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The QuEChERS approach in a novel application for the identification of antifungal compounds produced by lactic acid bacteria cultures

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ABSTRACT

Lactic Acid Bacteria (LAB) play an important role as natural food preservatives in many fermented food systems. To-date, characterisation of their diverse range of metabolites has been limited. Improved quantitation of low, medium and high concentration antifungal compounds is required, ensuring that both known and unknowns compounds are identified. This manuscript reports the first application of QuEChERS (quick, easy, cheap, effective, rugged and safe) for the extraction of natural antifungal metabolites in LAB cultures. The method provides improved individual recoveries (> 78%) for 15 known antifungal compounds, an improvement of 26% compared to previously reported techniques (> 52%). A protocol was developed that allowed LAB cultures to be easily assessed on a fully validated high performance liquid chromatography with ultra violet/diode array detection (HPLC-UV/DAD) method. Previously reported methods involving direct injection of filtered extracts and SPE clean-up, suffered from a rise in chromatographic baseline due to interfering matrix components, limiting accurate quantitation. This QuEChERS method removed these interfering matrix components to deliver clean chromatograms with greater recoveries (78.2–127.4%) and lower RSD values (2.5–10.8%) of all 15 antifungal compounds. The validated method was applied to LAB strains showing particularly strong antifungal activity and provided an increase in the number of compounds detected (both known and unknown) compared to previous techniques for the same strains, due to the improved recoveries now possible by this method. Confirmation of the compounds identified was performed by analysis on a liquid chromatography linear ion trap quadrupole Orbitrap hybrid Fourier transform mass spectrometer (LC-FTMS). This first application of QuEChERS to LAB cultures has significantly improved the analytical capabilities of antifungal compound profiling especially where the synergy of numerous compounds is suspected as producing the observed activity. LAB cultures can now be easily integrated into various food matrices, as natural food preservatives, now that a complete analyte profile is achievable.

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1. Introduction

Lactic acid bacteria (LAB) have long played a role as a biopreservative in various food and feed fermentation processes (dairy, meats, vegetables, sourdough and silage) [1–4]. LAB are classified as rod and coccus shaped gram-positive organisms that are non-motile, non-spore forming that cause fermentation of higher alcohols and carbohydrates to mainly form lactic acid [5]. The use of LAB for their antibacterial properties is well known and has been extensively studied [6]. Recently interest in LAB has explored their antifungal potential which is of considerable interest in food and feeds where fungal spoilage is a problem [7–9,3]. Food spoilage due to fungal contamination has led to the need for treatments using both physical (e.g. heating, drying, cold-storage, freeze-drying, modified atmosphere storage) and chemical methods (application separately of organic acids, benzoic acid, sodium benzoate) [10,7] to be applied to food and feed systems in an effort to combat such occurrences. Recently, public representatives and national food agencies have sought a reduction in chemical additives used in food preservation. This has prompted the search for the use of natural methods, bio-preservation [9]. Given their history of safe use in foods and feeds, LAB have considerable potential for exploitation within this context [8,11,12]. While the inhibition of spoilage and pathogenic bacteria is generally well understood in LAB, several authors have reported that the production of some metabolites by LAB has frequently resulted in a delay

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and reduction of fungal contamination of food and feed products [10,7,12,6,9]. To understand this, there is a need to obtain chemical profiles of the metabolites produced by LAB strains that contribute to this antifungal activity. For this to happen, more comprehensive and robust extraction and analytical methods need to be developed that quantitatively profile metabolites that give LAB cultures their antifungal activity.

Antifungal activity has been attributed to several compounds produced by LAB strains including organic acids [13–15]; low molecular weight compounds [16,2,11,17,18]; phenyllactic acids [2,11,19–26]; fatty acids [27,2,25,17,18]; cyclic dipeptides [20,21,24,28,11,29]; proteinaceous compounds [30–33]; reuterin [34–36] and other miscellaneous compounds (e.g. lactones [16]; nucleotides [11]) however this compilation may not be complete. Antifungal activity of these compounds has been assessed through assays that determine the minimal inhibitory concentration (MIC) required for the delay in the growth of the fungus in question to occur. Research to-date has shown that individual antimicrobial compound do not contribute completely to the total antifungal activity. This information has led to the idea that a synergy of compounds may account for the total observed activity as discussed by a few groups [16,20,27,25]. A study undertaken by Niku-Paavola et al. [16] showed that compounds (benzoic acid; methylhydantoin; mevalonolactone and cyclo(glycl-L-leucyl) when individually assessed at a concentration of 10 ppm (the concentration detected in *Lactobacillus plantarum* fraction) only inhibited the growth of *Pantoea agglomerans* by 10–15%. A 100% inhibition was achieved when the crude *L. plantarum* culture filtrate and the sephadex fractions containing an unknown number of low molecular mass compounds (<700 Da) were tested against *P. agglomerans*. Different combinations of the identified compounds were then combined. The combinations provided interesting results with increased antifungal activity observed with certain combinations such as (i) mevalonolactone (10 ppm) with lactic acid (1%) giving 60% inhibition and (ii) benzoic acid (10 ppm), methylhydantoin (10 ppm), mevalonolactone (10 ppm) with lactic acid (1%) giving 100% inhibition. Other combinations caused a decrease in antifungal activity (iii) benzoic acid (10 ppm), methylhydantoin (10 ppm) and mevalonolactone (10 ppm) gave 15% inhibition; (iv) methylhydantoin (10 ppm), mevalonolactone (10 ppm) and lactic acid (1%) gave 30% inhibition and (v) benzoic acid (10 ppm), methylhydantoin (10 ppm) and lactic acid (1%) gave 30% inhibition. Fractions found to contain up to 7 g/L lactic acid showed no inhibition but when 1% lactic acid was employed inhibition of 40% occurred. The data illustrated that compounds required for inhibition to occur against the target fungi depended on the fine balance of compound mixtures present. This balance can easily be undone by the presence of extra compounds, the lack of other compounds or the incorrect concentration of the correct suite of compounds, leading to antagonistic activity occurring [16]. This may explain why not all strains of LAB have antifungal activity. This study also found that against a different target fungus, *Fusarium avenaccum,* less inhibition was observed with both the crude *L. plantarum* culture filtrate and isolated fractions [16]. This and other studies [20,27,25] have indicated that the food researcher must obtain a comprehensive quantitative profile of the compounds present in each antifungal LAB strain.

Given their complex growth requirements, LAB strains generally have analytically-challenging matrices that require clean-up pre-analysis. Methods such as direct injection post-filtration [23]; liquid–liquid extraction (LLE) [19,20,22,18]; solid phase extraction (SPE) [23,21,11,2]; thin layer chromatography (TLC) [19] and semi preparative fractionations [19–21] have all been explored to extract and sometimes isolate both new and known antifungal compounds. In this manuscript, a new strategy for sample preparation was investigated. Anastassiades et al. [37] first developed this technique QuEChERS, for pesticide analysis of fruits and vegetables. Since then the methodology has been applied to detect numerous pesticides [37–39], drugs [40], veterinary drug residues [41,39], pharmaceuticals [42], natural metabolites [43], myotoxins [39,44], plant toxins [39] from various different matrices (fruit [38], vegetables [38], soil [42], animal tissue [41], blood [40], urine [40], foods [43], cereal [39], spices [44], dairy products [39,41], and honey [39]). Recently it has been applied to determine naturally occurring substances in the extraction of eight isoflavones in pluses [43]. QuEChERS is a rapid, straightforward sample preparation technique that requires a minimal number of steps. It can be applied to multi class multi-residue methods; it requires small sample size. The material cost is low and the reduced solvents volumes thus lead to less solvent waste making the method greener and cheaper than previous methods. The prerequisite of no specialised equipment or glassware is also an advantage to this technique. QuEChERS significantly improves laboratory efficiency and throughput. A batch of six pre-chopped sample extracts can be prepared in less than 30 min by a single analyst providing recoveries of greater than 85% with less than 5% RSD for a wide range of GC and LC amenable compounds [37]. The QuEChERS technique consists of taking a sample and performing an extraction/partition step by shaking with an organic solvent. Acetonitrile is most commonly used but ethyl acetate and acetone are also good alternatives. Sodium chloride is added to reduce polar interferences and magnesium sulphate, anhydrous helps facilitates solvent partitioning and improves the recovery of polar analytes. The organic layer is then transferred to dispersive SPE (dSPE) to remove any matrix interferences from the sample (Fig. 3).

The advantages of QuEChERS as an analytical tool are that it is quick (overall less time required per sample than older methods), easy (simple procedure with minimal maintenance), cheap (cost efficient), effective (high recoveries for multiple compounds), rugged (multiple matrices, instruments and user provide similar results) and safe (low waste generation) [42,37]. These advantages over traditional extraction methods make the application of QuEChERS to the LAB area appealing. In this paper, we propose a novel approach to improving the recoveries of antifungal compounds from LAB cultures, providing the first application of QuEChERS to extracting natural antifungal compounds from bacterial strains.

2. Material and methods

2.1. Reagents and materials

Antifungal compounds (Fig. 1) identified from the literature were mainly purchased from Sigma-Aldrich (Dublin, Ireland). These included 1,2-dihydroxybenzene; DL-Þ-hydroxyphenyllactic acid (OH-PLA); 4-hydroxybenzoic acid; 3,4-dihydroxyhydrocinnamic acid; vanillic acid; caffeic acid; 3-(4-hydroxyphenyl) – propionic acid; p-coumaric acid; 3-(4-hydroxy-3-methoxyphenyl)propanoic acid; benzoic acid; ferulic acid; salicylic acid; hydrocinnamic acid and methylcinnamic acid. The antifungal compound phenyllactic acid (PLA) was acquired from BaChem (Weil am Rhein, Germany). All analytes had a purity of $\geq 95\%$. The media for cultivating the LAB strains MRS (de Man, Rogosa and Sharpes) broth, the HPLC grade solvents (ethyl acetate (EA), acetonitrile (ACN), acetone (ACE) and water (H_2O)), drying agent (magnesium sulphate ($MgSO_4$)) and salt (sodium chloride (NaCl)) were obtained from Sigma-Aldrich (Dublin, Ireland). Acetic acid (AA) (eluent additive for LC–MS) was purchased from Fluka (Ireland). Formic acid (FA) ($\sim$99%) was bought from Fluka (Germany). Three dispersive SPE Kits: (1) Cat #: 5982-4956 (15 mL; 150 mg C18, 900 mg $MgSO_4$); (ii) Cat #: 5982-5256 (15 mL; 150 mg

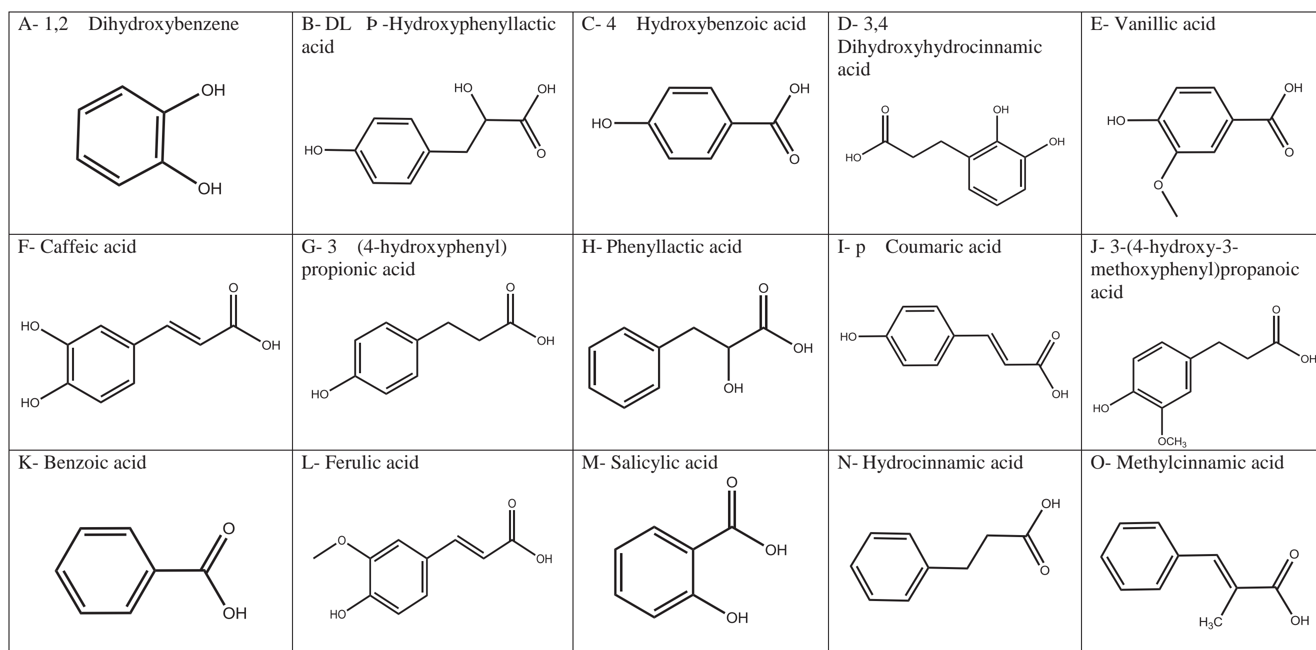

Fig. 1. Structure of the 15 previously reported antifungal compounds (Table 1).

primary secondary amine (PSA), 15 mg graphitised carbon black (GCB), 900 mg $MgSO_4$) and (iii) Cat #: 5982-5156 (15 mL; 150 mg PSA, 150 mg C18EC, 900 mg $MgSO_4$) were received from Agilent (Dublin, Ireland).

2.2. Standard solutions

An individual stock solution (2 mg/mL) of each of the 15 antifungal compounds (Fig. 1) was prepared by dissolving known amounts (4 mg) of each of the compounds in H_2O or ACN (2 mL) as required. From these 15 individual stock solutions a standard mix solution of 100 μg/mL (100 ppm) concentration was prepared by transferring 100 μL of each solution into a 4 mL vial. H_2O/ACN (90/10; 500 μL) was added to this vial bring the total volume to 2 mL.

Calibration curve standards (#1–5) at concentrations of 1 ppm, 5 ppm, 10 ppm, 30 ppm and 50 ppm were prepared weekly by accurately pipetting volumes of 10 μL, 50 μL, 100 μL, 300 μL and 500 μL respectively from the standard mix solution (100 ppm) into an amber vials (1.5 mL) and each vial was made up to a total volume of 1 mL with 90/10 (H_2O/ACN). Three standard controls (#6–8) at concentrations of 7.5 ppm, 20 ppm, 40 ppm were also prepared weekly by accurately pipetting volumes of 75 μL, 200 μL and 400 μL respectively from the standard mix solution (100 ppm) into an amber vials (1.5 mL) and each vial was made up to a total volume of 1 mL with 90/10 (H_2O/ACN).

Culture broth (MRS) uninoculated with LAB strain was used as control MRS broth. This was prepared as instructed by the manufacturer and pH adjusted to pH 4.3 (average LAB culture pH) with lactic acid and AA. The control MRS broth (10 mL) was then fortified with the 15 compound mix at the concentrations used for the preparation of both the calibration curve standards and three controls (see above). All weekly prepared standards and controls were stored between 2 and 4 °C in solution.

2.3. Extraction methods

For use in the QuEChERS optimisation studies, a control MRS broth (10 mL) was fortified at a concentration of 3 μg/mL, from the 15 standard mix solution (100 ppm; Section 2.2), mechanically shaken for 15 min. The following extraction methods were examined by direct injection (post-culture filtration) [23]; SPE [20]; LLE [18] and with the newly developed QuEChERS method.

2.3.1. Direct injection study

No sample preparation, except filtration was applied to the control MRS broth as previously reported by Armaforte et al. [23] in his methods when he profiled one antifungal compound, PLA in MRS broths. 1 mL of a 30 μg/mL fortified control MRS broth was filtered (0.2 μm; Machnery and Nagle) and placed in a vial. This study was carried out in triplicate and 10 μL of each was injected on the HPLC-UV/DAD system (Fig. 2(III)) and compared to the chromatogram generated from the separation of the 15 antifungal compounds standard mixture prepared in H_2O/ACN (90/10; Fig. 2(IV)).

2.3.2. Solid phase extraction (SPE) study

SPE was performed following the Strom et al. method [20]. Isolute, C18 EC cartridges (500 mg; 3 mL; International Sorbent Technology Ltd., Hengoed, United Kingdom) were preconditioned with ACN (3 mL) and H_2O (3 mL). Fortified control MRS broth (10 mL at a concentration of 3 μg/mL) was loaded onto the cartridge. A wash step of 5% aqueous ACN (3 mL) solution and an elute step of 95% aqueous ACN (3 mL) were employed. The wash and elute steps were collected separately to which dimethyl sulfoxide (DMSO; 100 μL) was added to each step. The wash and elute step were then dried under nitrogen (Turbovap LV evaporator). Each elution fraction was reconstituted with H_2O/ACN (90/10; 900 μL), filtered (0.22 μm; Machnery and Nagle), vialed and injected (10 μL) onto the HPLC-UV/DAD system. This study was performed in triplicate.

2.3.3. Liquid–liquid extraction (LLE) study

LLE was performed as per the Brosnan et al. method [18]. EA (10 mL) was used to extract 10 mL of fortified (3 μg/mL) control MRS broth, followed by two separate EA extractions (2 × 5 mL EA). The three organic layers were pooled and DMSO (100 μL) was added. This combined organic supernatant was dried under nitrogen (Turbovap LV evaporator), reconstituted with H_2O/ACN (90/10; 900 μL), filtered (0.2 μm pore size filter), vialled and injected (10 μL) onto the HPLC-UV/DAD system. This study was performed in triplicate.

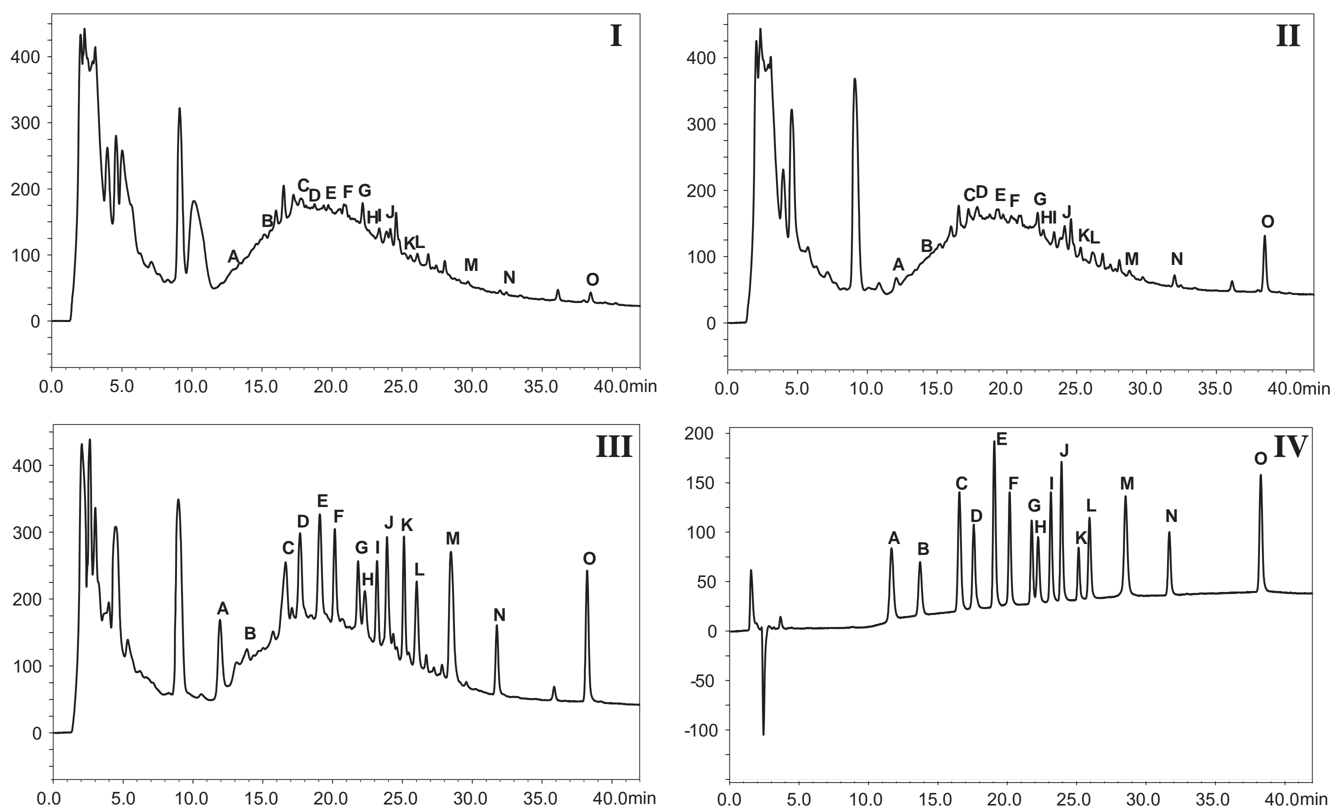

Fig. 2. Chromatographic separation of a standard mix (30 μg/mL; 10 μL injection) of previously reported antifungal compounds from LAB extracted/prepared using (I) SPE wash fraction [20] and (II) SPE elution fraction [20]. (III) Control MRS broth (direct injection, no sample preparation) and (IV) H_2O/ACN (90/10). Compounds listed in Table 1 and Fig. 1.

2.3.4. Quick, easy, cheap, effective, rugged and safe (QuEChERS) study

2.3.4.1. Optimisation of the QuEChERS dispersive SPE (dSPE) phase. Three dSPE phases were investigated ($n=3$). dSPE kits (#1) 150 mg C18, 900 mg $MgSO_4$; (#2) 150 mg PSA, 15 mg GCB, 900 mg $MgSO_4$ and (#3) 150 mg PSA, 150 mg C18EC, 900 mg $MgSO_4$ (Fig. 3(I)). Each of these parameters was performed in triplicate for each of the dSPE kits ($n=9$). 10 mL of EA was added to fortified control MRS broth (10 mL at a concentration of 3 μg/mL) preparations ($n=9$) and vortexed for 30 s. $MgSO_4$ (4 g) and NaCl (1 g) were then added and shaken for 1 min. The mixtures were then centrifuged for 10 min (3000 rpm) and the organic solvent supernatants was removed and added to the appropriate dSPE kit. Each was shaken for 1 min. The dSPE tubes were then centrifuged for 10 min (3000 rpm). Solvent (5 ml) was then transferred to a glass test tube containing DMSO (100 μL) and dried under nitrogen (Turbovap LV evaporator), reconstituted with H_2O/ACN (90/10; 900 μL) and syringe filtered (0.2 μm pore size filter) into vials. 10 μL was injected onto the HPLC-UV/DAD system.

2.3.4.2. Optimisation of the QuEChERS extraction solvent. Three different extraction solvents were examined (1) EA, (2) ACE and (3) ACN (Fig. 3(II)). 10 mL of the appropriate solvent was added to the fortified control MRS broth (10 mL at a concentration of 3 μg/mL) preparations ($n=9$) and vortexed for 30 s. $MgSO_4$ (4 g) and NaCl (1 g) were then added and shaken for 1 min. The mixtures were then centrifuged for 10 min (3000 rpm) and the organic solvent supernatants was removed and added to the optimised dSPE kit #1. Each was shaken for 1 min. The dSPE tubes were then centrifuged for 10 min (3000 rpm). Solvent (5 ml) was then transferred to a glass test tube containing DMSO (100 μL) and dried under nitrogen (Turbovap LV evaporator), reconstituted with H_2O/ACN (90/10; 900 μL) and syringe filtered (0.2 μm pore size filter) into vials. 10 μL was injected onto the HPLC-UV/DAD system.

2.3.4.3. Optimisation of the QuEChERS extraction solvent – influence of acid. Three parameters were examined ($n=3$) to determine the influence of acid on the extraction. (1) EA with 0% acid (Sections 2.3.4.2) (2) EA with 1% AA and (3) EA with 1% FA (Fig. 3(III)). 10 mL of each of the parameters (1–3) were added to the fortified control MRS broth (10 mL at a concentration of 3 μg/mL) preparations ($n=9$) and vortexed for 30 s. $MgSO_4$ (4 g) and NaCl (1 g) were then added and shaken for 1 min. The mixtures were then centrifuged for 10 min (3000 rpm) and the organic solvent supernatants were removed and added to the optimised dSPE kit #1. Each was shaken for 1 min. The dSPE tubes were then centrifuged for 10 min (3000 rpm). Solvent (5 ml) was then transferred to a glass test tube containing DMSO (100 μL) and dried under nitrogen (Turbovap LV evaporator), reconstituted with H_2O/ACN (90/10; 900 μL) and syringe filtered (0.2 μm pore size filter) into vials. 10 μL was injected onto the HPLC-UV/DAD system.

2.4. Application of the optimised QuEChERS protocol to LAB cultures

Culture broth (MRS; 10 mL) was prepared and autoclaved following the manufacturer's specifications. The selected antifungal strains showing strong antifungal activity (*Lactobacillus amylovorus* strain I, *L. plantarum* strain II and *Weisella Cibiara* strain III) were cultivated in the sterile MRS broth and incubated for 48 h at 37 °C. Culture broth (MRS; un-inoculated with a LAB strain) was treated under the same conditions. After incubation the samples are centrifuged (10,000 rpm for 10 min) and sterile filtered (0.45 μm pore size filter). 10 mL of the cell free supernatant (cfs) LAB culture was added to 10 mL of EA with 1% FA, 4 g $MgSO_4$ and 1 g of NaCl and shaken for 1 min. The mixture was centrifuged for 10 min (3000 rpm) and the organic solvent supernatant was removed and added to the optimised dSPE kit #1 (Section 2.3.4.1) and shaken for 1 min. The dSPE tube was then centrifuged for 10 min (3000 rpm). The solvent (5 mL) was transferred into a

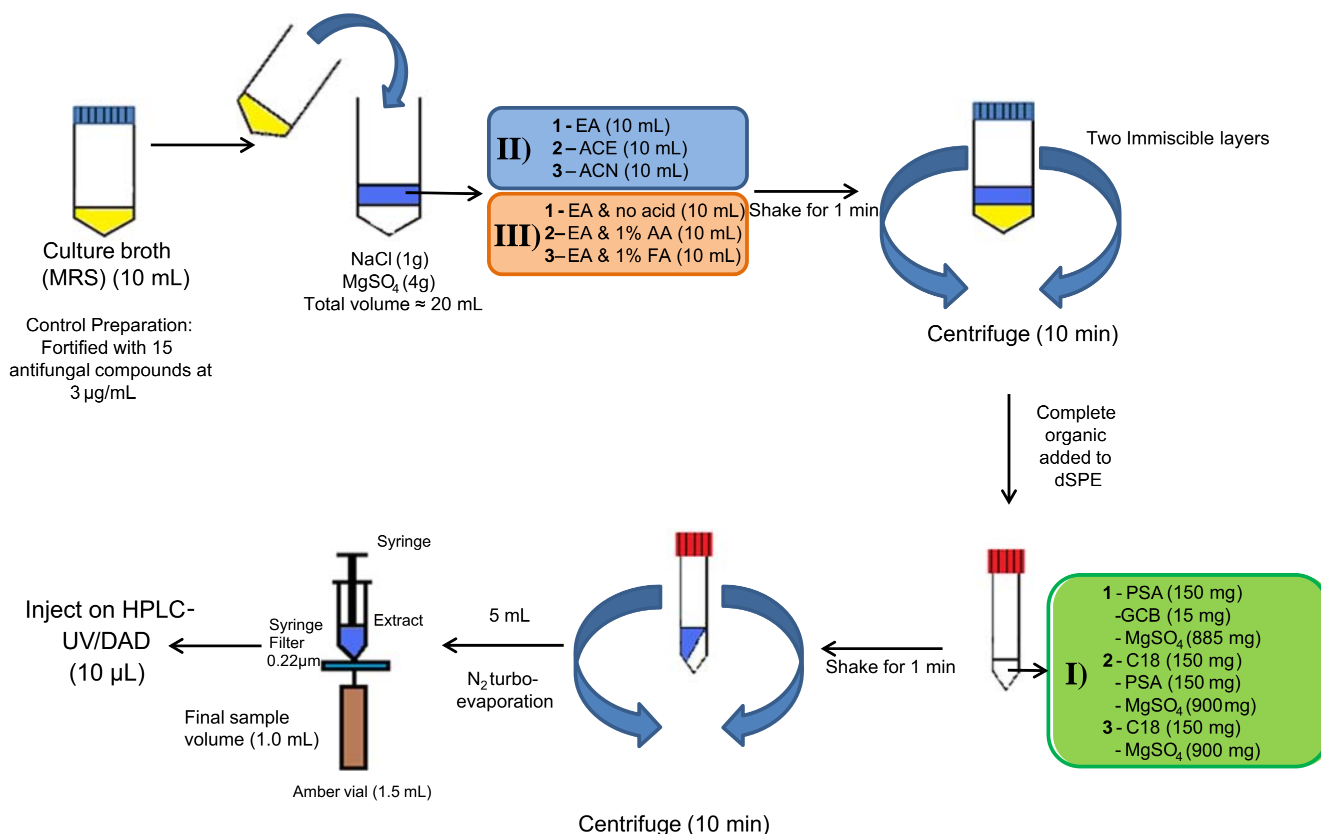

Fig. 3. Flow diagram outlining the optimisation steps involved in the LAB QuEChERS samples preparation method. Three parameters were assessed (I) dSPE phase selection, (II) extraction solvent selection and (III) optimised solvent with the addition of an acid for improved extraction.

labelled glass test tube to which DMSO (100 µL) added. This was dried under nitrogen (Turbovap LV evaporator), reconstituted to 1 mL with H_2O/ACN (90/10; 900 µL) and syringed filtered (0.2 µm pore size filter), into a HPLC amber vials (1.5 mL capacity). 10 µL was injected onto the HPLC-UV/DAD system (Fig. 3). This was performed in triplicate for each cfs LAB culture.

2.5. HPLC-UV/DAD analysis

A Shimadzu LC system (CMB-20A/LC-10AT) with photodiode array detector (SPD-M10A) achieved separation of the 15 compounds (Fig. 2(IV)) on a Gemini C18 column (150 × 2.0 mm^2, 5 µm; Phenomenex, Macclesfield, UK) equipped with a guard column (SecurityGuard™ Gemini C18 cartridge AF0-8497; 4 × 3.0 mm^2 ID; Phenomenex, Macclesfield, UK). The mobile phase composition was as follows solvent A) H_2O with 0.1% FA and solvent B) ACN with 0.1% FA; this was filtered through a pre-rinsed (ACN) 0.2 µm filter. A gradient flow was performed to ensure separation of compounds (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 kept at a temperature of 30 °C. A volume of 10 µL was injected and a wavelength of 210 nm was chosen as the universal λ_{max}.

2.6. Mass Spectrometry (MS) confirmation

The LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) was used to confirm the presence of the compounds identified by the HPLC-UV/DAD method. Fractions correlating to the peak retention times from the LAB culture extracts been examined by the HPLC-UV/DAD method were collected and infused into the MS to the mobile phase using a Tee union/T-junction. The method was operated in negative ionisation mode at 30,000 resolution with the following tune conditions – capillary temperature of 300 °C, capillary voltage −50 V, tube lens −110 V, sheath gas 45 arbitrary units and auxiliary gas 15 arbitrary units. A lock mass of m/z 59.013840 (present from the AA in the mobile phase) was integrated into the method to correct for mass shift. Weekly calibration as per the manufacturer's instructions insured robust high mass accuracy spectrum (< 2 ppm) to confirm the presence of all analytes.

2.7. Validation

A previously validated in-house method [45] which was performed in compliance with the EC [46] and ICH [47] guidelines taking into account specificity, linearity, limits of detection and quantitation, trueness and precision was used as the analytical method. Validation was completed by analysing five standard concentrations (1 ppm, 5 ppm, 10 ppm, 30 ppm, 50 µg/mL) in triplicate over three consecutive days and three controls (7.5 ppm, 20 ppm and 40 ppm) ran three times each over three consecutive days (Section 2.2). Signal to Noise (S/N) values of S/N = 3 was selected to determine the limit of detection (LOD) and S/N = 10 used to calculate the limit of quantitation (LOQ).

3. Discussion

Sample preparation (extraction and clean-up) is a prerequisite to analysing the majority of food matrices and in the area of LAB this process is a necessity. An earlier study undertaken by this group on a HPLC-UV/DAD system [45] assessed previously published extraction methods for this area [20,22,23,18] and applied modifications to these methods in an attempt to improve percentage recoveries for known antifungal compounds. This study

evaluated direct injection of the LAB cultures post-filtration, SPE clean-up of the LAB cultures and LLE of the LAB cultures [45]. This study found that the best average recoveries for the targeted antifungal compounds was 86.5% for LLE with EA with individual recoveries of greater than 55% for all of the studied antifungal compounds [45]. While the average % recovery was acceptable (86.5%) the individual recoveries of some compounds could be improved therefore an alternative sample extraction and matrix clean-up method(s) was investigated. Previously in 2006, minimal sample preparation with only LAB broths being filtered followed by direct injection onto a HPLC system was suggested by Armaforte et al. [23]. This limited method provided adequate separation, percentage recoveries (98.7%), quantitation and seemingly no matrix interference for one tested antifungal compound, PLA. A modified methodology of Armaforte et al. [45] was repeated as part of this study. Fig. 2(III) illustrates a typical chromatogram obtained from the HPLC analysis of a control MRS broth fortified (30 μg/mL) with 15 known antifungal compounds using a gradient elution which provided adequate separation of all the compounds. In comparison to the chromatogram (Fig. 2(IV)) obtained from the 15 standard mix prepared in HPLC mobile phase, a significant rise in the chromatographic baseline (not consistent amongst LAB cultures) was observed for the fortified control MRS broth, due to co-eluting matrix constituents present. At this concentration of 30 ppm which is at the higher end of the calibration range (1–50 ppm) antifungal compounds (known) can still be identified but at lower concentrations the rise in chromatographic baseline significantly interferes with the identification of both known and unknown antifungal compounds. This rise in the chromatographic baseline decreases the linear calibration range achievable plus increases the RSD values obtained from the multiple studies completed involving fortified known standards (Section 2.2). For HPLC-UV/DAD methods this rise in chromatographic baseline can be clearly observed in the chromatogram and considered during the analyses. A concern would arise when such a sample preparation technique is transferred to LC–MS or LC–MS/MS where this rise in chromatographic baseline would most probably equate to significant ion suppression or enhancement [48,40], leading to inaccurate quantitation (data to be published elsewhere).

When developing the method for the quantitation of PLA; Armaforte et al. [23] also assessed the well-known and commonly applied Strom et al. [20] SPE method. A partitioning of the compound PLA was observed between wash and elute step with a percentage recovery of 10.5% in the elute step (95% aqueous ACN) and 63.2% in the wash step (5% aqueous ACN). The partition of this compound between both wash and elute steps is of concern as in general the elute step is kept for testing and the wash step is discarded. This SPE methodology leads to very low recoveries for compounds produced by LAB cultures making the identification difficult for both known and unknown compounds plus leads to inaccurate quantitation of compounds, unless accurate recoveries are known for each compound. Upon examination of this SPE methodology with 15 antifungal compounds (Fig. 1) fortified in control MRS broth similar quantitative recoveries were obtained with 12 of the 15 antifungal compounds being detected in both the wash and elute steps. Average percentage recoveries for the 15 compounds was extremely low with 12.5% in wash step and 24.2% in the elute step. Chromatographic rise in chromatographic baseline with both SPE wash and elution fractions, similar to that obtained when analysing filtered crude MRS cultures (Fig. 2(III)) is also a concern, as this indicates that the Strom et al. [20] SPE methodology is not removing key matrix interferences (Fig. 2 (I) and (II)). These conclusions were supported with large RSD values (3.7–74.7%) when the fortified standard mix was spiked into control MRS broth tested during intraday and intermediate precision studies.

To solve both the antifungal analyte recovery variations and the chromatographic quantitation issue due to baseline matrix rise in the LAB field, the QuEChERS samples preparation technique was examined and optimised. The QuEChERS methodology is a streamlined approach that makes it easier and less expensive for scientists to examine target analyte residues in food matrices.

3.1. QuEChERS

3.1.1. Method development

Fig. 3 shows the progression of steps undertaken to assess if QuEChERS could be applied to determine naturally occurring antifungal compounds from LAB cultures. Three studies were considered in the method development and assessment of this extraction methodology (I) dSPE phase selection (Section 2.3.4.1), (II) extraction solvent selection (Section 2.3.4.2) and (III) addition of an acid to the optimised extraction solvent (Section 2.3.4.3).

3.1.2. (I) dSPE phase selection

The first parameter assessed was a selection of the dSPE phases: dSPE kits (#1) 150 mg C18, 900 mg $MgSO_4$; (#2) 150 mg PSA, 15 mg GCB, 900 mg $MgSO_4$ and (#3) 150 mg PSA, 150 mg C18EC, 900 mg $MgSO_4$. C18 dSPE is mainly used to removes long chain fatty compounds, sterols and other non-polar interferences [41]. PSA dSPE is used in the removal of sugars and fatty acids, organic acids, lipids and some pigments [37]. When PSA is used in combination with C18, additional lipids and sterols can be removed [38]. $MgSO_4$ anhydrous is present with the dSPE phase to removes any residual water from the organic phase.

Table 1 outlines the percentage recoveries results for each of the 15 individual compounds. PSA dSPE provided recoveries from 1.1–123.2% for 12 of the 15 compounds detected and unacceptable RSD values from 3.7% to 46.4%. Compounds (3-(4-hydroxy-3-methoxyphenyl)propanoic acid, salicylic acid, hydrocinnamic acid and methylcinnamic acid) were not detected using this PSA dSPE phase. PSA and C18 dSPE gave recoveries from 1% to 114.8% for 10 of the 15 compounds with unacceptable RSD values ranging from 7.9% to 115.4%. Compounds (vanillic acid, 3-(4-hydroxy-3-methoxyphenyl)propanoic acid, salicylic acid, hydrocinnamic acid and methylcinnamic acid) were not detected using the PSA and C18 dSPE phase. C18 dSPE provided recoveries for all 15 ranging from 26.2% to 144.0% with RSD values ranging between 3.1% and 18.4%. Therefore C18 dSPE was chosen as the most appropriate phase for use as an extraction method. The result is not a surprise over PSA or PSA/C18 as PSA is known to remove organic acids from samples which would explain the low recoveries when PSA was employed [37].

3.1.3. (II) Extraction solvent selection

Solvent extraction techniques are designed to remove as much as possible of the desired analyte from the sample matrix. Solvent selection is important so as to minimise co-extracting compounds and sample clean-up is necessary to reduce interferences. Matrix interferences can contaminate analytical instrumentation (e.g. HPLC detector flow cells and ion sources) and complicate analyte identification and quantification. EA was chosen as the extraction solvent initially due to its efficiency during LLE compared with ACN for the compounds of interest [45]. As ACN is the most commonly selected extraction solvent with QuEChERS methodologies its application was compared to EA and ACE to determine if indeed EA is the best solvent to choose for these compounds. Table 1 shows the percentage recoveries for the solvents trialled. EA did indeed show the best average recoveries (103.0%) similar to that observed using LLE. ACE provided the next best average recoveries with individual recoveries ranging from 10.9% to 103.6% and with RSD values ranging from 0.1% to 14.7%. ACN gave

Table 1
Percentage recovery for compounds fortified into control MRS broth extracted using parameters outlined in Fig. 3 and compared to the optimised LLE methodology [45].

Compound name	Solvent dSPE	EA PSA % recovery	EA PSA RSD (n=3)	EA PSA/C18 % recovery	EA PSA/C18 RSD (n=3)	EA C18 % recovery	EA C18 RSD (n=3)	ACE C18 % recovery	ACE C18 RSD (n=3)	ACN C18 % recovery	ACN C18 RSD (n=3)	EA and 1% AA C18 % recovery	EA and 1% AA C18 RSD (n=3)	EA and 1% FA C18 % recovery	EA and 1% FA C18 RSD (n=3)	EA* N/A* % recovery	EA* N/A* RSD (n=3)
A)	1,2-Dihydroxybenzene	68.5	46.4	56.6	42.9	106.3	10.8	68.6	8.2	63.7	6.1	39.8	15.1	90.1	4.0	52.1	1.7
B)	DL-P-Hydroxyphenyllactic acid	35.8	22.8	22.7	28.3	69.7	18.4	74.3	12.4	60.8	1.2	55.8	7.2	78.2	10.6	52.5	3.8
C)	4-Hydroxybenzoic acid	3.3	13.9	2.5	115.4	90.5	15.9	103.6	14.7	52.2	16.8	44.3	1.5	93.4	4.9	78.6	7.0
D)	3,4-Dihydroxyhydrocinnamic acid	28.6	27.4	70.0	10.9	129.8	13.8	86.3	7.0	72.1	3.6	55.0	3.6	119.4	4.1	96.0	3.9
E)	Vanillic acid	1.1	9.0	N/D	N/D	111.8	14.1	59.4	2.9	49.4	1.3	75.9	2.5	111.3	8.2	93.7	0.9
F)	Caffeic acid	3.2	8.8	3.0	40.6	79.9	8.8	91.1	6.3	80.8	3.1	82.3	2.4	91.7	2.8	81.1	0.1
G)	3-(4-hydroxyphenyl)-propionic acid	123.2	23.6	114.8	18.7	130.3	14.3	67.0	2.7	42.8	1.2	100.0	16.2	114.5	7.3	117.4	0.9
H)	Phenyllactic acid	4.6	5.5	1.0	28.8	26.2	5.7	10.9	4.2	4.7	2.6	N/D	N/D	103.9	6.7	64.3	3.5
I)	p-Coumaric acid	15.9	16.0	14.2	61.8	125.1	6.8	89.8	3.4	82.9	1.4	84.3	0.9	91.7	6.5	94.1	1.0
J)	3-(4-hydroxy-3-methoxyphenyl) propanoic acid	N/D	N/D	N/D	N/D	78.1	5.2	80.5	8.7	64.9	2.6	119.1	2.3	87.3	10.0	74.7	0.1
K)	Benzoic acid	10.9	3.7	9.9	29.0	107.5	9.5	76.9	2.3	69.7	3.5	45.1	2.2	109.5	3.3	124.6	1.2
L)	Ferulic acid	13.8	4.5	13.5	7.9	133.3	4.8	67.2	3.1	61.4	10.2	94.6	0.6	101.2	9.3	114.4	0.6
M)	Salicylic acid	N/D	N/D	N/D	N/D	144.0	4.7	62.5	2.0	46.4	1.7	61.2	0.5	127.4	10.8	96.7	0.7
N)	Hydrocinnamic acid	0.5	36.9	N/D	N/D	114.1	3.1	81.7	4.0	74.9	5.6	59.6	13.4	86.7	5.8	94.4	0.4
O)	Methylcinnamic acid	N/D	N/D	N/D	N/D	97.7	5.5	70.9	0.1	63.6	2.7	52.7	1.1	85.7	2.5	62.3	1.6
Average % recovery		**25.8**	**18.2**	**30.8**	**38.4**	**103.0**	**9.4**	**72.7**	**5.5**	**59.4**	**4.2**	**69.3**	**5.0**	**99.5**	**6.5**	**86.5**	**1.8**

N/D concentration not determined.

* LLE method.

the lowest recoveries for all compounds ranging from 4.7% to 80.8% and RSD values ranging from 1.2% to 16.8%. The method with EA as the solvent and C18 as the dSPE showed the greatest promise as an extraction technique but the low recoveries for PLA in all three solvents giving recoveries of 26.2%, 10.9% and 4.7% for EA, ACE and ACN respectively are of concern.

3.1.4. (III) Addition of an acid

Many modifications have been employed within QuEChERS to improve the recoveries of certain compounds which show low recoveries. AOAC 2007.01 [49] method employs 1% AA in ACN and sodium acetate buffer to protect base sensitive analytes from degradation and provide superior recovery of pH sensitive compounds. 1% AA is the most commonly chosen acid to pH adjust a sample but 1% FA has also been used [44]. For this next part of the optimisation study, the separate addition of 1% AA and 1% FA was compared to the addition of 0% acid. Table 1 shows that EA with 1% FA provided better average percentage recoveries (99.5%) than EA with 1% AA (69.3%). EA with 0% acid (103.9%) gave similar recoveries to EA with 1% FA. Fig. 4(I) shows the results for the individual compounds. As can be seen from looking at the influence of the acid to the individual compounds the results are indeed quiet similar for EA with 1% FA and EA with 0% addition of an acid with only one major difference being observed. PLA which shows a percentage recovery of 26.2% with an extraction involving EA with 0% acid shows a percentage recovery of 103.9% with the addition of EA with 1% FA. This improvement gives an average percentage recovery of 99.5% for all 15 antifungal compounds and an individual percentage recovery range from 78.2% to 127.4% with RSD values ranging from 2.5% to 10.8% when EA with 1% FA is employed.

3.2. QuEChERS vs LLE

The application of the optimised QuEChERS (EA with 1% FA, C18) provides an excellent extraction method for the determination of the 15 antifungal compounds. This optimised QuEChERS method was compared to the previously optimised LLE method (EA), (Table 1). Results show the average recovery has increased to 99.5% compared to the 86.5% previously determined for the LLE methodology. In particular, compound OH-PLA show a much improved percentage recovery (Fig. 4(II)) of 78.2% for the QuEChERS extraction methodology compared to 52.5% for the LLE method. Average RSD values have increased from 1.8% in the LLE method to 6.5% in the QuEChERS method but these RSD values are still at an acceptably low level. Improved recoveries can be seen for most compounds except coumaric acid (94.1% to 91.7%) and hydrocinnamic acid (94.4% to 86.7%), both having < 9% reductions. The values obtained fall within those achieved by other users of QuEChERS (Lehotay et al. [50]) with average % recoveries of 98% and RSD of 10% for pesticides in fruit and vegetables [41,43,44].

The chromatographic profile of Brosnan et al. [18] LLE extraction (Fig. 5(I)) and QuEChERS (Fig. 5(II)) both illustrate that no chromatographic rise in chromatographic baseline was observed from co-eluting matrix interferences. Overall the QuEChERS method has significantly improved the laboratory efficiency and throughput in relation to LAB cultures. Batch analysis time, solvent use and waste generation are greatly reduced by employing the QuEChERS technique over the LLE methodology. The number of LLE samples per hour that can be extracted is limited by the amount of glassware available. QuEChERS eliminates this issue and also reduces the final solvent evaporation volume from 20 mL (LLE) to 5 mL (QuEChERS) reducing time and N_2 costs and usage. The improved antifungal compound recoveries ranging from 78–127% with RSD's < 11% for the wide range of antifungal compounds has also been obtained through application of the QuEChERS technique.

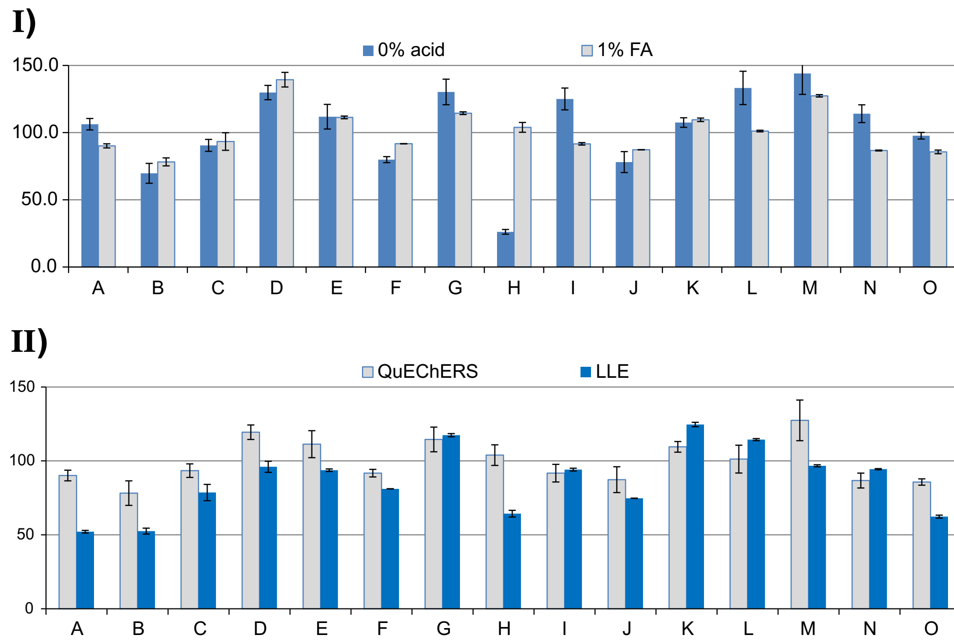

Fig. 4. Graphical representation of the average % recovery values obtained for the parameters tested. The individual values obtained for each individual compound examined under the corresponding factors (I) addition of an acid and (II) optimised QuEChERS versus optimised LLE [18]. Data can also be found in Table 1.

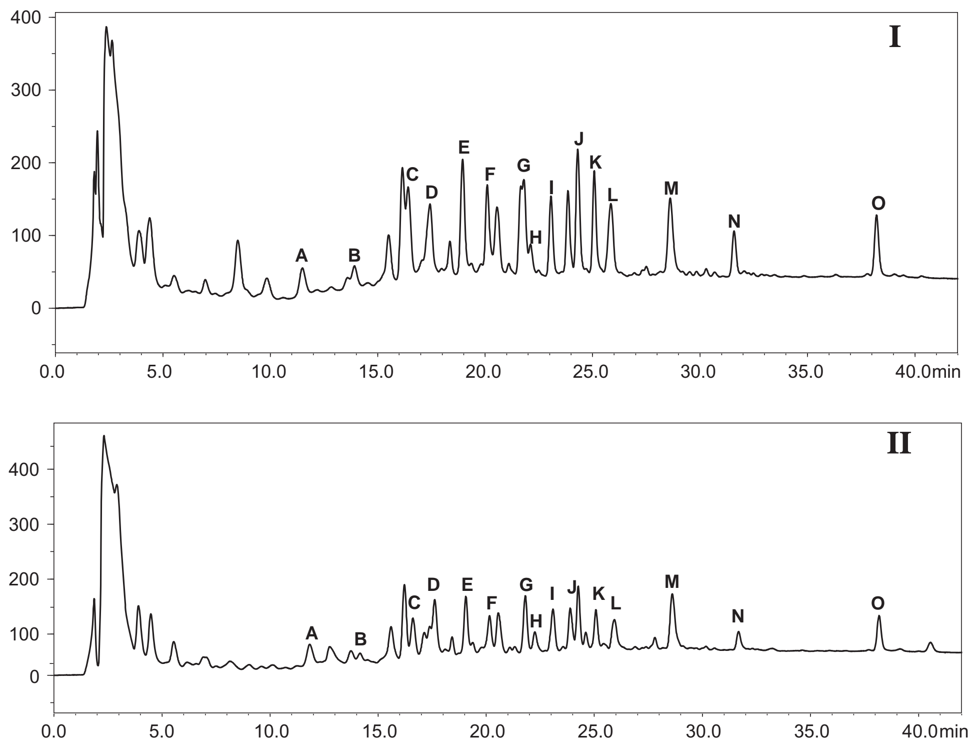

Fig. 5. Chromatographic profile of a standard mix (30 μg/mL; 10 μL injection) of previously reported antifungal compounds from LAB extracted by (I) the optimised LLE (EA) method [18] and (II) the optimised QuEChERS methodology (EA + 1% FA; C18). Chromatographic conditions as described in Section 2.5. % recovery data can be seen in Table 1.

This methodology will ensure that the best possible recoveries will be obtained for both known and unknown antifungal compounds present within LAB cultures.

3.3. Sample application

This first application of QuEChERS in the detection of antifungal compounds was applied to three LAB strains showing strong antifungal activity, *L. amylovorus* A, *L. plantarum* B, *Weisella cibaria* C. Antifungal compounds present can be easily identified and quantified from these antifungal LAB cultures. High mass accuracy data was obtained by Orbitrap MS infusion with a T-junction to the mobile phase of the collected fractions confirming the presence of the identified compounds. The quantities of the compounds detected for *L. amylovorus* A, *L. plantarum* B, *W. cibaria* C can be seen in Table 2. Twelve compounds were detected for *L. amylovorus* A strain compared to nine compounds that were detected by the LLE method. Compound A) 1,2-dihydroxybenzene; D) 3,4-dihydroxyhydrocinnamic acid; and F) caffeic acid were newly identified. The increased number of compounds detected can most likely be attributed to both the

Table 2
Analyte concentration (μg/mL) detected for 3 LAB cultures showing strong antifungal activity (I) *Lactobacillus amylovorus* A, (II) *Lactobacillus plantarum* B and (III) *Weisella cibaria* C.

Compound name		*Lactobacillus amylovorus A* (μg/mL)	*Lactobacillus plantarum B* (μg/mL)	*Weisella cibaria C* (μg/mL)
A)	1,2-Dihydroxybenzene	0.07	0.19	
B)	DL-Þ-Hydroxyphenyllactic acid	2.37	19.35	2.06
C)	4-Hydroxybenzoic acid	5.46	4.19	4.69
D)	3,4-Dihydroxyhydrocinnamic acid	1.09	0.75	0.84
E)	Vanillic acid			
F)	Caffeic acid	2.00	2.23	2.12
G)	3-(4-hydroxyphenyl) – propionic acid	6.33		4.68
H)	Phenyllactic acid	21.95	49.35	6.65
I)	p-Coumaric acid	0.81	0.74	0.70
J)	3-(4-hydroxy-3-methoxyphenyl)propanoic acid	4.44	4.30	3.96
K)	Benzoic acid	7.65	30.08	3.13
L)	Ferulic acid	1.47	1.62	2.32
M)	Salicylic acid			
N)	Hydrocinnamic acid	0.01		
O)	Methylcinnamic acid			
Average concentration		**4.47**	**11.28**	**3.11**

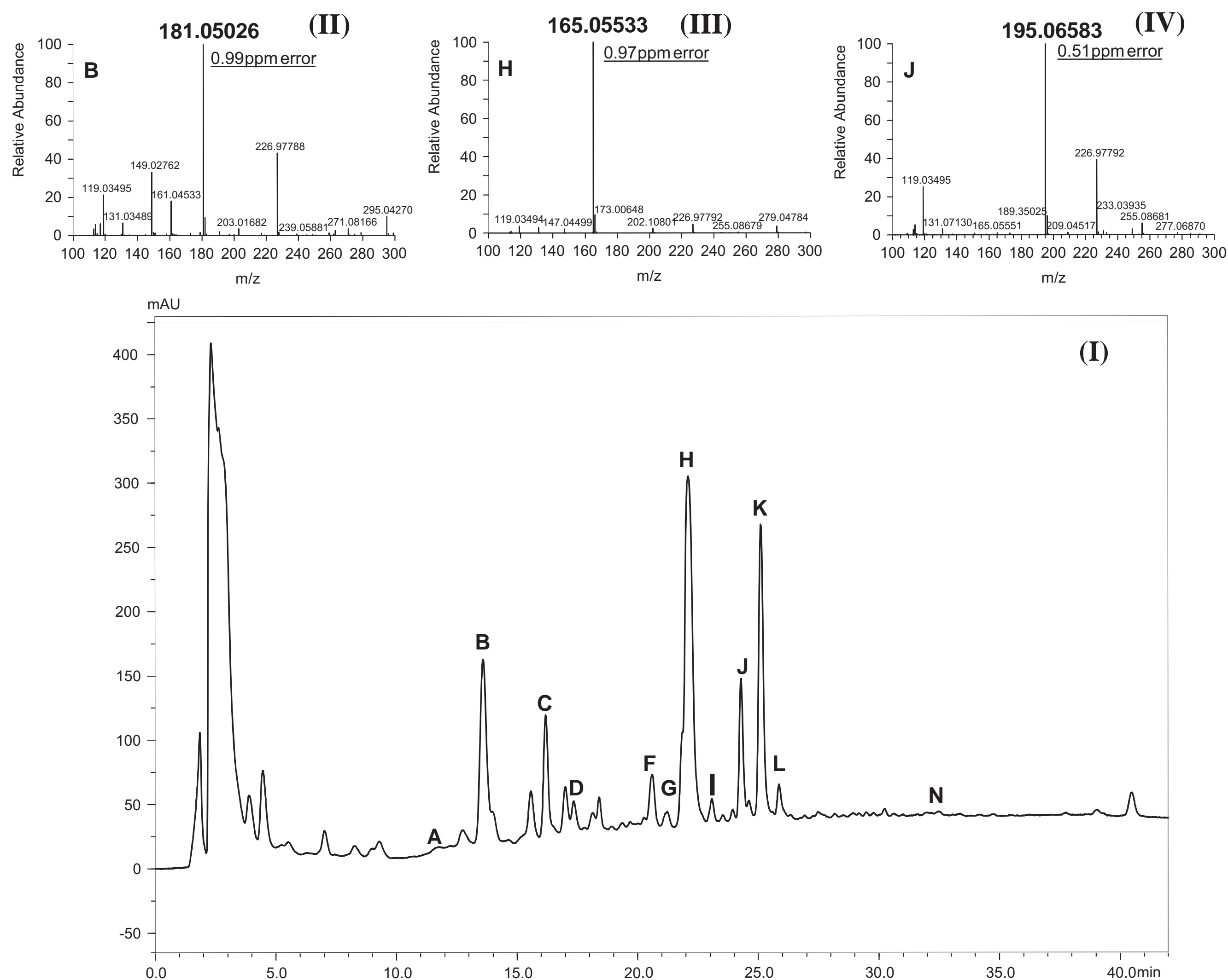

Fig. 6. Chromatographic profile of *Lactobacillus plantarum* strain showing antifungal activity (I); high mass accuracy spectrum of compound B) OH-PLA (II); high mass accuracy spectrum of compound H) PLA (III); high mass accuracy spectrum of compound J) 3-(4-hydroxy-3-methoxyphenyl)propanoic acid (IV). Chromatographic conditions as described in Section 2.5.

improved chromatograms and higher percentage recoveries using the QuEChERS extraction technique. The same results are observed for the *L. plantarum* B and *W. cibaria* C cultures with increased concentration of compounds being detected along with the number of compounds being identified increasing from 8 to 10 for *L. plantarum* B strain and from 9 to 10 for the *W. cibaria* C strain. Fig. 6(I) shows a chromatogram

of the *L. amylovorus* A with the high mass accuracy spectrums acquired for the fractions collected at peaks B, H and J to confirm their identities by LC-FTMS. Compound B – OH-PLA gave a ppm error of 0.99 (Fig. 6(II)), Compound H – PLA gave a ppm error of 0.97 (Fig. 6 (III)) and the lowest ppm error was shown by Compound J 3-(4-hydroxy-3-methoxyphenyl)propanoic acid with a ppm error of 0.51 (Fig. 6(IV)). These results confirm the improvement QuEChERS samples preparation can play in analytical chemistry supporting improved accuracy in sample profiling and quantitation for HPLC-UV/DAD and LC–MS, LC–MS/MS and LC-FTMS techniques.

4. Conclusion

The development of an efficient and effective QuEChERS-based HPLC-UV/DAD method to determine 15 naturally produced antifungal compounds from LAB has been established. This is the first application of QuEChERS in the detection of antifungal compounds and compounds from a bacterial strain. The method achieved high quality results allowing for the removal of interfering matrix components which were an issue with direct injection and SPE techniques. The application also provided improved overall recoveries of the tested antifungal compounds when compared to the optimised LLE method. The low cost, high throughput, minimal labour, small waste generation, minimal laboratory ware requirements, high compound recoveries, the ease of use, plus the potential to easily apply to various food matrices has secured QuEChERS as a very attractive methodology for use in the LAB field.

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