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A comprehensive investigation into sample extraction and method validation for the identification of antifungal compounds produced by lactic acid bacteria using HPLC-UV/DAD†

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High Performance Liquid Chromatography with Ultra Violet/Diode Array Detection (HPLC-UV/DAD) is an analytical tool that has the potential to develop and advance analytical research and routine analyses in industrially-important Lactic Acid Bacteria (LAB) with the aim of detecting metabolites with antifungal capabilities. Diode array detection is a multi-wavelength detection system that enables the simultaneous collection of absorption data from a range of UV and visible (UV/vis) wavelengths. The majority of antifungal organic compounds have characteristic UV/vis absorption spectra due to the presence of chromophoric groups or structures, this feature can be utilised to assist in identifying and quantifying the components (known and unknown) as they pass through the detector. HPLC-UV/DAD is robust, cheap to purchase and run, easy to maintain, the software is user friendly and automated for quantitation and the methodology can be easily switched/up-scaled to semi-preparative applications for target analyte isolation and purification. However inefficient sample preparation has become the limiting factor for the routine use of HPLC-UV/DAD in the LAB area. This manuscript will outline the development and validation of an HPLC-UV/DAD method that addresses all of the factors that has limited the routine use of this technology for LAB samples. The method has been developed to quantitatively profile fifteen antifungal compounds in LAB strains in a run time of 40 minutes, allowing the assessment of crude samples (direct injection), eliminating sample matrix chromatographic baseline rise effects through the optimisation of liquid–liquid extraction (LLE with average recoveries = 87%) and assessing a widely applied SPE methodology that is shown to yield poor recoveries for the suite of target analytes. The method reported is validated (linear calibration curve: 1–50 μg mL^{-1}; $R^2 \geq 0.999$; intra-day repeatability gave RSD values ($n = 3$) ranging from 0–8.8%; inter-day repeatability gave RSD values ($n = 9$) ranging from 0.3–7.9%; LODs of 0.2–0.6 μg mL^{-1} and LOQs of 0.5–0.9 μg mL^{-1} following LLE applied to a range of crude culture broths and extracted LAB cultures, and all results were confirmed against a liquid chromatography (LC) linear ion trap quadrupole (LTQ) Orbitrap hybrid Fourier transform mass spectrometer (FTMS). In summary, through this methodology, HPLC-UV/DAD can be routinely used as a quantitative screening technique for both culture broth (*e.g.* de Man, Rosaga and Sharpe (MRS)) and LLE-extracted LAB cultures.

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1. Introduction

Research into antifungal lactic acid bacteria (LAB) has increased in the past decade and shows that many LAB strains have the potential to combat the proliferation of fungi in various food and feed materials.[1–7] In particular the demand to reduce fungal contamination in foods has seen an increased demand by the consumer for the replacement of artificial chemical preservatives with natural biopreservatives.[1,4,8,9] LAB, due to their long history of safe use in food and feed fermentations,[10,11] have received both GRAS (Generally Recognised as Safe) status and QPS (Qualified Presumption of Safety) status in the EU and thus have good potential for future exploitation as antifungal biocontrol agents.

The sources for antifungal LAB strains vary from sourdough,[12,13] beer,[14] grass silage,[14–16] kimichi,[17,18] cereal environments,[19] and the gastrointestinal tracts of various mammals.[20] Banks of LAB have been compiled in a number of laboratories

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and tested with the aim to find the best antifungal strains for exploitation.[1]

Studies into the applications of antifungal LAB strains are quite diverse and typically involve both *in vitro* and *in situ* food model studies where a known fungus (*e.g. Fusarium*, *Penicillium*, *Aspergillus*, *Monilia*, *Pichia*, *Kluyveromyces*, *Rhodotorula*, *Debaryomyces*, *Zygosaccharomyces*, *Candida* and *Saccharomyces*) is tested against the antifungal LAB strain and where successful, applied to a final food (bread, cheese, yogurt) or beverage (fruit juice/fermented drink) product. The most promising areas where these applications have seen success include: (i) breads;[6,7,13,19,21] (ii) dairy products;[22,23] (iii) fruits and vegetables;[2,17,24,25] (iv) silage;[15] (v) seafood[26] and (vi) beverages – orange juice[22,27] and fermented seaweed drinks.[28]

The diversity of compounds that have been shown to provide antifungal activity include organic acids;[12,29,30] phenyllactic acid;[7,13,15,16,19,25,31–37] fatty acids;[15,20,36–38] cyclic dipeptides;[7,19,31,32,39,40] proteinaceous compounds;[5,41–43] reuterin[44–46] nucleosides,[19] lactones[14] and other low molecular weight compounds.[14,15,19,20,37] The development of analytical methods to improve the identification of the antifungal metabolites produced by bacteria and also to determine their concentrations is very important, but is frequently hampered by the large diversity of these compounds especially those produced by LAB.

HPLC coupled to ultra-violet (UV) and ultra-violet diode array (UV/DAD) detectors have been previously applied to the analysis of antifungal LAB with the development of both analytical and semi-preparative methods. However the level of analytical validation and application using these techniques has been quite limited and no publication has developed a comprehensive protocol that could be readily adapted into a LAB research or regulatory laboratory. In 2004, Valerio *et al.*[33] developed a HPLC-UV method for phenyllactic acid (PLA) and hydroxyphenyllactic acid (OH-PLA) and applied the method to 29 LAB strains. In 2006 Armaforte *et al.*[34] developed a HPLC-UV method for just one compound PLA, which included chromatographic figures and a more comprehensive method validation however, a protocol optimised for one target analyte is not sufficient in the LAB area. Similarly in 2010, Mu *et al.*[35] developed a HPLC-DAD methodology for OH-PLA only and applied it to profile *Lactobacillus* sp. SK007 broth grown in MRS.

Two LC-MS methods have been developed to date however this manuscript sets out to prove that HPLC-UV/DAD is sufficiently capable as the cost effective, routine and quantitative technique for the profiling of LAB strains whereas LC-MS technology is more expensive and is prone to ion suppression effects.[47] In food research and surveillance laboratories, LC-MS is often used as a secondary technique for confirmation and for more forensic investigation of the profile of samples. However LC-MS can also be used as a competent analytical tool and in 2011 Ndagano *et al.*[36] identified one compound, PLA using LC-MS in the strains *L. plantarum* VE56, *W. cibaria* FMF4B16 and *W. paramesenteroides* LC11 for antifungal compounds. In 2012, an LC-FTMS method for the quantitative profiling of antifungal compounds was published from this group where 25 compounds were chromatographically separated in 13 min.[37] The MS detection technology used was a LTQ Orbitrap FTMS, where both high resolution and high mass accuracy data was generated for both targeted and unknown compounds. Detailed method validation was included for all the compounds, spiked into mobile phase, crude MRS broth and after sample preparation (LLE). This methodology was applied to numerous LAB strains[20,23] and is used as a standardisation for this manuscript's HPLC-UV/DAD methodology.

HPLC-UV has also been used as a semi-preparative technique for the isolation of antifungal compounds.[15,19,25,31,32] These methods used HPLC-UV to guide fractionation. These fractions are then tested by an antifungal assays to detect fractions with antifungal activity. Once a fraction with activity was found the main compound(s) were identified by MS and Nuclear Magnetic Resonance (NMR). Due to the nature of this initial work the applied chromatography gradients were not optimised by using matrix matched standard mixes, on either a matched analytical column or on the semi-preparative columns due to the lack of knowledge of the compounds present. Knowledge of these compounds now has shown these methods to be inefficient. These gradients do not ensure that the target analytes are chromatographically resolved and that specific fractions would yield individually isolated compounds.

This manuscript therefore outlines an extensive study that was carried out to illustrate the analytical capabilities that the chromatographic technique, HPLC-UV/DAD, can play in LAB research. Use of DAD detector has major advantage for biological sample analysis; firstly it allows for the best wavelength(s) to be selected for individual compounds within the analysis (λ_{max}) and then allows for better detection based on peak purity capability. Biological samples can contain numerous components some of these may potentially co-elute with peaks of interest. This is of major concern with using such technologies as the peak shape of a compound may not clearly show the presence of another peak, therefore absorbance rationing at several wavelengths is particularly helpful in deciding whether the peak represents a single compound or, is in fact, a composite peak. A pure compound will produce a peak with spectra that has the same slope across the peak. If interferences from co-eluting compounds are present they will produce a composite spectrum which will give various degrees of spectral dissimilarity across the peak. This is the basis for peak purity determination. The percentage of similar spectra is a numerical measure of spectral homogeneity for the chromatographic peak and is reported as the Peak Purity Index (PPI). A homogeneous peak will produce a PPI of 100%; a PPI less than 100 suggests that only a fraction of the total number of spectra, matches the spectrum at the apex leading to the potential of a co-eluting compound. A thorough method validation was carried out that also addressed all of the factors that have limited the routine use of this technology in this analytical field. The method has been developed to quantitatively profile fifteen antifungal compounds in LAB strains in a run time of 40 minutes, allowing the assessment of both crude samples (direct injection) and LLE extracts. A throughput of 24 samples per day is possible with this methodology. Future work may look at employing UHPLC technology to retain separation but shorten method run time and increase method throughput. This method also assessed

and eliminated sample matrix chromatographic baseline rise effects through optimising the LLE procedure and assessed existing SPE methodologies that were shown to yield poor analyte recoveries for the suite of target analytes. The analytical results obtained from this validated HPLC-UV/DAD methodology were confirmed against a state-of-the-art MS technology, an Orbitrap LTQ-FTMS.

2. Experimental

2.1. Materials and chemicals

Analytical standards (A) 1,2-dihydroxybenzene; (B) DL-*p*-hydroxyphenyllactic acid (OH-PLA); (C) 4-hydroxybenzoic acid; (D) 3,4-dihydroxyhydrocinnamic acid; (E) vanillic acid; (F) caffeic acid; (G) 3-(4-hydroxyphenyl)propionic acid; (I) *p*-coumaric acid; (J) 3-(4-hydroxy-3-methoxyphenyl)propanoic acid; (K) benzoic acid; (L) ferulic acid; (M) salicylic acid; (N) hydrocinnamic acid; (O) methylcinnamic acid and MRS broth (used for cultivation of the LAB) were purchased from Sigma Aldrich (Dublin, Ireland). (H) Phenyllactic acid (PLA) was obtained from BaChem (Weil am Rhein, Germany). The purity of all analytes was 95% or higher. HPLC grade ethyl acetate (EA), acetonitrile (ACN) and water (H_2O) were obtained from Sigma Aldrich (Dublin, Ireland). Formic acid was from Fluka (Germany).

Stock solutions (2 mg mL^{-1}) of the individual antifungal compounds ($n = 15$) were prepared by dissolving 4 mg in 2 mL of H_2O or ACN following solubility guidelines as outlined in their material safety data sheets. 100 μL of each of the fifteen stock solution (2 mg mL^{-1}) was combined and 500 μL of H_2O–ACN (90/10) added to prepare a 100 ppm mixture stock solution (2 mL). 10 μL, 50 μL, 100 μL, 300 μL and 500 μL of the 100 ppm mixture stock solution were individually made up in amber vials to prepare the calibration curve standards of 1 ppm, 5 ppm, 10 ppm, 30 ppm and 50 ppm respectively. In the same manner controls of 7.5 ppm (75 μL), 20 ppm (200 μL) and 40 ppm (400 μL) were prepared.

2.2. Instrumentation and analytical conditions

2.2.1. HPLC-UV/DAD separation. The chromatographic system used was a Shimadzu LC system (CMB-20A/LC-10AT) equipped with an autosampler, a quaternary pump and photodiode array detector (SPD-M10A). A Phenomenex Gemini C18 column (150 × 2.0 mm, 5 μm; Macclesfield, UK) with a Phenomenex guard column (AF0-8497; Macclesfield, UK) was kept at 30 °C temperature. The mobile phase was composed of (A) H_2O with 0.1% formic acid and (B) ACN with 0.1% formic acid; this was filtered through a 0.2 μm filter. A gradient flow was optimised (0 min – 5% (B); 5 min – 10% (B); 10 min – 30% (B); 20 min – 30% (B); 30 min – 40% (B); 35 min – 40% (B); 40 min – 95% (B); 45 min – 95% (B)) at a flow rate of 0.2 mL min^{-1}. Injection volume was 10 μL. $\lambda_{max} = 210$ nm however a λ range of between 200–350 nm was also applied for DAD spectra generation.

2.2.2. MS. Fractions from the HPLC-UV/DAD analysis were collected and infused into the LTQ Orbitrap XL FTMS (Thermo Fisher Scientific, Hemel Hempstead, UK). The instrument was operated in negative ionisation mode at 30 000 resolution. The method tune conditions were as follows: capillary temperature of 300 °C, capillary voltage −50 V, tube lens −110 V, sheath gas 45 arb and auxiliary gas 15 arb. The instrument was calibrated weekly as per manufactures instructions to ensure accurate high mass data (<2 ppm) was obtained for all targeted analytes. A lock mass for acetic acid (m/z 59.013840) which was present consistently in the mobile phase was applied to the tune method to ensure reproducible instrument high mass accuracy. The high mass accuracy spectrum showing precursor and product ions with mass accuracies of <2 ppm confirm unequivocally the presence of target analytes.

2.3. Lactic acid bacteria (LAB) strain

The *Lactobacillus amylovorus* strain I, *Lactobacillus plantarum* strain II and *Weissella cibaria* strain III were grown in MRS broth and incubated at 37 °C for 48 hours. Cell free supernatant (cfs) was prepared by centrifugation (10 000 rpm for 10 minutes) and sterile filtration (0.45 μm pore size filter). Un-inoculated crude MRS broth was also prepared in the same manner as the LAB strains, for use as a blank matrix.

2.4. Extraction procedures

For each extraction procedure examined; 10 mL of cfs was spiked (3 μg mL^{-1}) with a standard mix of 15 antifungal compounds. All fractions collected from extractions (Fig. 3 and 5) were dried with 100 μL of DSMO added to the sample (DMSO keeps the analyte in solvent throughout solvent evaporation). The fractions were evaporated using nitrogen (Turbovap LV evaporator), samples were then reconstituted with water–acetonitrile (900 μL; 90/10; v/v), filtered (0.22 μm; Machinery and Nagle) and placed in individual amber vials (1.5 mL). 10 μL of the prepared extract was then injected onto a HPLC-UV/DAD system. During standard, control, blank and sample sequences, all vials were kept at 4 °C. Fig. 3 and 5 outlines the steps taken for the various extraction protocols.

2.4.1. Direct injection.[34] The spiked cfs was filtered (0.22 μm; Machinery and Nagle) prior to injection (10 μL) onto the analytical column as outlined in Section 2.2.1.

2.4.2. Solid phase extraction (SPE)

2.4.2.1. Ström et al. 2002 (ref. 31) (Fig. 3(A)) This method involved using Isolute, C18 EC cartridges (500 mg; 3 mL; International Sorbent Technology Ltd., Hengoed, United Kingdom). ACN (3 mL) and H_2O (3 mL) were added to pre-condition the cartridge. Spiked cfs was then loaded onto the cartridge. A 5% aqueous ACN solution (3 mL) was used to wash the cartridge and a 95% aqueous ACN (3 mL) was applied to elute the compounds. Both wash and elute fractions were collected and dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.4.3. Liquid liquid extraction (LLE)

2.4.3.1. Valerio et al. 2004 (ref. 33) (Fig. 3(B)) Spiked cfs was pH adjusted to pH 2 by the addition of 10 M formic acid (5 mL). This solution was then extracted with EA (4 × 30 mL). The organic layers were combined (~120 mL) and anhydrous sodium sulphate (Na_2SO_4, 4 g) was added to remove any

residual water. The solution was then filtered through a Whatman #4 filter paper and dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.4.3.2. Brosnan et al. 2012 (ref. 37) (Fig. 3(C)) Spiked cfs was extracted with EA (1 × 10 mL followed by 2 × 5 mL portions). The organic layers were combined (~20 mL) and dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.4.3.3. LLE modification #1 (Fig. 5). This extraction follows Section 2.4.3.1 with the modification that smaller volumes of EA (1 × 15 mL followed by 2 × 5 mL portions) were used. The organic layers were combined (~25 mL) and dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.4.3.4. LLE modification #2 (Fig. 5). This extraction follows Section 2.4.3.2 with the modification that after the organic layers were combined (~20 mL), anhydrous magnesium sulphate ($MgSO_4$, 4 g) was added to remove any residual water. This was then decanted and dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.4.3.5. LLE modification #3 (Fig. 5). This extraction is a modification to the method described in Section 2.4.3.2 with the spiked cfs being pH adjusted to pH 2 by the addition of concentrated formic acid (1 mL). The solution was then extracted with EA (1 × 10 mL followed by 2 × 5 mL portions). The organic layers were combined (~20 mL) and dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.4.3.6. LLE modification #4 (Fig. 5). This extraction combines modifications from Section 2.4.3.4 and 2.4.3.5. The pH was adjusted to pH 2 by the addition of concentrated formic acid (1 mL). The solution was then was extracted with EA (1 × 10 mL followed by 2 × 5 mL portions). The organic layers were combined (~20 mL) and anhydrous $MgSO_4$ (4 g) was added to remove any residual water. The EA supernatant was then dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.4.3.7 LLE modification #5 (Fig. 5). Spiked cfs was extracted with ACN and 3 g sodium chloride (NaCl) (1 × 10 mL portion) followed by ACN (2 × 5 mL portions). The organic layers were combined (~20 mL) and anhydrous $MgSO_4$ (4 g) was added to remove any residual water. The solution was then dried under nitrogen as outlined in Section 2.4 and analysed as outlined in Section 2.2.1.

2.5. Method validation

Validation is a prerequisite in all method developments and it was carried out in accordance with the EC[48] and ICH[49] guidelines. Calibration curves were generated by analysing five different concentrations (1 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1}, 30 μg mL^{-1}, and 50 μg mL^{-1}) of standard mixes in triplicate on 3 consecutive days. The calibration curves were then constructed as peak area *versus* the standard concentrations and the linear relationship determined. Sample extracts were quantified using the standard calibration curve. Limit of detection (LOD) and limit of quantitation (LOQ) were determined by measuring signal to noise (S/N) values by LOD ($S/N = 3$) and LOQ ($S/N = 10$) respectively following the serial dilutions of a standard mix. Three different concentrations (controls #1; 7.5 μg mL^{-1}, control #2; 20 μg mL^{-1} and control #3; 40 μg mL^{-1}) were picked corresponding to the lower, middle and upper sections of the calibration curve. These were injected three times within the same day ($n = 3$) and three times over three successive days ($n = 9$). The peak areas were quantified against generated calibration curves and the relative errors were calculated and compared to the true values. The relative standard deviation as percentages for the intra-day results and inter-day data was also calculated for all standard and control points.

3. Results and discussion

Use of HPLC for the identification and quantification within the area of LAB has been underutilised. To-date only three HPLC-UV and HPLC-UV/DAD methods have been published in peer-reviewed journals.[33,34,50] The number of compounds detected by these methods is quiet limited (1–2 compounds) with an incomplete level of validation details being provided. ESI Table S2† summarises these published methodologies. LAB strains contain numerous compounds which are believed to act synergistically. Therefore a method which only identifies 1–2 compounds is not advantageous if progress within the area is to be made. HPLC-DAD has found more frequent use as a semi-preparative technique for the isolation of antifungal compounds[15,19,25,31,32] with isolated fractions being by NMR spectroscopy, gas chromatography (GC) and MS. For all of these published methods no information was given on the purity of the fractions generated from the chosen semi-preparative chromatography or as to how the semi-preparative mobile phase, column or gradient conditions were chosen to achieve pure fractions of the target analytes. The authors in this study found that reported semi-preparative chromatographic conditions (linear gradients) when repeated on an analytical column (Phenomenex Gemini C18, Agilent Eclipse XDB-C18, Supelco Discovery C18, GL Sciences InertSustain C18), did not produce the chromatographic peak resolution that would be necessary for definitive and selective compounds isolation (unpublished data to be published elsewhere). Therefore a study was initiated:

(1) To achieve complete chromatographic separation of 15 known antifungal analytes so that these compounds could be quantitatively identified. The optimised gradient should then facilitate the identification of new compounds (having similar structural features), chromatographically resolved from the 15 antifungal standards. Therefore both known and new analytes can be profiled during LAB strains screening campaigns.

(2) To develop and validate an HPLC-UV/DAD method for each of the chosen 15 antifungal compounds. To demonstrate the analytical capabilities that an optimised chromatographic technique HPLC-UV/DAD method can play in the research field of LAB for the quantitative profiling of known compounds and in the identification of new compounds.

(3) To address all of the factors that have limited the routine use of HPLC-UV/DAD for the screening of LAB samples allowing:

(a) The assessment of crude sample/extracts through direct injection.

(b) Eliminate sample matrix chromatographic baseline effects through optimised LLE, and,

(c) Assess an existing and widely applied SPE methodology that indicates poor recoveries for the suite of target analytes found in LAB strains.

(4) To confirm all generated HPLC-UV/DAD results against a previously validated state-of-the-art MS technology, a LTQ Orbitrap FTMS.

3.1. Chromatographic separation of fifteen antifungal compounds

Fig. 1 outlines the structures of fifteen previously reported antifungal compounds that were used as standards for the chromatographic separation and method development using a HPLC-UV/DAD. The aim was to generate a HPLC-UV/DAD analytical method with excellent chromatographic separation and resolution of the target analytes to allow the quantitative identification of both known and unknown compounds. Fig. 2(I) outlined the optimised chromatographic separation of a standard mix (30 μg mL^{-1}; 10 μL injection) of previously reported antifungal compounds from LAB prepared in H_2O–ACN (90/10). This chromatography shows fifteen distinctly separated compounds (1,2-dihydroxybenzene, OH-PLA, 4-hydroxybenzoic acid, 3,4-dihydroxyhydrocinnamic acid, vanillic acid, caffeic acid, 3-(4-hydroxyphenyl)propionic acid, PLA, coumaric acid, 3-(4-hydroxy-3-methoxyphenyl)propanoic acid, benzoic acid, ferulic acid, salicylic acid, hydrocinnamic acid, methylcinnamic acid). Compounds are stated in order of elution for the optimised chromatographic conditions. It was observed during method development that changing the mobile phase solvent used (ACN or methanol); the mobile phase additive (formic acid, trifluoroacetic acid or acetic acid) or the column (4 × C18 columns assessed) while using the same gradient conditions changed the elution order of compounds. This is an important consideration for semi-preparative scale-up and the fractionation/purification of target analytes.

Fig. 2(II) illustrated a typical chromatogram generated after spiking (30 μg mL^{-1}; 10 μL) the fifteen antifungal standards into crude MRS broth, filtering the broth and injecting it directly into the HPLC. No selective sample preparation was carried out so as to reduce samples matrix effects. From the start of peak A (1,2-dihydroxybenzene) to after the elution of peak N (hydrocinnamic acid), a region of increased baseline rise due to matrix constituents was observed. While the baseline rise is always present, the extent of this baseline rise varies between crude culture broths and is unpredictable. Direct injection of broth was suggested by Armaforte *et al.*[34] as a simple and rapid method for identification of PLA using a short modified gradient. However Armaforte *et al.*[34] method was optimised for the identification of only one compound (PLA) so the method has no consideration for the samples matrix influences that occur when the chromatography is developed to identify numerous antifungal compounds. As a norm elevated chromatographic baselines should be minimized or eliminated

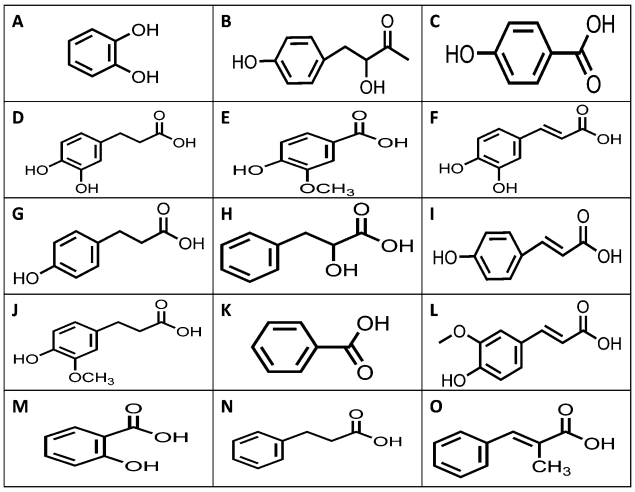

Fig. 1 Structure of the 15 previously reported antifungal compounds (listed in order of chromatographic elution): (A) 1,2-dihydroxybenzene (110.11 g mol^{-1}); (B) DL-*p*-hydroxyphenyllactic acid (OH-PLA) (182.17 g mol^{-1}); (C) 4-hydroxybenzoic acid (138.12 g mol^{-1}); (D) 3,4-dihydroxyhydrocinnamic acid (182.17 g mol^{-1}); (E) vanillic acid (168.15 g mol^{-1}); (F) caffeic acid (180.16 g mol^{-1}); (G) 3-(4-hydroxyphenyl)propionic acid (166.17 g mol^{-1}); (H) phenyllactic acid (PLA) (166.17 g mol^{-1}); (I) coumaric acid (164.16 g mol^{-1}); (J) 3-(4-hydroxy-3-methoxyphenyl)propanoic acid (196.26 g mol^{-1}); (K) benzoic acid (122.12 g mol^{-1}); (L) ferulic acid (194.18 g mol^{-1}); (M) salicylic acid (138.12 g mol^{-1}); (N) hydrocinnamic acid (150.17 g mol^{-1}); (O) methylcinnamic acid (162.19 g mol^{-1}).

prior to samples analysis. Through the assessment of sample preparation techniques this raised baseline was eliminated with appropriate sample preparation; indicating the baseline rise was probably a result of the complex matrix constituents from the bacteria growth medium (MRS). A review of the literature indicated that in LC application there is very little discussion on chromatographic baseline rises or humps. However, in GC applications, baseline rises or humps can be an issue. EPA method 8000C (ref. 51) suggests that through suitable sample preparation clean up, dilution of samples, the use of pre-columns and/or inserts or selection of an appropriately selective detector can reduce or eliminate these baseline rises or humps.

However this chromatography (Fig. 2(II)) while not ideal chromatographically, still allows the analyst to identify and semi-quantify all fifteen antifungal standards and identify numerous other unknown peaks. This ability to qualitatively profile crude MRS broth samples using HPLC-UV/DAD is most beneficial and time efficient to food chemist when (i) tracking the antifungal profile of MRS broths, (ii) assessing new growth media recipes, (iii) tracking the antifungal profile of compounds after their integration into the food material. This ability to screen crude matrices cannot be succeeded consistently and routinely using electrospray tandem LC-MS due to intense ion suppression throughout regions of the chromatogram.[47]

3.2. Method validation

Validation of the method was carried out in compliance with the EC and ICH guidelines taking into account specificity,

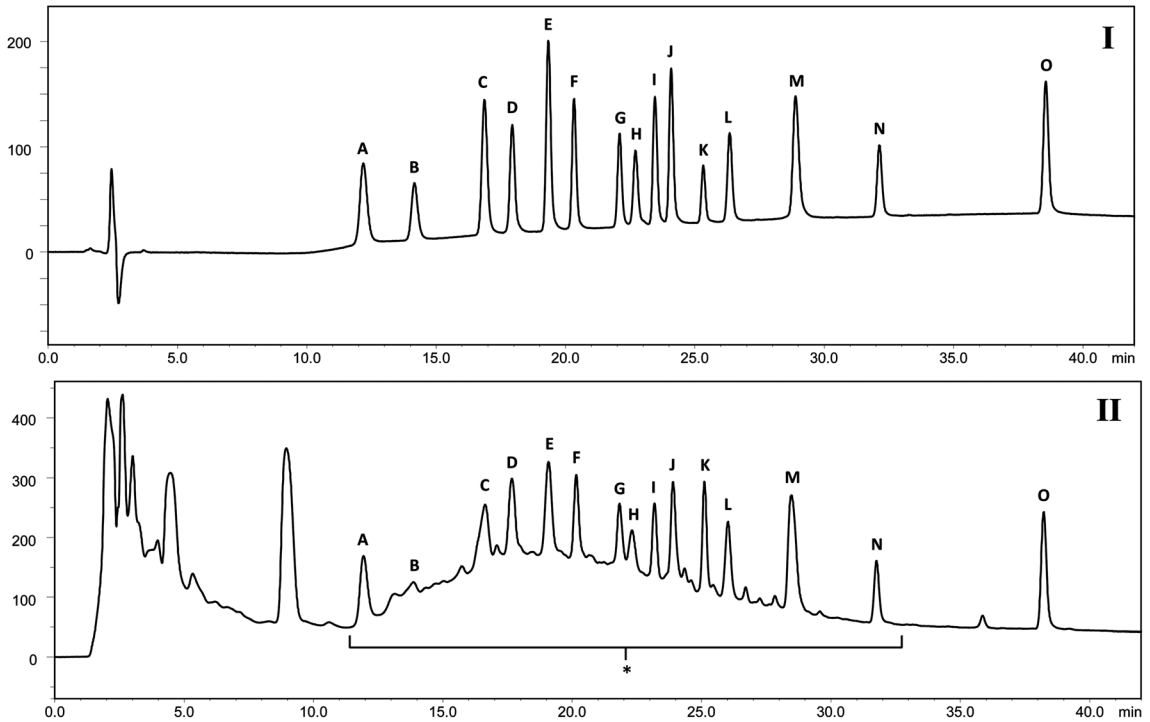

Fig. 2 Chromatographic separation of a standard mix (30 μg mL^{-1}; 10 μL injection) of previously reported antifungal compounds from LAB prepared in (I) water–acetonitrile (90/10) and (II) crude MRS broth (filtered 0.2 μm; no LLE extraction; direct injection). (A) 1,2-dihydroxybenzene (12.17 min); (B) OH-PLA (14.15 min); (C) 4-hydroxybenzoic acid (16.86 min); (D) 3,4-dihydroxyhydrocinnamic acid (17.93 min); (E) vanillic acid (19.33 min); (F) caffeic acid (20.32 min); (G) 3-(4-hydroxyphenyl)propionic acid (22.08 min); (H) PLA (22.69 min); (I) coumaric acid (23.45 min); (J) 3-(4-hydroxy-3-methoxyphenyl)propanoic acid (24.07 min); (K) benzoic acid(25.31 min); (L) ferulic acid (26.34 min); (M) salicylic acid (28.88 min); (N) hydrocinnamic acid (32.12 min); (O) methylcinnamic acid (38.562 min). Chromatographic conditions: Phenomenex Gemini C18 (150 × 2.1 mm, 5 μm); mobile phase (A) H_2O + 0.1% formic acid, mobile phase (B) MeCN + 0.1% formic acid; gradient conditions: 0 min – 5% (B); 5 min – 10% (B); 10 min – 30% (B); 20 min – 30% (B); 30 min – 40% (B); 35 min – 40% (B); 40 min – 95% (B); 45 min – 95% (B) with an equilibration time of 20 min; 0.2 mL min^{-1}; λ_{max} = 210 nm.* Region where there is an increase in baseline rise due to matrix constituents.

linearity, limits of detection and quantitation, trueness and precision, as outlined in previous papers from our group.[50,52–58]

3.2.1. Specificity. With HPLC-UV/DAD both retention time and DAD spectra are the only tools available as identification marker. The EC council directive 2002/657/EC (implementing the council directive 96/23/EC) suggests that retention time should not vary more than ±2.5%, for LC with other detection techniques. For this HPLC-UV/DAD method, retention times were recorded over 3 days with three repeat injections per day of five standards and three controls in the range of (1–50 μg mL^{-1}) spiked into blank (uninoculated) MRS broth (n = 72). The average retention time variation for all 15 antifungal standards range between 0.1–0.3%, well below the recommended value of ±2.5%.

3.2.2. Linearity. The linear range for all the standard antifungal compounds was between 1–50 μg mL^{-1}. Table 1 and ESI Table S1† shows the linear range, equation of line and correlation coefficient for each compound prepared in mobile phase (H_2O–ACN; 90/10). This method showed good linearity with correlation coefficients of ≥0.999 for all compounds.

3.2.3. Limits of detection and quantitation. Determination of the limit of detection (LOD) and limit of quantitation (LOQ) was completed by measuring the signal to noise (S/N) ratio's and applying a S/N = 3 for LOD values and S/N = 10 for LOQ values. Table 1 shows LOD and LOQ values for all the compounds.

3.2.4. Precision of standard calibration curves. Precision results were good for this method, Table 1 and ESI Table S1† shows a list of the relative standard deviations (RSD) for the concentrations analysed in the calibration curve (1–50 μg mL^{-1}) for all 15 compounds. Percentage RSD values are small ranging from 0–8.7% (intra-day) and from 0.3–7.9% (inter-day) for all antifungal compounds (Table 1). ESI Table S1† outlined the specific RSD for each of the standard concentrations (1, 5, 10, 30 and 50 μg mL^{-1}). As expected the higher concentration standards (5, 10, 30 and 50 μg mL^{-1}) gave the lowest RSDs for both intra-day (0.0–3.9%) and inter-day (0.3–4.4%) studies. The lowest concentration standard (1 μg mL^{-1}) gave the higher RSDs for both intra-day (1.2–8.7%) and inter-day (3.0–7.9%) studies.

3.2.5. Trueness and precision of the spiked controls. Trueness is defined as the comparison between a spiked standard(s) of known concentration(s) examined as an unknown sample(s) and qualitatively compared to the known concentration(s). This is expressed as the relative error (RE) which indicates the extent of the deviation from the true concentration as a percentage. In this study, three controls were prepared:

Table 1 Linear calibration curve data for antifungal compounds

Antifungal compound	Equation of line	Correlation coefficient (R^2)	Linear range[a] (μg mL^{-1})	Intra-day RSD[b] (%)	Inter-day RSD[c] (%)	LOQ[d] (μg mL^{-1})	LOD[e] (μg mL^{-1})
(A) 1,2-Dihydroxybenzene	$y = 49\,229x + 35\,200$	1.000	1–50	0.32–5.7	0.34–5.7	0.8	0.5
(B) DL-*p*-Hydroxyphenyllactic acid	$y = 30\,959x + 1285$	1.000	1–50	0.07–7.2	0.68–7.9	0.5	0.2
(C) 4-Hydroxybenzoic acid	$y = 63\,307x + 45\,144$	1.000	1–50	0.99–2.1	0.93–3.2	0.5	0.2
(D) 3,4-Dihydroxyhydrocinnamic acid	$y = 45\,311x + 23\,772$	0.999	1–50	0.04–3.5	0.69–3.2	0.5	0.2
(E) Vanillic acid	$y = 74\,265x + 58\,429$	0.999	1–50	0.13–2.2	0.66–3.0	0.5	0.2
(F) Caffeic acid	$y = 47\,634x + 15\,727$	1.000	1–50	0.79–1.8	0.84–4.3	0.5	0.2
(G) 3-(4-Hydroxyphenyl)propionic acid	$y = 34\,793x + 11\,800$	1.000	1–50	0.45–3.5	0.81–5.3	0.6	0.3
(H) Phenyllactic acid	$y = 31\,199x + 9843$	1.000	1–50	0.31–4.9	0.87–6.4	0.8	0.5
(I) *p*-Coumaric acid	$y = 42\,453x + 42\,357$	0.999	1–50	0.64–7.3	1.03–6.9	0.6	0.3
(J) 3-(4-Hydroxy-3-methoxyphenyl) propanoic acid	$y = 53\,725x + 34\,875$	1.000	1–50	0.06–4.2	0.87–4.6	0.5	0.2
(K) Benzoic acid	$y = 20\,318x + 1430$	1.000	1–50	0.08–3.5	0.82–6.4	0.6	0.3
(L) Ferulic acid	$y = 35\,918x + 22\,679$	1.000	1–50	0.02–8.8	1.07–6.9	0.9	0.6
(M) Salicylic acid	$y = 61\,383x + 57\,587$	0.999	1–50	0.20–1.6	1.4–7.0	0.6	0.3
N) Hydrocinnamic acid	$y = 28\,721x + 18\,766$	1.000	1–50	0.06–5.6	0.91–7.6	0.9	0.6
(O) Methylcinnamic acid	$y = 58\,763x + 45\,565$	0.999	1–50	0.23–2.5	1.2–3.4	0.6	0.3

[a] 10 μL injection. [b] Average relative standard deviation ($n = 3$) calculated on the same day. [c] Average relative standard deviation ($n = 9$) calculated over 3 days. [d] Limit of quantitation ($S/N = 10$). [e] Limit of detection ($S/N = 3$).

control #1 (7.5 μg mL^{-1}), control #2 (20 μg mL^{-1}) and control #3 (40 μg mL^{-1}). These were injected three times within the same day ($n = 3$) and nine times over three successive days ($n = 9$). The peak areas obtained were quantified against corresponding calibration curves and the relative error calculated and compared to the true value (Table 2). A RE range of −0.5 to 6.4% for control #1 (average = 3.0%), −1.8 to 2.0% for control #2 (average = 0.2%) and −1.4 to 1.8% for control #3 (average = −0.1%) was obtained for all compounds analysed (Table 2). The corresponding RSD as percentages for both the intra-day and inter-day runs was also calculated for the three controls (Table 2). The intra-day RSD ranged from 0.4–3.4% for control #1 (averaging 1.4%), 0.2–1.8% for control #2 (averaging 1.1%), 0.1–0.8% for control #3 (averaging 0.4%) while the inter-day RSD ranged from 0.7–3.4% for control #1 (averaging 1.55%), 0.8–3.0% for control #2 (averaging 1.4%), 0.4–1.9% for control #3 (averaging 1.0%) (Table 2).

3.3 Extraction assessment of previously published methods

Recovery experiments were carried out for the extraction of the fifteen target antifungal analytes spiked into MRS broth, these results are summarised in Table 3 outlining the percentage recoveries and the corresponding RSD's generated following previously published extraction methods.[31,33,37] ESI Fig. S1† illustrates the data generated as a bar chart. Poor recoveries were obtained following the well-known and widely applied Ström *et al.*[31] SPE method. Fig. 3 outlines this SPE method involving the use of an Isolute C18 EC cartridge. The wash fraction was typically disposed to waste. For this study this fraction was kept, re-concentrated and analysed and yielded average recoveries ($n = 3$) ranging from 0–35% for the suite of 15 antifungal compounds. The elution fraction yielded average recoveries ($n = 3$) ranging from 0–77%. Thirteen of the 15 compounds had average recoveries <32% from the elution fraction which is an astonishingly poor recovery for a SPE protocol. The baseline rise observed in Section 3.1 (Fig. 2(II)) is present in the SPE wash and elute step (Fig. 4(Ia) and (Ib)) and is the major contributing factor to these large RSD values as integration of the peaks was more difficult to obtain due to baseline rise. The average percentage recoveries for all 15 compounds were extremely poor at 13% and 24% for the wash and elution SPE steps (Table 3). Armaforte *et al.* method[34] also assessed the performance of the Ström *et al.*[31] SPE method and similarly observed partitioning between the wash and elute steps with a percentage recovery of 11% in elute and 63% in wash step for PLA.

Improved recoveries for the 15 antifungal standards spiked into MRS broth were obtained following the extraction of broth using LLE methods from previous publications (Fig. 3, Table 3 and ESI Fig. S1†). Average recoveries ranging from 29–121% were obtained applying the Valerio *et al.*[33] LLE method. The combined EA supernatant of 120 mLs was notably substantial and impracticable and took almost 210 min to evaporate with nitrogen using a turbo-evaporator. The RSD for thirteen of the 15 antifungal compounds ranged from 29 to 43% which is well above the 20% threshold.[48]

The Brosnan *et al.*[37] LLE extraction method showed similar percentage recoveries to the Valerio *et al.*[33] methods ranging from 52–125% (Table 3 and ESI Fig. S1†). This method involved an improved procedure with the volume of the LLE solvent greatly reduced by 83% and the time required to evaporate the supernatant with nitrogen reduced by 87% from 210 min to 30 min (Fig. 3). Precision results were excellent for this method. The RSD for each of the 15 target compounds was also significantly reduced and ranged from 0.1–7.0%. These values were not achievable for both the Ström *et al.*[31] SPE and Valerio *et al.*[33] LLE methods.

Table 2 Trueness and precision data

Antifungal compound	T. conc.[a] (μg mL^{-1})	M. conc.[b] (μg mL^{-1})	RE[c] (%)	Intra-day RSD[d] (%)	Inter-day RSD[e] (%)
(A) 1,2-Dihydroxybenzene	7.5	7.5	−0.5	3.4	2.4
	20	20	−0.7	0.2	0.8
	40	40	0.1	0.4	0.5
(B) DL-*p*-Hydroxyphenyllactic acid	7.5	7.6	1.7	0.5	1.0
	20	20	−1.8	1.0	0.9
	40	39	−1.4	0.5	0.7
(C) 4-Hydroxybenzoic acid	7.5	7.7	3.3	1.9	1.4
	20	20	0.9	0.8	1.0
	40	40	0.6	0.6	0.8
(D) 3,4-Dihydroxyhydrocinnamic acid	7.5	7.9	4.7	1.2	1.4
	20	20	2.0	0.5	0.9
	40	40	0.3	0.1	0.4
(E) Vanillic acid	7.5	7.8	4.1	0.9	0.7
	20	20	1.8	1.0	1.1
	40	39	−0.8	0.2	0.4
(F) Caffeic acid	7.5	7.6	1.5	0.7	1.0
	20	20	−0.9	1.0	1.4
	40	40	−0.2	0.1	0.5
(G) 3-(4-Hydroxyphenyl)propionic acid	7.5	7.8	3.5	1.1	1.0
	20	20	−1.4	1.0	1.2
	40	40	−0.3	0.2	0.4
(H) Phenyllactic acid	7.5	7.7	3.1	2.5	1.6
	20	20	−1.2	1.7	1.8
	40	40	−0.4	0.5	0.9
(I) *p*-Coumaric acid	7.5	7.6	0.8	0.6	3.4
	20	20	1.3	1.6	3.0
	40	41	1.8	0.4	3.0
(J) 3-(4-Hydroxy-3-methoxyphenyl) propanoic acid	7.5	7.8	3.9	0.4	0.8
	20	20	1.0	0.9	1.2
	40	40	−0.2	0.3	0.8
(K) Benzoic acid	7.5	7.6	2.5	1.5	2.8
	20	20	1.1	1.5	1.3
	40	40	−0.4	0.3	0.7
(L) Ferulic acid	7.5	7.8	1.2	2.7	1.7
	20	20	−1.9	1.1	1.1
	40	40	−0.7	0.3	1.2
(M) Salicylic acid	7.5	8.0	6.4	0.6	1.8
	20	20	1.1	0.7	2.6
	40	40	0.3	0.5	1.9
(N) Hydrocinnamic acid	7.5	7.7	2.5	1.7	1.3
	20	20	1.1	1.9	1.3
	40	40	−0.4	0.8	1.7
(O) Methylcinnamic acid	7.5	7.9	5.0	1.1	0.8
	20	20	1.4	1.8	1.5
	40	40	−0.1	0.5	1.3

[a] True concentration. [b] Measured concentration (10 μL injection). [c] Relative error. [d] Average relative standard deviation ($n = 3$) calculated on same day for the three controls. [e] Average relative standard deviation ($n = 9$) calculated for the three controls over 3 days.

In summary the Brosnan *et al.*[37] extraction method gave an average percentage recovery of 87% and an average percentage RSD of 1.8% for all fifteen antifungal compounds, followed by the Valerio *et al.*[33] method which gave an average percentage recovery of 83% and an average RSD of 30%. The Ström *et al.*[31] SPE elution fraction gave an average percentage recovery of 24% and an average RSD of 14% for fourteen antifungal compounds, not showing any peak for OH-PLA. The Ström *et al.*[31] SPE wash fraction gave an average percentage recovery of 13% and an average RSD of 26% for thirteen antifungal compounds, not showing any peaks for 1,2-dihydroxybenzene and salicylic acid.

3.4. Modification of extraction methods

Following assessment of the recovery experiments of the previously published methods (Fig. 3) and the recovery data (Table 3 and ESI Fig. S1†) obtained from the extraction methods, it was decided to eliminate SPE for now as a technique

Table 3 Percentage recovery for compounds spiked into crude MRS broth extracted using previously published extraction methods[31,33,37]

	Ström *et al.* 2002 (ref. 31) (wash)		Ström *et al.* 2002 (ref. 31) (elute)		Valerio *et al.* 2004 (ref. 33)		Brosnan *et al.* 2012 (ref. 37)	
Compound name	% recovery	RSD ($n = 3$)	% recovery	RSD ($n = 3$)	% recovery	RSD ($n = 3$)	% recovery	RSD ($n = 3$)
(A) 1,2-Dihydroxybenzene			16	8.7	56	33	52	1.7
(B) DL-*p*-Hydroxyphenyllactic acid	5.7	50			55	35	53	3.8
(C) 4-Hydroxybenzoic acid	30	3.7	24	6.3	45	33	79	7.0
(D) 3,4-Dihydroxyhydrocinnamic acid	21	44	33	5.1	106	3.2	96	3.9
(E) Vanillic acid	1.1	24	4.7	39	62	29	94	0.9
(F) Caffeic acid	4.8	27	9.2	13	121	43	81	0.1
(G) 3-(4-Hydroxyphenyl)propionic acid	35	4.2	42	9.1	108	4.2	117	0.9
(H) Phenyllactic acid	6.5	75	12	27	80	36	64	3.5
(I) *p*-Coumaric acid	4.6	37	12	14	109	31	94	1.0
(J) 3-(4-Hydroxy-3-methoxyphenyl) propanoic acid	11.2	21	17	15	89	39	75	0.1
(K) Benzoic acid	9.1	21	31	8.5	138	29	125	1.2
(L) Ferulic acid	19	21	31	18	111	31	114	0.6
(M) Salicylic acid			6.6	6.5	29	33	97	0.7
(N) Hydrocinnamic acid	5.0	10	25	8.4	67	33	94	0.4
(O) Methylcinnamic acid	9.9	6.2	77	14	71	34	62	1.6
Average	13	26	24	14	83	30	87	1.8

for the rapid and selective extraction of antifungal compounds from LAB broths. Hence modifications to the LLE methods were undertaken in an attempt to improve the methods further (Fig. 5). Table 4 and ESI Fig. S2† display the percentage recovery for the modifications trialled. ESI Fig. S3 and S4† show the chromatography generated by the modification extractions.

Regardless of the modification made, overall the average recoveries for LLE Modifications (#1 was 76%, #2 was 71%, #3 was 80%, #4 was 72% and #5 was 72%) are lower than the previously published LLE methods; Valerio *et al.*[33] and Brosnan *et al.*[37] (83% and 87% respectively). As can be clearly seen from the graphical representation of ESI Fig. S2,† improvements

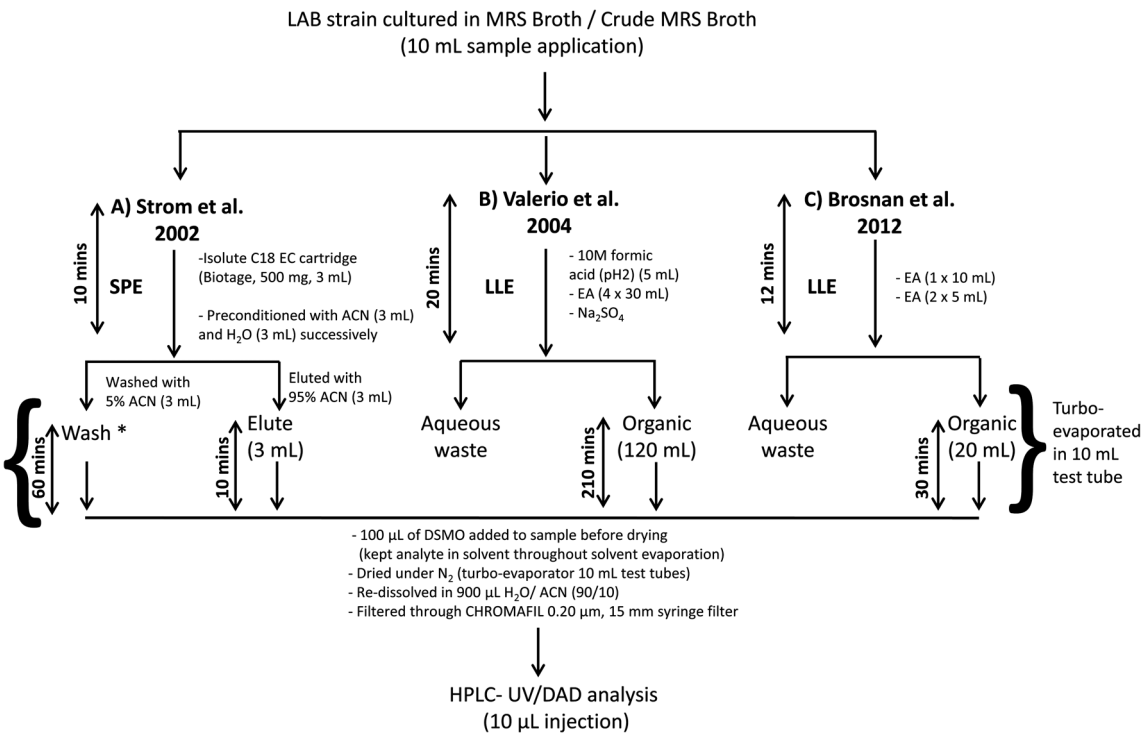

Fig. 3 Flow diagram of published methods (Ström *et al.*;[31] Valerio *et al.*;[33] Brosnan *et al.*[37]) sample preparation procedures for the extraction of a crude MRS broth sample and/or LAB strain cultured in MRS broth, showing volumes and time required for the methods. * Wash step discarded in Ström *et al.*[31] method, kept for comparison in this study.

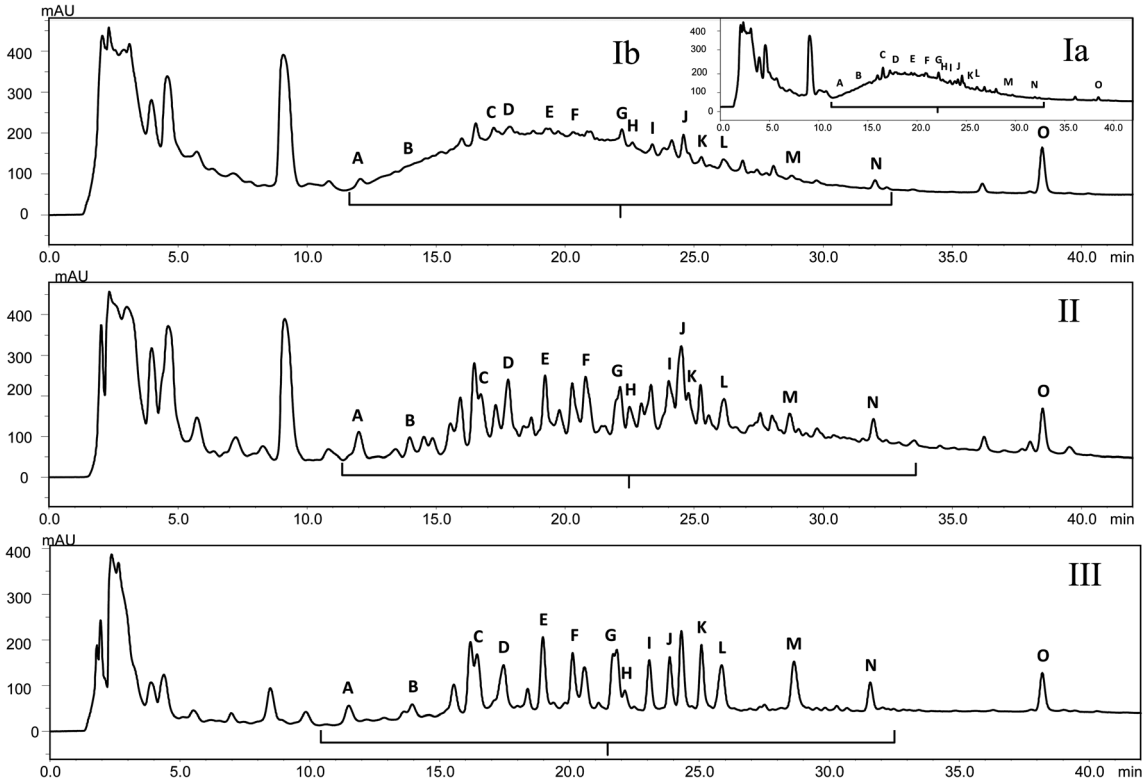

Fig. 4 Chromatographic profiles of spiked crude MRS broth (30 μg mL^{-1}): (A) 1,2-dihydroxybenzene; (B) OH-PLA; (C) 4-hydroxybenzoic acid; (D) 3,4-dihydroxyhydrocinnamic acid; (E) vanillic acid; (F) caffeic acid; (G) 3-(4-hydroxyphenyl)propionic acid; (H) PLA; (I) *p*-coumaric acid; (J) 3-(4-hydroxy-3-methoxyphenyl)propanoic acid; (K) benzoic acid; (L) ferulic acid; (M) salicylic acid; (N) hydrocinnamic acid; (O) methylcinnamic acid extracted by published methods outlined in Fig. 3. (Ia) Ström *et al*. (SPE wash);[31] (Ib) Ström *et al*. (SPE elute);[31] (II) Valerio *et al*. (LLE);[33] (III) Brosnan *et al*. (LLE).[37] Chromatographic conditions as described in Fig. 2. Percentage recovery data can be seen in ESI Fig. S1† and Table 3.

from certain compounds by these modifications can be seen but when choosing the best overall LLE method the Brosnan *et al.*[37] still appears to provide the optimum extraction for the 15 antifungal compounds.

3.5. Application of best extraction method to antifungal LAB strains

Brosnan *et al.* method was chosen as the extraction method to proceed with as it gave the highest average percentage recoveries (87%) and the lowest RSD average (1.8%). It also provided the best range with greater than 51% recovered for all 15 antifungal compounds. Three LAB strains (*Lactobacillus amylovorus* strain I, *Lactobacillus plantarum* strain II and *Weissella cibaria* strain III) which show strong antifungal activity were extracted by the Brosnan *et al.*[37] method and injected on the HPLC-UV/DAD method. The antifungal compounds present can be identified and quantified from these antifungal strains (Table 5). OH-PLA, 4-hydroxybenzoic acid, caffeic acid, 3-(4-hydroxyphenyl)propionic acid, PLA, coumaric acid, 3-(4-hydroxy-3-methoxyphenyl)propanoic acid, benzoic acid, ferulic acid and hydrocinnamic acid were identified at different concentration by the three strains. *Lactobacillus amylovorus* strain I – identified 2.1 μg mL^{-1} of OH-PLA, 3.4 μg mL^{-1} of 4-hydroxybenzoic acid, 6.1 μg mL^{-1} of 3-(4-hydroxyphenyl)propionic acid, 20 μg mL^{-1} of PLA, 0.7 μg mL^{-1} coumaric acid, 0.1 μg mL^{-1} of 3-(4-hydroxy-3-methoxyphenyl) propanoic acid, 7.5 μg mL^{-1} of benzoic acid, 1.3 μg mL^{-1} of ferulic acid and 0.03 μg mL^{-1} of hydrocinnamic acid. *Lactobacillus plantarum* strain II identified – 4.8 μg mL^{-1} of OH-PLA, 1.03 μg mL^{-1} of 4-hydroxybenzoic acid, 0.04 μg mL^{-1} of caffeic acid, 43 μg mL^{-1} of PLA, 0.46 μg mL^{-1} of coumaric acid, 0.13 μg mL^{-1} of 3-(4-hydroxy-3-methoxyphenyl)propanoic acid 29 μg mL^{-1} of benzoic acid and 1.2 μg mL^{-1} of ferulic acid. *Weissella cibaria* strain III identified 1.5 μg mL^{-1} of OH-PLA, 3.4 μg mL^{-1} 4-hydroxybenzoic acid, 0.07 μg mL^{-1} of caffeic acid, 7.1 μg mL^{-1} of 3-(4-hydroxyphenyl)propionic acid, 5.4 μg mL^{-1} of PLA, 0.88 μg mL^{-1} of coumaric acid, 2.2 μg mL^{-1} of 3-(4-hydroxy-3-methoxyphenyl)propanoic acid, 2.8 μg mL^{-1} of benzoic acid and 2 μg mL^{-1} of ferulic acid.

Fractions were collected from the HPLC-UV/DAD method and infused into the Thermo LTQ Orbitrap hybrid FTMS. Tune conditions as used in Brosnan *et al.*[37] method. High mass accuracy data was obtained confirming the presence of the identified compounds. Fig. 6(I) shows the chromatographic profile of *Weissella cibaria* strain III. The high mass accuracy spectrums of compound (C) 4-hydroxybenzoic acid ($[M - H]^-$ *m*/*z* 137.02389) showing a ppm error of 0.15 ppm; fragmentation of the parent mass ($[M - H]^-$ *m*/*z* 137.02389) @ 30 CID produced the C_6H_5O fragment ($[M - H]^-$ *m*/*z* 93.03407) showing a ppm error of 0.34 ppm (Fig. 6(II)). Compound (G) 3-(4-

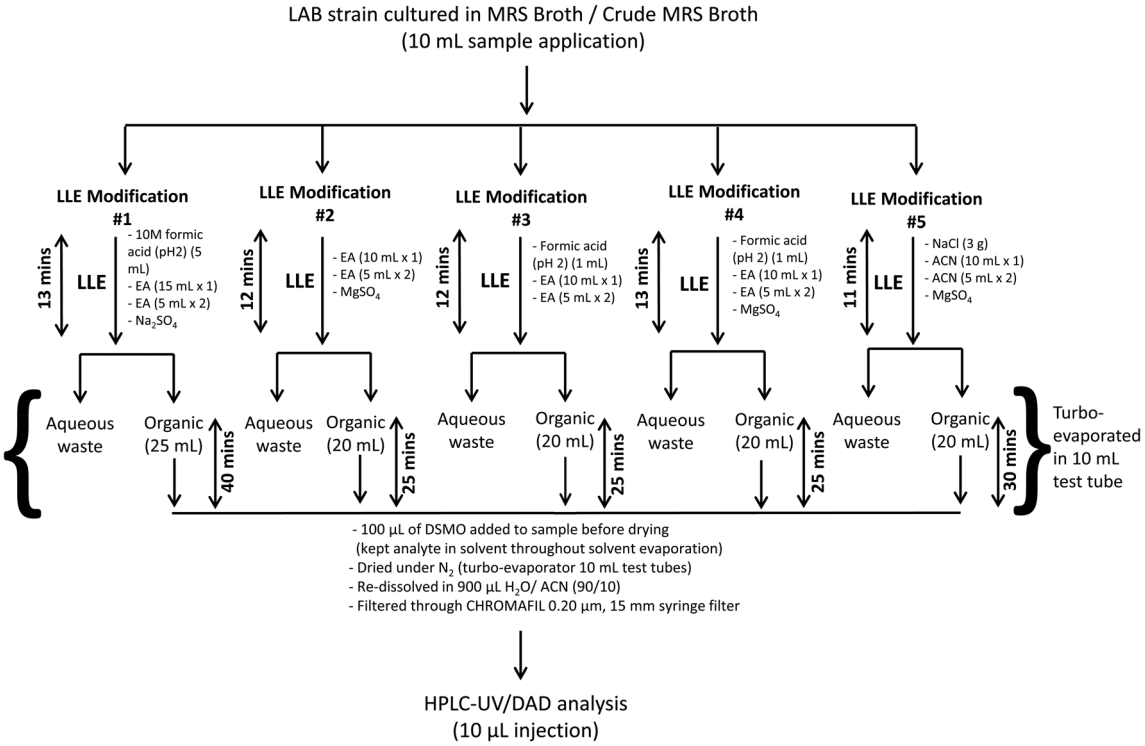

Fig. 5 Flow diagram of the modified sample preparation procedures used for the extraction of a crude MRS broth sample and/or LAB strain cultured in MRS broth, showing volumes and time required for each of the methods. These methods were modifications of the previously published methods outlined in Fig. 3.

Table 4 Percentage recovery for compounds spiked into crude MRS broth extracted applying modifications (see Fig. 5) to previously published extraction methods. * Compounds level too low for detection or overshadowed by broad matrix co-elutants (unknown)

	LLE modification #1		LLE modification #2		LLE modification #3		LLE modification #4		LLE modification #5	
	2 × 15 mL		EA + $MgSO_4$		EA-pH2 (1% FA)		EA pH2 (1% FA + $MgSO_4$)		ACN NaCl	
Compound name	% recovery	RSD ($n = 3$)	% recovery	RSD ($n = 3$)	% recovery	RSD ($n = 3$)	% recovery	RSD ($n = 3$)	% recovery	RSD ($n = 3$)
(A) 1,2-Dihydroxybenzene	2.6	5.2	86	4.7	*	*	*	*	2.7	8.4
(B) DL-*p*-Hydroxyphenyllactic acid	36	12	66	16	24	27	*	*	*	*
(C) 4-Hydroxybenzoic acid	41	37	94	9.9	34	29	96	1.1	0.6	7.2
(D) 3,4-Dihydroxyhydrocinnamic acid	3.0	9.5	87	10	142	32	62	2.9	143	0.1
(E) Vanillic acid	82	2.2	53	8.8	86	35	33	49	5.8	7.1
(F) Caffeic acid	85	1.2	55	11	76	36	96	0.6	114	7.5
(G) 3-(4-Hydroxyphenyl)propionic acid	155	34	85	1.4	98	34	129	25	66	12
(H) Phenyllactic acid	10	14	13	13	73	36	42	73	3.4	2.4
(I) *p*-Coumaric acid	85	3.5	60	16	62	35	91	2.2	84	0.4
(J) 3-(4-Hydroxy-3-methoxyphenyl) propanoic acid	78	1.1	70	16	82	36	114	0.8	134	0.5
(K) Benzoic acid	168	0.5	139	1.4	94	26	45	32	183	1.8
(L) Ferulic acid	141	1.7	94	13	109	36	72	54	120	2.0
(M) Salicylic acid	94	0.4	5.8	18	*	*	58	9.9	56	14
(N) Hydrocinnamic acid	87	0.1	74	26	83	37	60	11	3.2	9.7
(O) Methylcinnamic acid	65	1.5	79	21	72	38	38	29	81	0.2
Average	76	8.2	71	12	80	34	72	22	71	5.3

Table 5 Analyte concentration (μg mL^{-1}) detected within 3 antifungal LAB strains (I) *Lactobacillus amylovorus*, (II) *Lactobacillus plantarum* and (III) *Weissella cibaria*

	Lactobacillus amylovorus (I) (μg mL^{-1})	*Lactobacillus plantarum* (II) (μg mL^{-1})	*Weissella cibaria* (III) (μg mL^{-1})
(A) 1,2-Dihydroxybenzene			
(B) DL-*p*-Hydroxyphenyllactic acid	2.1	4.8	1.5
(C) 4-Hydroxybenzoic acid	3.4	1.03	3.4
(D) 3,4-Dihydroxyhydrocinnamic acid			
(E) Vanillic acid			
(F) Caffeic acid		0.04	0.07
(G) 3-(4-Hydroxyphenyl)propionic acid	6.1		7.1
(H) Phenyllactic acid	20	43	5.4
(I) *p*-Coumaric acid	0.7	0.46	0.88
(J) 3-(4-Hydroxy-3-methoxyphenyl)propanoic acid	0.11	0.13	216
(K) Benzoic acid	7.5	29	2.8
(L) Ferulic acid	1.3	1.2	2
(M) Salicylic acid			
(N) Hydrocinnamic acid	0.03		
(O) Methylcinnamic acid			
Average	2.7	5.3	1.5

hydroxyphenyl)-propionic acid ([M − H]$^-$ *m*/*z* 165.05522), showed a ppm error of 0.30 ppm; fragmentation of the parent mass ([M − H]$^-$ *m*/*z* 165.05522) @ 30 CID produced the C_8H_9O fragment ([M − H]$^-$ *m*/*z* 121.06530) showing a ppm error of 0.25 ppm (Fig. 6(III)). Compound (H) phenyllactic acid ([M − H]$^-$ *m*/*z* 165.05529) showing a ppm error of 0.73 ppm; fragmentation of the parent mass ([M − H]$^-$ *m*/*z* 165.05529) @ 30 CID produced the $C_9H_7O_2$ fragment ([M − H]$^-$ *m*/*z* 147.04473) showing a ppm

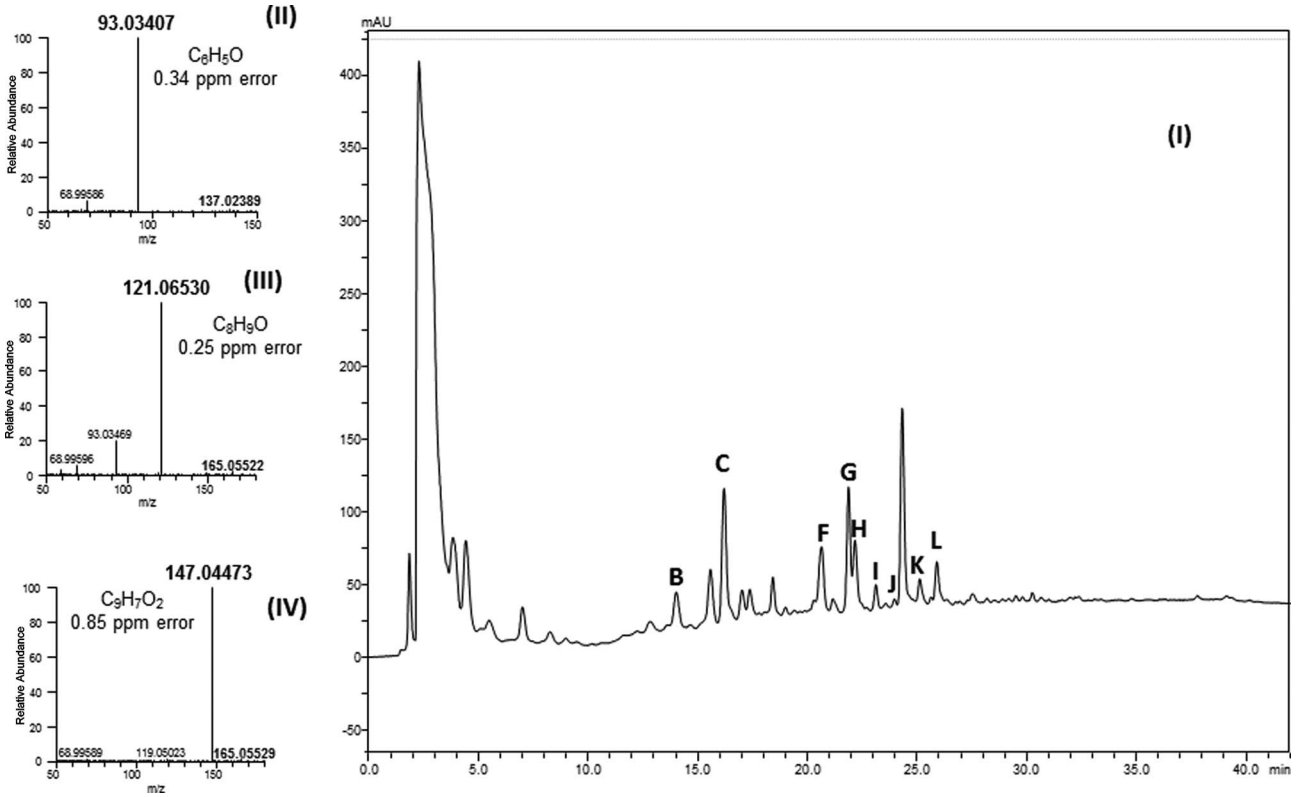

Fig. 6 Chromatographic profile of: (I) *Weissella cibaria* strain C showing antifungal activity; (II) high mass accuracy spectrum of compound (C) 4-hydroxybenzoic acid ([M − H]$^-$ *m*/*z* 137.02389), 0.15 ppm error; fragment @ 30 CID ([M − H]$^-$ *m*/*z* 93.03407, C_6H_5O, 0.34 ppm error); (III); high mass accuracy spectrum of compound (G) 3-(4-hydroxyphenyl)propionic acid ([M − H]$^-$ *m*/*z* 165.05522), 0.30 ppm error; fragment @ 30 CID ([M − H]$^-$ *m*/*z* 121.06530, C_8H_9O, 0.25 ppm error) (IV); high mass accuracy spectrum of compound (H) phenyllactic acid ([M − H]$^-$ *m*/*z* 165.05529), 0.73 ppm error; fragment @ 30 CID ([M − H]$^-$ *m*/*z* 147.04473, $C_9H_7O_2$, 0.85 ppm error).

error of 0.85 ppm (Fig. 6(IV)). PPM errors of less than 1.0 ppm were obtained for all parent masses and fragments providing unequivocal identification of the compounds present.

4. Conclusion

A HPLC-UV/DAD method has been developed and validated that allows for the separation and quantification of 15 antifungal compounds originating in LAB. The principle advantages of validating HPLC-UV/DAD methods for use in the LAB field are that this technology is relatively cheap to purchase for food testing and LAB research labs. It is robust, can be easily maintained, the software can be automated for compound identification (λ_{max}) and quantitation, applicability to a wide spectrum of compounds with chromophore functionalities and it is easy to upgrade to semi-preparative applications. This method details to date the most comprehensive method (15 compounds) to be chromatographically separated within this area. It shows the importance and the need for the optimisation of chromatographic conditions to ensure correct detection of peaks, identification of unknowns and allow for purer fractions to be collected if required. A detailed examination of the sample preparation techniques was also undertaken to evaluate their efficiency. The previous extraction method of Brosnan *et al.*[37] provided the best percentage recoveries (average 87%) and reduced baseline rise due to matrix constituents leading to better overall results for identification and quantification of compounds. These improvements will lead to advances within this area with the potential to increase the number of compounds currently known through better percentage recoveries from the extraction technique and better separation of compounds using optimised chromatography.

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