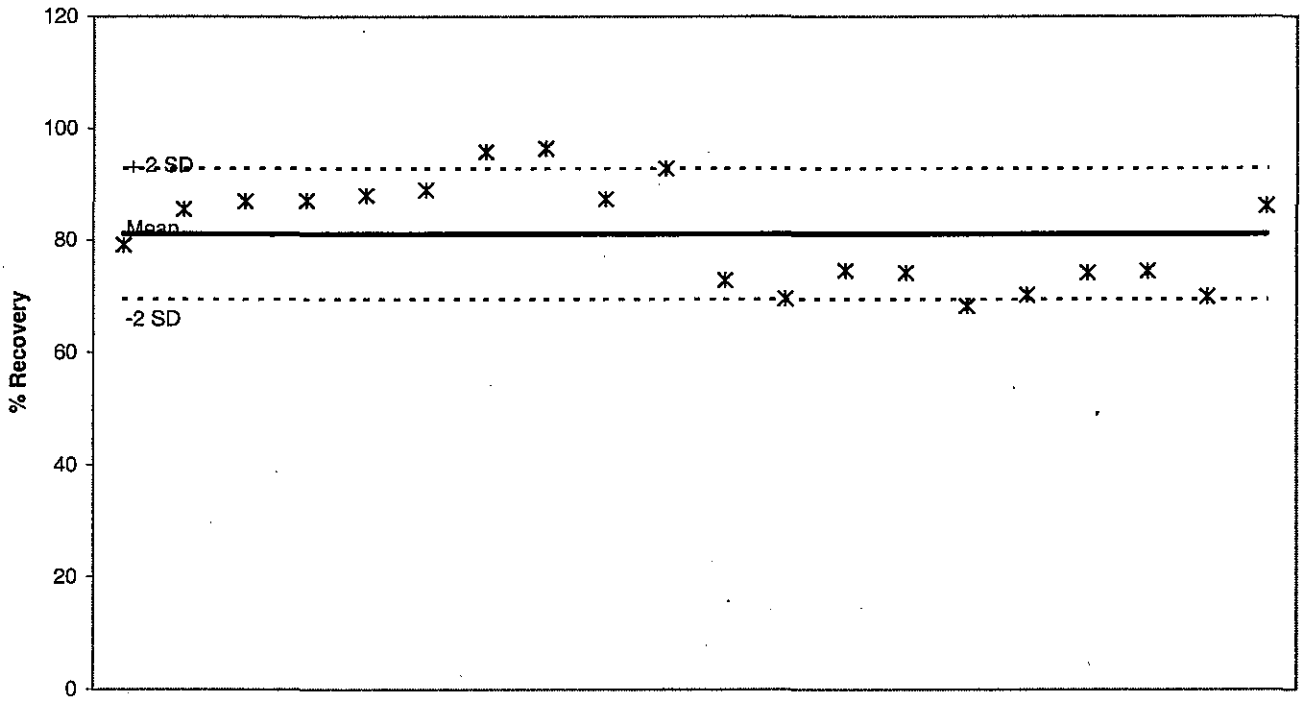
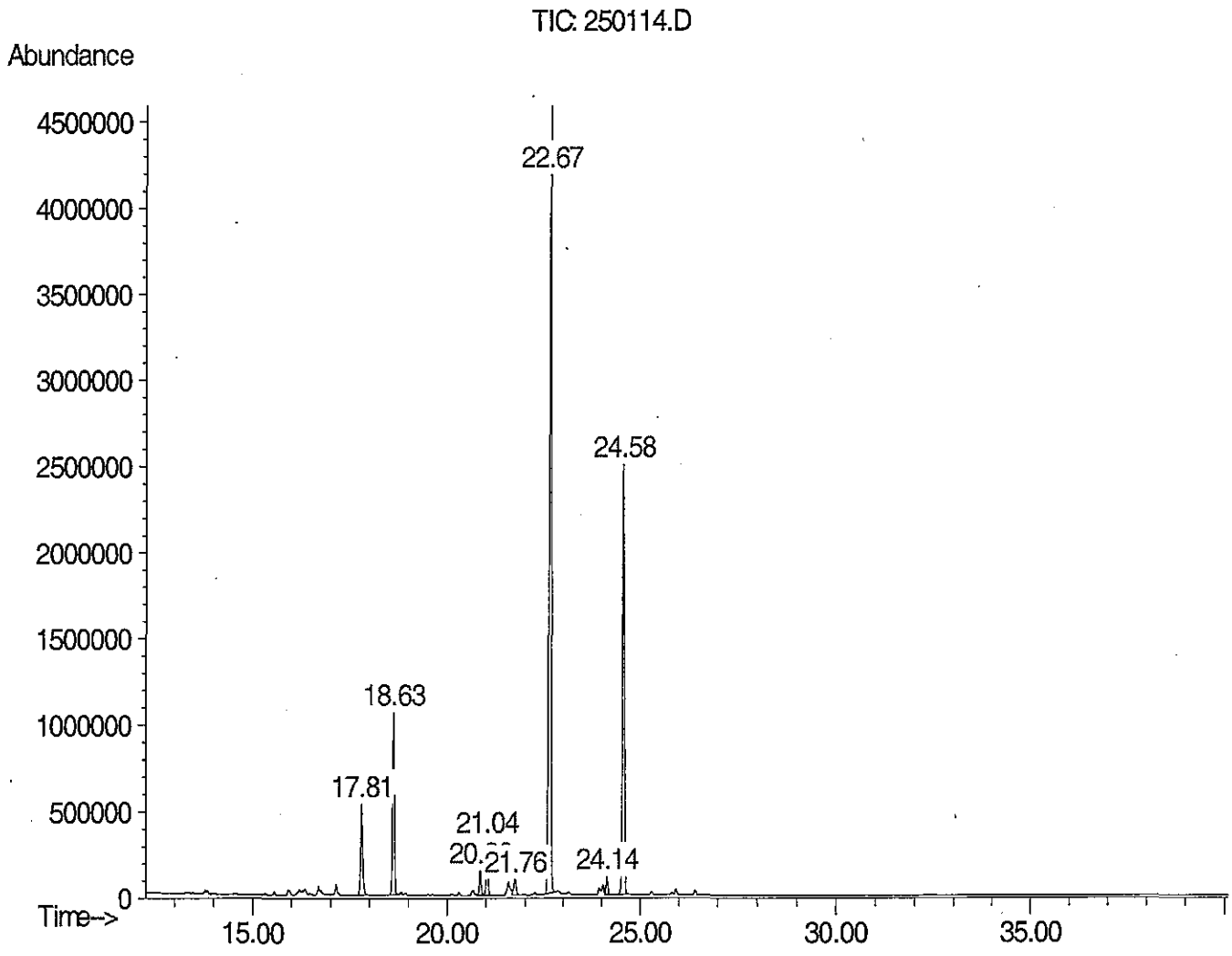


Figure 7. GC-MS analysis of IHVM extract.



[Peak identification: Fructose (20.9 and 21.1 min) and glucose (21.8, 22.7 and 24.6 min).

Author	**D Roberts**
Reviewed by	**M J Scotter**
Date	**28/02/05**
Issue authorisation	
Position	**Project Manager**
Signature	
Date	

Implementation date:_____ Copy No. (optional):_____

PROCEDURAL HISTORY

Issue	Date Issued	Changes
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

1. Scope and field of application

This standard operating procedure (SOP) has been developed for the determination of sucrose esters in foodstuffs.

2. Principle

Food samples are homogenised with a mixture of tetrahydrofuran and ethyl acetate, and centrifuged. Free sucrose and other potential interfering components are removed by partition with saturated sodium sulfate. The sucrose esters are hydrolysed with sodium hydroxide to liberate free sucrose, which is then acid hydrolysed to glucose and fructose and analysed by GC-MS following silylation.

3. Safety aspects associated with this method

This method involves the use of several hazardous chemicals and procedures likely to produce an increased risk to the operator. If in doubt always consult the appropriate safety advisor. Chemicals and equipment should be handled in accordance with the relevant COSHH assessment. Before performing this procedure staff should have read and understood the following:

COSHH Risk Assessment No. XXX.

1-Butanol. Flammable. Vapour/air mixture may be explosive. Harmful by inhalation, ingestion or skin absorption. Irritant. Narcotic. CAS No. 71-31-3.

Ethyl Acetate. Stable. Incompatible with various plastics, strong oxidizing agents. Highly flammable. Vapour/air mixtures explosive. May be moisture sensitive. Harmful if swallowed in quantity. Vapours may cause drowsiness. Irritant. CAS No. 141-78-6.

Fructose. May cause skin or eye irritation. Inhalation of powder may cause respiratory irritation. CAS No. 57-48-7.

Fucose. (6-deoxy-D-galactose) May be harmful by inhalation, ingestion or skin absorption. CAS No. 3615-37-0.

Glucose. Inhalation of powder may cause respiratory irritation. CAS No.50-99-7.

Cyclohexane. Highly flammable. Vapour/air mixture explosive. Harmful by ingestion, inhalation and skin contact. Degreases. Irritating to skin and eyes. Vapour may irritate respiratory system, and is narcotic in high concentrations. Possible risk of irreversible effects if exposure is prolonged or repeated. CAS No.110-82-7.

Hydrochloric acid. May evolve toxic fumes in fire. Causes severe burns to eyes and skin. If ingested causes severe internal irritation and damage. Extremely irritating, harmful vapour. CAS No.7647-01-0.

Silylation reagent(s). Tri-Sil® Z (Perbio Science UK Ltd., Cramlington). Highly flammable liquid and vapour. Causes damage to kidneys, nervous system, liver, heart. May be harmful by inhalation or ingestion. Contains pyridine (CAS No. 110-86-1) and trimethylsilylimidazole (CAS No. 18156-74-6).

Sodium hydroxide. Corrosive. Causes burns. Exothermic in contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves and eye/face protection. In case of accident or if feel unwell, seek medical advice immediately. CAS No. 1310-73-2.

Sodium sulfate. May be harmful by inhalation, ingestion or skin absorption. Causes eye and skin irritation. CAS No. 7757-82-6.

Sucrose. Inhalation of powder may cause respiratory irritation. CAS No. 57-50-1.

Tetrahydrofuran. Highly flammable. Harmful. may form explosive peroxides. Harmful if swallowed. Irritating to eyes, respiratory system and skin. Keep away from sources of ignition - No smoking. Take precautionary measures against static discharges. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. CAS No. 109-99-9.

4. General Precautions

Protective clothing including buttoned laboratory coat, safety spectacles and gloves (latex or similar) should be worn at all times. Extractions should be carried out in a properly ventilated fume cupboard.

This method involves the use of several hazardous chemicals and procedures likely to produce an increased risk to the operator. Operators should familiarise themselves with any risks attributable to the chemicals and reagents used in this procedure. If in doubt always consult the appropriate safety advisor.

UNDER THE CONTROL OF SUBSTANCES HAZARDOUS TO HEALTH (COSHH) REGULATIONS. THIS METHOD MUST BE ASSESSED FOR ANY RISK TO HEALTH TO OPERATORS THROUGH ITS USE. A FULL ASSESSMENT REQUIRES COMPLETION OF COSHH DOCUMENTS THAT ARE TO BE KEPT ON FILE AND ACCESSIBLE TO STAFF.

BEFORE STARTING THIS METHOD ANYONE USING THIS PROCEDURE MUST HAVE A COPY OF COSHH DOCUMENTS

Particular attention is drawn to some organic solvents and chemicals which may be toxic and/or flammable. Contact by inhalation, skin absorption or ingestion must be avoided. Work in a fume cupboard if necessary.

4.1 First Aid

Any injury must be reported, in the first instance, to a qualified First-Aider and recorded in the accident book kept with all first aid kits. The First-Aider will decide on further action. All accidents, incidents and near misses should be reported on form CSL/ACC>REP (Report of an accident, dangerous occurrence or near miss).

5. Reagents

Reagents should be of recognized analytical grade unless specified otherwise.

5.1. 1-Butanol
5.2. Ethyl Acetate
5.3. Fructose
5.4. Fucose
5.5. Glucose
5.6. Cyclohexane
5.7. Hydrochloric acid
5.8. Sodium Hydroxide
5.9. Sodium Sulfate
5.10. Silylation reagent, Tri-Sil® Z (Perbio Science UK Ltd., Cramlington).
5.11. Sucrose
5.12. Tetrahydrofuran

5.13. Water, HPLC grade

6. Apparatus

In addition to standard laboratory glassware:

6.1. Homogeniser, e.g. Ultra-Turrax with 5mm dispersion tool

6.2. Vials, glass screw-top 40ml

6.3. Vials, glass screw-top 12ml

6.4. Vials, glass screw-top 4 ml

6.5. Vials, glass, crimp-cap 250µl for GC analysis

6.6. Safety pipettes or microlitre syringes, calibrated, up to 500ul capacity

6.7. Laboratory shaker

6.8. Centrifuge, capable of 1000 x *g*

6.9. Thermally controlled heating block and nitrogen blow-dry apparatus

6.10. GC-MS

Capillary GC with headspace autosampler, split/splitless injector capable of injecting 1ul, MS detector and PC data processing facility. The following have been found to be suitable:

Column	Agilent HP-5 MS fused silica column, 30m x 0.25mm x 0.25µm
Oven program	80°C initial temp, hold for 1 min. then 5°C/min to 200°C, hold for 3 mins.
Injection volume	1µl
Split mode	Splitless
Inlet temp	280°C
Carrier gas	Helium at 1ml/min
Tranfer line temp.	250°C
MS detection	Selected ion impact mode, ions monitored (m/z) 204 and 217

7. Reagent Solutions

7.1. Tetrahydrofuran: ethyl acetate (7:3 v/v)

Mix 140ml of tetrahydrofuran (THF) with 60 ml ethyl acetate (EtOAc).

7.2. Saturated sodium sulfate solution

7.3. Butanol-1-ol: Cyclohexane (1:1)

7.4. Mix Butan-1-ol and Cyclohexane in equal volumes.

7.5. Hydrochloric acid, 6M

7.6. Sodium hydroxide, 4M

7.7. Standards

7.7.1. Stock solutions at 10mg/ml in water of the following

- Fucose
- Sucrose
- Fructose and Glucose (may be required to check peak ID and retention time)

7.7.2. From fucose stock solution, prepare internal standard spiking solution in water at 0.1 mg/ml.

7.7.3. From sucrose stock prepare standards in water at

- 1 mg/ml
- 0.1 mg/ml

7.7.4. Calibration Standards. Suggested calibration standard range shown in Table 1.

Table 1. Calibration standards composition

Standard conc. after deriv. (µg/ml)	Sucrose in 4ml vial (µg)	1 mg/ml fucose stock (µl)	10 mg/ml sucrose stock (µl)	100 µg/ml sucrose intermediate (µl)
0	0	10	0	0
4	1	10	0	10
20	5	10	0	50
40	10	10	10	0
10	25	10	25	0
200	50	10	50	0
500	125	10	125	0

8. Procedure

All of the procedures listed below must be carried out with minimal delay between steps.

8.1. Weigh 1 ± 0.1 g foodstuff homogenate directly into a 40 ml glass vial and record the weight to 0.1 g.

8.2. Add 10 ± 0.1 ml of THF: EtOAc (7.1) to the homogenate and blend using the Ultra Turrax at 4,000 to 10,000 rpm for 30 seconds.

8.3. Place the vial on a the shaker for 30 minutes.

8.4. Centrifuge the extract at 700-1000 x *g* at 20°C for 5 minutes. Carefully transfer the supernatant to a 40 ml vial.

8.5. Add 10 ml of saturated sodium sulfate solution and place on the shaker for 20 minutes.

8.6. Centrifuge the extract at 700-1000 x *g* at 20°C for 5 minutes and transfer the supernatant to a 12 ml vial.

8.7. Place the vial on the N_2 blow-dry apparatus and evaporate to dryness at a temperature of not more than 70 ± 2°C. Do not allow the residue to remain in the dry state any longer than is necessary.

8.8. Add 2 ml butanol: hexane (7.3), cap and shake to dissolve.

8.9. Hydrolyse by adding 2 ml 4M sodium hydroxide solution and shaking for 40 minutes.

8.10. Using a Pasteur pipette transfer the lower aqueous layer into a separate 4 ml vial. Discard the upper solvent layer.

8.11. Derivatisation

Transfer 100 ul of the aqueous sample extract into a clean 4 ml vial, add 100 ul of fucose internal standard solution, 125 ul of 6M hydrochloric acid and mix well.

8.12. Blown down at 50°C under nitrogen gas until a dry residue is produced. (*The addition of a small volume (1-2ml) of acetonitrile to the aqueous extracts aids evaporation).*

8.13. Add 250 µl of Tri-Sil® Z derivatising agent to each 4 ml vial. Cap the vial and heat in a hot block inside a fume cupboard at 70°C for 30 minutes. Allow the samples to cool and transfer into vials suitable for GC-MS analysis.

8.14. Treat the calibration standards (Table 1) in the same way from paragraph 8.18.

9. Calculation of sucrose levels in samples

Identify the analyte and internal standard peaks on the basis of their retention time and measure the respective peak areas. Due to isomeric effects there are at least 2 peaks for each of the analytes of interest. The 2 largest peaks for each analyte are integrated and the areas summed to give a single area value for each analyte:

- fucose internal standard (m/z 204 at ~18.5 and ~19.5 mins.)
- fructose (m/z 217 at ~21.6 and ~21.8 mins.)
- glucose (m/z 204 at ~23.5 and ~25.4 mins.)
- Divide the analyte peak areas by the internal standard peak areas to obtain the peak area ratios.

10. Calibration

Plot the peak area ratio values derived for calibration standards against the concentration of sucrose in µg/ml. The correlation coefficient should be 0.990 or better. Calculate the peak area ratios for the extracts. From these calculate sucrose concentration from the regression equation of the calibration graph.

10.1. Calculation of sucose ester equivalents

Calculate the sucrose ester content from the equation:

Sucrose ester content (mg/kg) = Sucrose content (mg/kg) x 4.5

Note: The theoretical sucrose contents of SuEs are based on mono-, di-, tri- and poly-ester contents of SuEs, and are crude estimates taken from the manufacturers specifications. Therefore, the most appropriate way to express the results i.e. in terms of SuE content based on sucrose measured, will be to apply a conversion factor, based on the sucrose contents of available standard SuEs. Theoretically, SuEs over the entire HLB range could contain between 22.3 and 52.2% sucrose by weight. In the extreme hypothetical case, the use of the wrong conversion factor would incur an error of ca. 2.3 times (or reciprocal thereof, depending which way round it is applied), which is clearly not acceptable. The calculated arithmetic mean conversion factor conveniently falls around the centre of the HLB scale and could therefore be used as an 'average' conversion factor, the incurred error notwithstanding. However, given that the main purpose of determining the SuE content of foods is for public protection, the use of a conversion factor for higher esters (low HLB) would seem appropriate because it would incur an overestimation of the SuE content. Thus a factor of **4.5** is recommended to convert from sucrose content to SuE content (i.e. approximately 100/22.3). If however, for a particular food analysis, the identity of the SuE added is known then a different conversion factor can be used but this should be stated in the test report.

11. Quality Assurance

11.1. Operator training

When this method is to be used by an operator for the first time it must be validated for in-house repeatability and reproducibility. The full procedure should be carried out on a suitable in-house validation matrix (IHVM). A minimum of four replicates

of the IHVM will be analysed. All results should fall within the ±2 STDEV boundaries for the IHVM control charts.

11.2. Quality Control of Method During Use

A batch consisting of up to 10 samples should always include a reagent blank and IHVM sample.

11.3. Limit of detection (LOD).

A limit of detection for this method is suggested at 6 mg/kg for sucrose, equivalent to 27 mg/kg sucrose ester. This is calculated from an analyte peak with a signal to noise ratio of 5.

11.4. Limit of quantitation (LOQ).

A limit of quantitation for this method is suggested at 12 mg/kg for equivalent to 54 mg/kg sucrose ester. This is calculated from an analyte peak with a signal to noise ratio of 10.

ANNEX 2

Details of experimental work carried out during the preliminary stages of method development.

1. *Carbohydrate assays*

The phenol-sulfuric acid carbohydrate assay, which has been used to determine SuE in milkfat was examined [15]. Phenol (8 g) was dispersed in water (10 ml), forming a two-phase liquid system. This was mixed to a milky consistency and an aliquot (50 ul) was added to the test sample (1ml) along with sulfuric acid (2 ml). The sulfuric acid caused all non-reducing sugars (sucrose) to convert to reducing sugars (glucose). After sitting for 10 min the absorbance at 420 nm was measured, which was proportional to the carbohydrate concentration. Using a sucrose standard, an acceptable colour change was produced from yellow (sulfuric acid) to dark red (pale yellow 0.1 mg, orange 2.5 mg, red 25 mg, dark red 100 mg). However, since this method required the use of a corrosive and toxic reagent mixture it was discarded and the anthrone-sulfuric acid assay was investigated.

2. The anthrone test is recommended for identifying pure SuEs [25]. Filtered sucrose solution (1 ml) was added to anthrone reagent (2 ml of 0.4% anthrone in 95% sulfuric acid) in a glass vial and left for 20 min for the colour to fully develop. Hydrolysed reducing sugars (glucose) react with anthrone under acidic conditions to yield a blue-green colour. Calibration was achieved using aqueous sucrose solutions. The absorbance of the solution was measured at 620 nm after cooling (Figure 1). While measurement of the dark blue colour intensity by spectrophotometry was imprecise, visual comparison was used for range finding. Extracts were diluted with water until the

colour was within the instrument dynamic range. The anthrone-sulfuric acid procedure is reported to be subject to interference from inorganic ions such as Fe^{n+}, NO_3^- and Cl^- [24]. Moreover, the method was observed to be inadequate when a moderate amount of organic matter was present, where charring of organic material produce carbon, which masked the colour.

3. *Derivatization for GC analysis*

Silylation is a well established derivatisation technique for sugars and their esters. While it was possible to analyse C_{12}-tri-esters and C_{18} mono-esters as pure standards, in more complex matrices the derivatisation process was less successful because the derivatives were unstable. Moreover, the complexity of the inherent esterification patterns in the sucrose esters (mono- through to octa- substitution with $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids) did not allow sufficient chromatographic scope for a method producing more than one peak per structure. The silylation systems investigated were:

- TMSI (Trimethylsilylimidazole) as *Tri-Sil*® *Z* proprietary reagent can be used where traces of water may be present as in sugar syrups. It is available for use as a one-step derivatisation mixture comprising TMSI: pyridine (1:4).

- HMDS (Hexamethyldisilazane) and TMCS (trimethylchlorosilane) as *Tri-Sil*® proprietary reagent, available as a mixture of HMDS:TMCS:Pyridine (2:1:10).

- BSTFA (bis(trimethylsilyl)trifluoroacetamide).

- TMS-Oximes. An oxime is formed between the aldehyde of a reducing sugar and the highly active amine group of hydroxylamine, which is subsequently silylated.

4. Solutions of sucrose and SuEs (50 µl of 20 mg/ml) were dried in vials (5 ml) at 50°C. These were heated with silylation reagent at ca. 70C for a specified time period, cooled and transferred into smaller crimp-cap vials for GC analysis. Derivatised extracts were injected without dilution directly on to the GC system.

5. The various derivatives were analysed by GC-FID on a Carlo-Erba Mega series capillary GC (Fisons Instruments, Altrincham, UK) or by GC-MS on a HP 5890 gas chromatograph detection interfaced to a 5971 mass-selective detector controlled by ChemStation software (Agilent, Bracknell). The column was a HP-1 (100% methylsilicone, Agilent, Bracknell)). The injector was set at 250°C and the oven temperature was programmed initially at 100°C for 2 min then ramped at 5°C/min to 350°C. Helium carrier gas was delivered at 1 ml/min. For MS, electron impact (EI) spectra were obtained at 75 eV with the mass-selective detector at 0.76 scan/s over the m/z range 75-600.

6. *Derivatization of SuE standards*

GC has been used successfully with lower mass sucrose esters with relatively short-chain fatty acids, some of which (C_2-C_{12}) are found as natural products in e.g. tobacco leaves [28,29]. While Chortyk [22] has reported that intact mono-tetra octanoyl esters (SuE C_8) may be analysed after derivatization, application of this technique to food grade SuEs esterified with C_{16}-C_{18} fatty acids was problematic. The monoesters eluted readily, whereas higher esters were not eluted even though there was scope within the

column temperature ramp for higher ester classes to be moved through the GC column. It was therefore concluded that it was more the constraints of injection / derivatization protocols that prevent higher esters being detected rather than chromatographic elution. Using the smallest mass SuE food grade products (L-595, L-1695) which incorporate lauric acid (C_{12}), it was possible to demonstrate derivatization and elution of SuEs containing groups up to tri-esters, thereby indicating the derivatisation process was not prone to steric hindrance if multiple fatty acids were attached. Other techniques such as heating the syringe and sample prior to manual injection were not tried as these were considered as impractical for the long term aim of producing a readily transferable method.

7. Using this approach, it was possible to characterise a small number of food grade $C_{12:0}$, SuEs L-595 and L-1695, which contained poly- and mono-esters respectively and covering the HLB range 5 to 16 (Figure 2). Although, these SuEs are used in powdered milk and detergents respectively, it was considered unlikely that they wiould be observed in UK foods. Since only mono- and di-esters of the larger C_{16} and C_{18} esters could be eluted, it was not possible to fully characterise any other commercial SuE mixes using this method (Figure 3).

8. *Derivatization of sucrose*

An oxime of sucrose eluted as a single peak earlier in the chromatogram than a standard sucrose-TMS derivative. However, a small peak due to sucrose-TMS eluted *ca.* 7 minutes after the larger oxime peak (at 29 minutes), suggesting incomplete conversion to the oxime before silylation. As a result this relatively complex derivatization

approach was not pursued. Because of its ease of use and efficiency, subsequent method validation was carried out with TMSI (*Tri-Sil® Z*).

9. *Solid phase extraction (SPE)*

Development of a cleanup procedure that could encompass the available range of SuEs and the main food co-extractives, particularly lipid, was predictably very challenging analytically. For example, 100 ul of DCM containing 10 mg of Mono-P was adsorbed onto an Oasis (C_{18}) cartridge, eluted with methanol and detected using anthrone reagent. Attempts at dissolving P-1570 at 10 mg/ml (methanol/water 1:1) were only successful when the solution was warmed; after addition to the SPE cartridge a milky precipitate was formed which blocked the column frit. It has been reported that SuE can be added in water-methanol (1:1) onto C_{18} SPE, washed with the same solvent, and eluting SAIB and SuE with methanol-THF (1:1) [19]. However, this method appeared to be limited to monoesters at concentrations of < 2 mg/ml. Since the SuEs of most interest were not soluble in the high aqueous content solvents generally required for C_8 or C_{18} RP-SPE, it was concluded that SPE using these phases was not a viable option for the primary cleanup step.

10. Whilst the physical properties of SuE are more suited to normal phase retention, there is a compromise to be made in obtaining the analytes in a suitable non-polar solvent. The diol phase has been reported to offer better elution recovery than bare silica or neutral alumina [16,26]. The elution profile described by Uematsu *et al.* [19] for silica SPE was investigated using diol stationary phase in place of silica, which gave poor recovery. When using the anthrone test to determine elution, it was essential to maintain tight elution bands since all of the related components produced similar colour changes.

DCM was found to be suitable for dissolving and transferring the SuEs onto diol SPE, adsorbing the lipids and SuEs and washing. Solvent polarity was increased using ethyl ether : hexane (1:1) for removal of the lipid fraction. THF was suitable for elution of SuEs and methanol eluted any retained free sucrose. EtOAc used alone eluted trace amounts of SuE, therefore mixtures of EtOAc/THF were expected to elute SuEs with limited retention. Various solvent mixtures such as ether/EtOAc 3:7, EtOAc/Methanol 1:1 were examined but abandoned in favour of single solvent elution with THF, allowing sucrose to be retained.

11. The developed clean up procedure was applied to the analysis of high-fat meat products such as pork pies and sausage rolls. Using THF/EtOAc (7:3) solvent, approximately 25% w/w of the food extract comprised lipid, which in turn contained the SuE. It was therefore necessary to remove the SuEs from the lipid or to extract these sequentially from the food. Two systems were examined; the first smaller scale system for extracts containing little fat or that had been defatted, and the second a system for use with high fat content samples without prior defatting. The actual quantities of sorbent/solvent/sample needed to achieve cleanup were interrelated. Diol cartridges (5 g) could adsorb a suitable weight of lipid, such that the entire extract from 20 g of food extract in 20 ml DCM could be adsorbed. Elution volumes of 40 ml were found to be satisfactory on this scale and rotary evaporation was necessary to remove solvent. Smaller scale systems were found to worked equally well and a 2 g cartridge with 20 ml elution volumes or a 1 g cartridge with 10 ml elution volumes gave equivalent separation.

12. Relatively concentrated SuE solutions were manipulated in order to achieve detection by TLC and GC systems, which in turn created a problem for the more sensitive anthrone reagent, as this required a concentration of only 50 ug/ml to effect an intense blue colour. In sample extracts where brown residues were observed, formation of the blue colour was obscured. After hydrolysis, this effect was reduced by passage of opaque and brown solutions through an unconditioned Oasis SPE cartridge. However, there is a danger that such matrix suppression could be interpreted as a negative result. The suppression appeared to be linked to sample mass, with smaller scale extracts giving the blue anthrone colour, while in scaled-up extractions colour formation was suppressed.

13. *General conclusions*

Sucrose esters proved extremely difficult to manipulate due to widely differing physical properties. The use of n-BuOH/CHX solvent systems allowed quantitative transfer of all SuEs without compromising solubility of either very hydrophilic SuEs such as sucrose monolaurate L-1695 (HLB 16) or hydrophobic SuEs such as sucrose polysterate S-170 (HLB 1). Other solvents systems such as EtOAc, THF or DCM (or combinations thereof) required a compromise between solubility of SuEs and compatibility with subsequent hydrolysis and cleanup steps. While it was feasible to measure intact SuEs, demonstrated to an extent with L-595 and L-1695, the apparent technical complexity associated with their isolation and measurement made this approach inappropriate for a simplistic method.

14. The development and combination of steps to achieve sufficient cleanup and hydrolysis of SuEs to sucrose were key to the development of a robust method. The biphasic

system used to remove free sucrose and to hydrolyse the SuEs *in situ*. The use of the anthrone reagent carbohydrate test was of more limited use as this assay is not specific to sucrose and was readily disrupted by the presence of co-extractants, but could perhaps find use as a screening tool to be used beside GC-MS. GC-MS of sucrose is a well documented procedure which was applied successfully to the determination of SuEs via the hydrolysis product and derivatization with TMSI (*Tri-Sil® Z*) but required further refinement for quantitative analysis.

REFERENCES

See main report

FIGURES

Figure 1. Sucrose calibration curve using anthrone reagent.

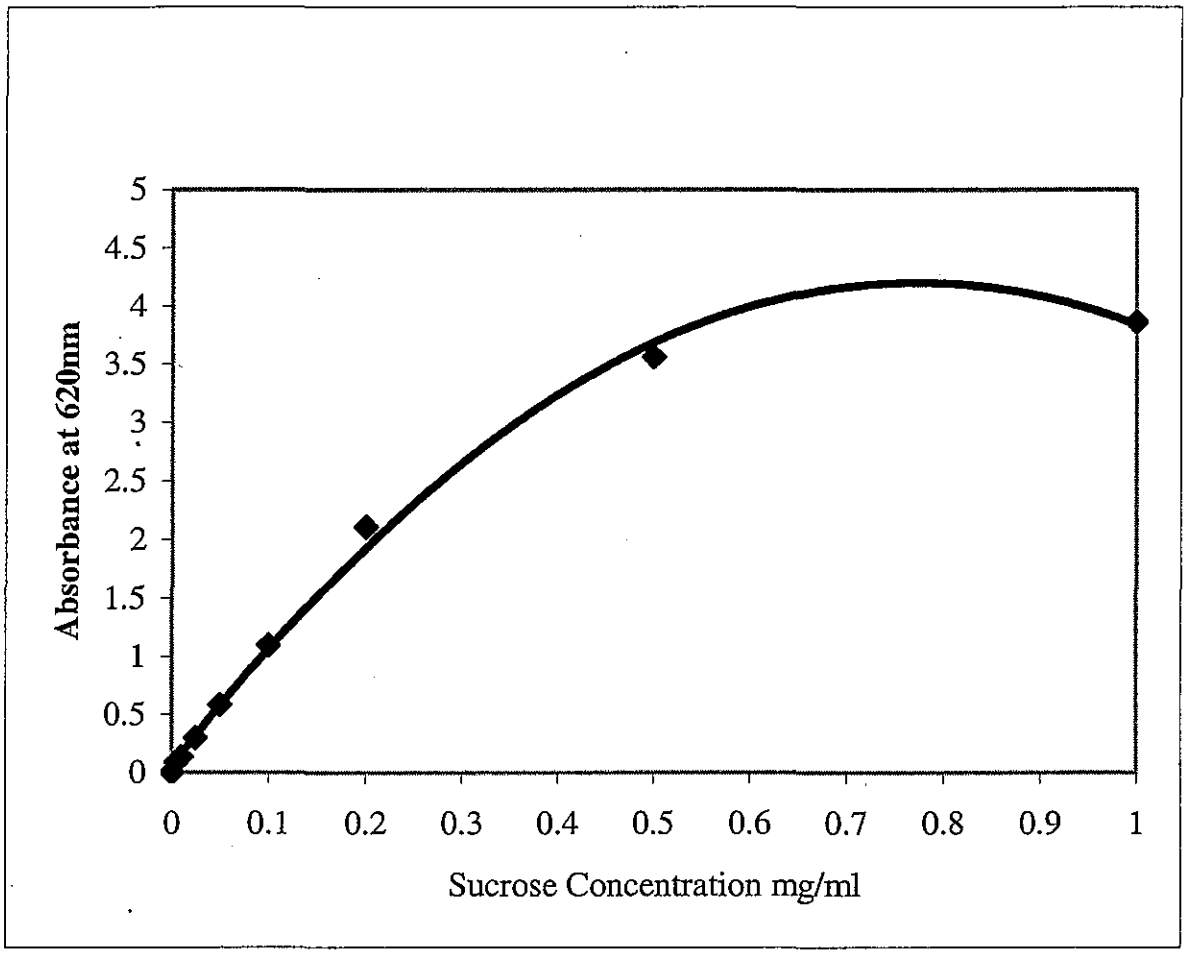
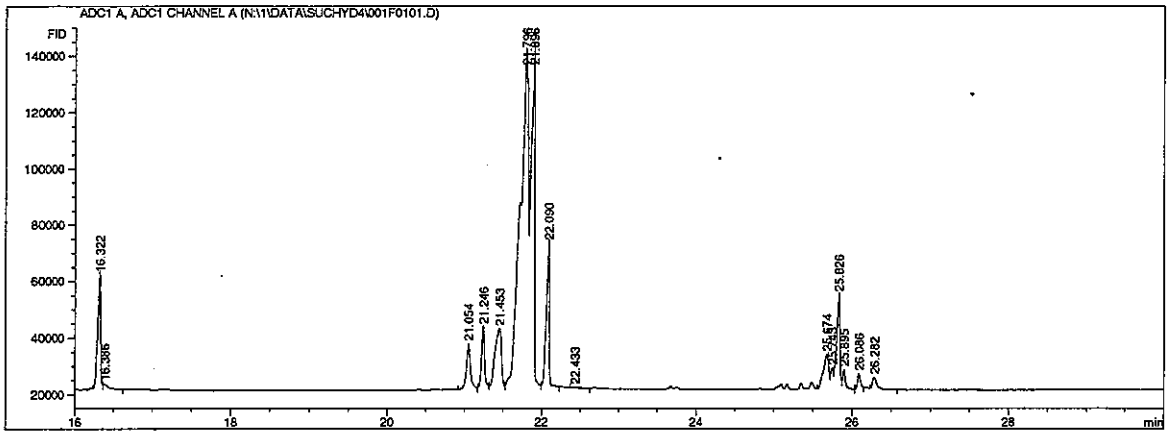


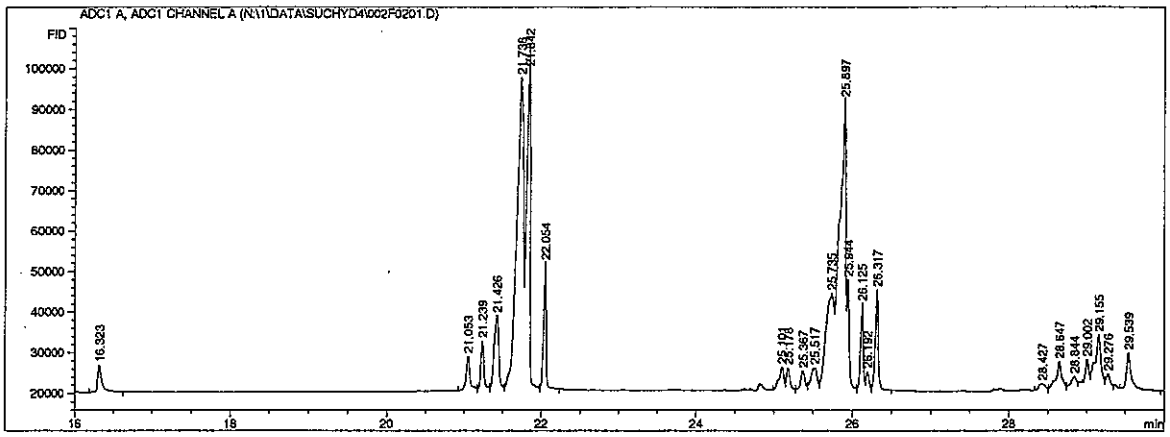
Figure 2. Characterisation of Esters by GC-FID.

A) Sucrose Ester L-1695 (HLB-16, 95% Lauric acid)



Sucrose	Mono-esters	Di-esters	Triesters
6%	83%	11%	Absent

B) Sucrose Ester L-595 (HLB-5, 95% Lauric acid)

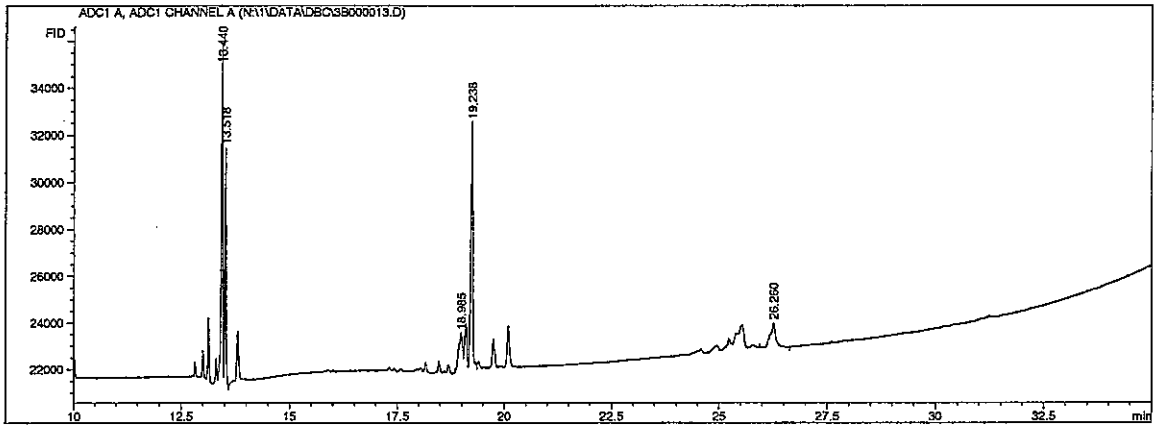


Sucrose	Mono-esters	Di-esters	Triesters
1%	42%	45%	12%

[Component identity; peaks at 16.3 min = sucrose, 21-22 min = sucrose monolaurate isomers, 25-26 min = sucrose dilaurates, 28-30 min = sucrose trilaurates. All observed as silyl ethers after BSTFA/DMF treatment].

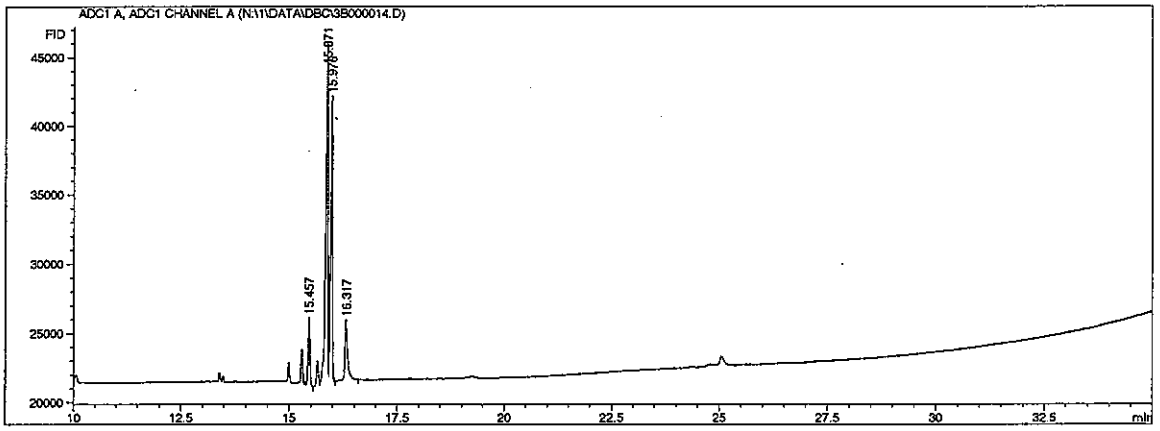
Figure 3. GC-FID analysis of sucrose fatty acid esters.

A) L-595 Lauric acid $C_{12:0}$ (mono:di: tri esters, 30:40:30%)



Mono-L Di-L Tri-L

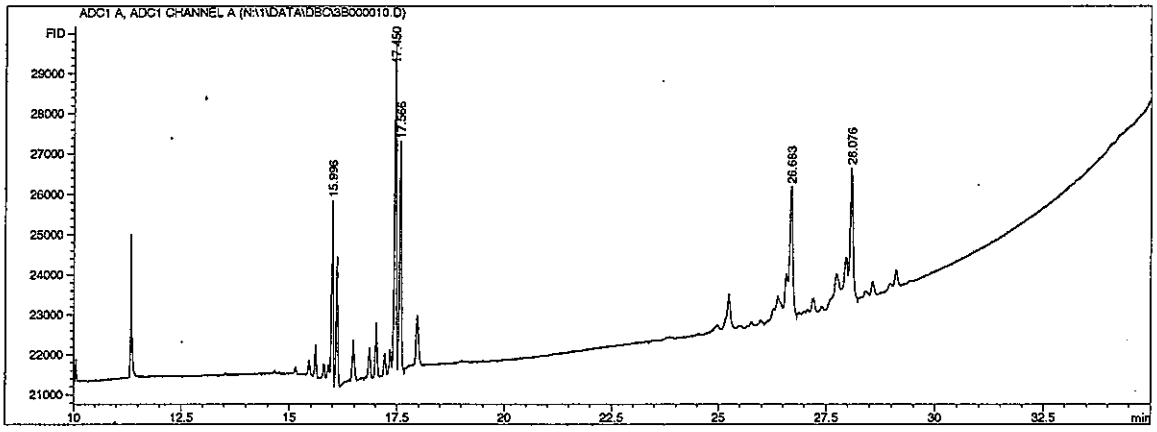
B) Mono-P Palmitic acid $C_{16:0}$ (mono:di, 95:5%)



Mono-P

Figure 3. (cont.)

C) S-1570 Mixed palmitic and stearic $C_{18:0}$ acids (mono-P:mono-S:di-P:Di-S, 15:59:5:15)



Sucrose Mono-P Mono-S Di-P Di-S

D) S-170 Mixed palmitic and stearic $C_{18:0}$ acids predominately higher esters