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A Thin-layer Chromatography Autographic Method for the Detection of Inhibitors of the *Salmonella* PhoQ–PhoQ Regulatory System

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ABSTRACT:

Introduction – The PhoP–PhoQ system from *Salmonella enterica* serovar Typhimurium controls the expression of factors that are critical for the bacterial entry into host cells and the bacterial intramacrophage survival. Therefore it constitutes an interesting target to search for compounds that would control *Salmonella* virulence. Localisation of such compounds in complex matrixes could be facilitated by thin-layer chromatography (TLC) bioautography.

Objective – To develop a TLC bioautography to detect inhibitors of the PhoP–PhoQ regulatory system in complex matrixes.

Methods – The TLC plates were covered by a staining solution containing agar, Luria-Bertani medium, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), kanamycin and a *S. typhimurium* strain that harbours a reporter transcriptional *lacZ*-fusion to an archetypal PhoP-activated gene *virK*. After solidification, the plate was incubated at 37°C for 16 h.

Results – A bioautographic assay suitable for the localisation of inhibitors of the PhoP–PhoQ system activity in *S. enterica* serovar Typhimurium present in a complex matrix is described. The assay was used to analyse a series of hydrolysed extracts prepared by alkaline treatment of crude plant extracts. Bioassay-guided analysis of the fractions by NMR spectroscopy and MS led to the identification of linolenic and linoleic acids as inhibitory input signals of the PhoP–PhoQ system.

Conclusion – A practical tool is introduced that facilitates detection of inhibitors of the *Salmonella* PhoP–PhoQ regulatory system. The assay convenience is illustrated with the identification of the first naturally occurring organic compounds that down-regulate a PhoP–PhoQ regulatory system from a hydrolysed extract. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: Bioautography; linoleic acid; linolenic acid; PhoP–PhoQ; *Salmonella*

Introduction

The PhoP–PhoQ system from *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) consists of an orthodox two-component regulatory system (TCS), comprised of PhoQ, a transmembrane sensor with bifunctional (histidine-kinase/phosphatase) activity and by PhoP, a cytoplasmic response regulator (Castelli *et al.*, 2000). *Salmonella typhimurium* responds to environmental magnesium deprivation by inducing the transcription of the PhoP–PhoQ regulon (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996). The regulon governed by the PhoP–PhoQ TCS includes genes that are critical for Mg^{2+} homeostasis (Soncini *et al.*, 1996) and those that provoke modifications of the lipopolysaccharide (LPS), which determine bacterial susceptibility to cationic anti-microbial peptides (Soncini *et al.*, 1996; Guo *et al.*, 1997; Groisman, 1998; Gunn *et al.*, 1998). This TCS controls the expression of essential virulence factors (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996; Blanc-Potard and Groisman, 1997; Guo *et al.*, 1997, 1998), which are critical for the bacterial entry mechanism into the host cell (Aguirre *et al.*, 2006; Deiwick *et al.*, 1999; Osborne and Coombes, 2011). Expression of the PhoP–PhoQ regulon is also necessary for intramacrophage survival, resistance to acid pH, modification of antigen presentation, formation of intracellular spacious vacuoles and alteration of macrophage cell death (Groisman, 2001).

Taking into consideration the relevant role of the PhoP–PhoQ system in the adaptive capacity of *S. typhimurium* to either host or non-host environmental challenges, and the fact that TCSs can be found only in prokaryotes, low eukaryotes and plants (García Véscovi *et al.*, 2010), the PhoP–PhoQ system constitutes an ideal target to search for new compounds that would prevent and/or control *Salmonella* virulence.

The chemical transformation of the components of natural crude extracts represents a clear way of altering the properties of those natural mixtures. Sometimes, however, unintended chemical alteration occurs during extraction or purification. In solution, natural products may react with the extraction solvent, or be activated by the solvent to react with other components present in the solution (Maltese *et al.*, 2009). In other cases chemical alteration is deliberate as in the case of chemically modified extracts (Lopez *et al.*, 2007; Ramallo *et al.*, 2011).

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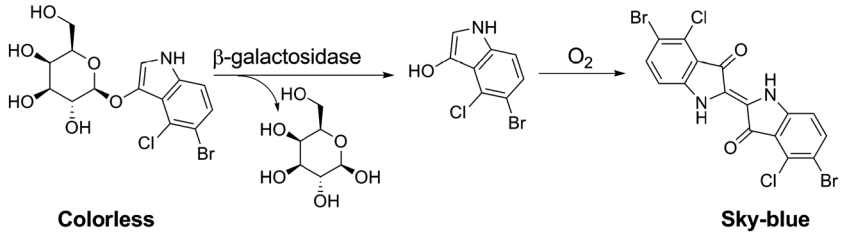

Figure 1. Hydrolysis and dimerisation of the colourless β-galactosidase substrate X-gal to produce the blue product 5,5′-dibromo-4,4′-dichloro-indigo.

Subsequent bioautographic TLC of the crude extracts and the chemically modified products then allows for a targeted isolation of bioactive compounds. In this present case a bioautographic-guided assay enabled isolation of inhibitors of the PhoP–PhoQ regulatory system activity in *Salmonella* adsorbed on TLC plates.

Experimental

Plant material

Whole-plant samples of *Urtica urens* L. Utricaceae (ID MO010), *Lamium amplexicaule* L. Laminaceae (ID MO001), *Brassica rapa* L. Brasicaceae (ID MO003) and *Sida rhombifolia* L. Malvaceae (ID MO030) were collected at the flowering stage in Pergamino, Buenos Aires province, Argentina, during November 2005. Voucher specimens were deposited at the Universidad Nacional de Rosario Herbarium. In all cases the sample was oven dried (at 65°C for 72 h) and milled to a fine powder.

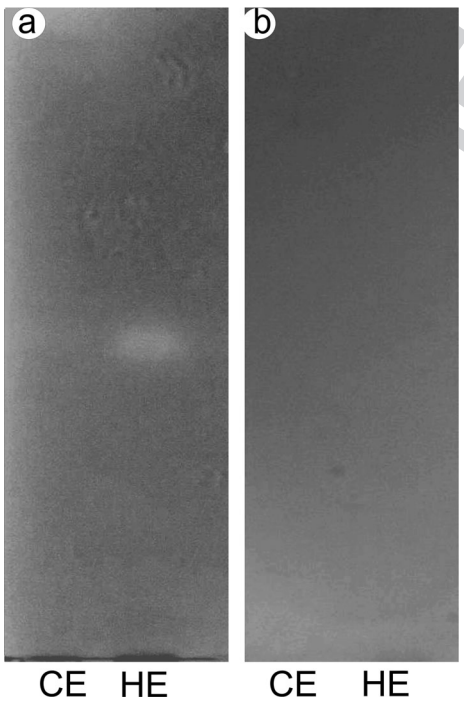

Figure 2. Bioautography assay of the crude (CE) and hydrolysed (HE) extracts of *L. amplexicaule*. Twenty micrograms of the samples were spotted on a TLC plate that was developed in DCM:MeOH (94:6). After development the solvent was removed and the plate was revealed by bioautography as described in the Methods section. (a) Bioautography carried out with the *S. typhimurium* strain that harbours a reporter transcriptional *lacZ*-fusion to a PhoP-activated gene *virK*. (b) Bioautography carried out with the *S. typhimurium* strain that harbours a reporter transcriptional *lacZ*-fusion to a PhoP-unrelated gene *tppB*.

Bacterial strains

Two bacterial strains, derived from *S. typhimurium* wild-type (ATCC 14028 s), that is, *virK*::*lacZ* (EG9532) (García Véscovi *et al*., 1996) and *tppB*::*MudI* (PB3062) (Gibson *et al*., 1987) were grown at 37°C in Luria-Bertani (LB) broth with shaking for 18 h.

Other material and methods

Silica gel 60 F254 TLC aluminium sheets and pH-indicator strips were purchased from Merck (Darmstadt, Germany). Cell-culture media reagents were from Difco (Buenos Aires, Argentina). Agar was purchased from Britania (Buenos Aires, Argentina). X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) was purchased from LabScientific, Inc. (Highlands, NJ,

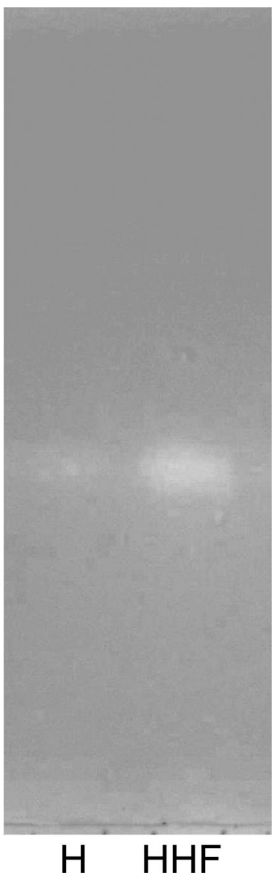

Figure 3. Bioautography assay of the hexane fraction (H) and hydrolysed hexane fraction (HHF) of *L. amplexicaule*. Three micrograms of the samples were spotted on a TLC plate that was developed in DCM:MeOH (94:6). After development the solvent was removed and the plate was revealed by bioautography with a *S.* Typhimurium strain that harbours a reporter transcriptional *lacZ*-fusion to a PhoP-activated gene *virK* as described in the Methods section.

USA). Phosphomolybdic acid was purchased from Anedra (Buenos Aires, Argentina). Palmitic acid (16:0), palmitoleic acid (16:1D^9), stearic acid (18:0), g-linolenic acid (18:3D^6) and polyethylene glycol 4'000 were purchased from Sigma-Aldrich (St Gallen, Switzerland). Ninhydrin, 2-aminoethylborinate, kanamycin, linoleic acid (18:2D^9) and oleic acid (18:1D^9) were purchased from Sigma-Aldrich (St Louis, MO, USA). Stearic and palmitic acids were diluted in tergitol 10% to a final stock concentration of 50 mg/mL, and unsaturated fatty acids (palmitoleic, oleic, linoleic and linolenic) were diluted in absolute ethanol to a final stock concentration of 100 mg/mL.

^{1}H-NMR spectra of samples (30 mg/mL $CDCl_3$ with ~~trimethylsilyl~~) were recorded on a Bruker Avance II at 300 MHz and high-resolution mass spectra were recorded on a Bruker micrOTOF-Q II spectrometer (Bruker-Daltonics) in MeOH. The MS parameters were: source type, electrospray ionization (ESI); ion polarity, negative; set nebuliser, 0.4 bar; set dry heater, 180°C; set dry gas, 4.0 L/ min; set capillary, 4500 V; set end plate offset, −500 V; set collision cell RF, 150.0 Vpp.

Preparation of crude extracts

The powdered material from each plant (200 g) was extracted separately in refluxing methanol (4 L) for 45 min, three times. The filtered extracts for each sample were combined and evaporated to dryness under reduced pressure by rotary evaporation.

Solvent partitioning of *Lamium amplexicaule* methanol extract

Dried crude *L. amplexicaule* extract (26.83 g) was suspended in H_2O: MeOH (90:10) (1 L) and extracted with hexane (2 × 500 mL). The MeOH was removed from the aqueous phase by rotary evaporation and the resulting aqueous fraction was extracted with dichloromethane (3 × 350 mL). The organic fractions were dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed by rotary evaporation to obtain the hexane fraction (3.7 g, 13.7% yield) and the dichloromethane fraction (2.1 g, 7.7% yield).

Chemical modification

Aliquots (50 mg) of the crude methanol plant extracts and of the dichloromethane and hexane fractions of *L. amplexicaule* were each stirred for 3 h in a refluxing 3.5% w/v ethanol solution of KOH (1 mL). The excess of reagent was neutralised by addition of 2 N HCl.

In order to detect changes produced by the alkaline treatment in refluxing ethanol, and not merely produced by the refluxing ethanol, aliquots (50 mg) of all the extracts and fractions were also refluxed in ethanol (1 mL) for 3 h prior to analysis.

Staining solution

Luria-Bertani (LB) medium containing agar was sterilised in an autoclave (121°C, 1 atm, 20'). When the process finished, the liquid medium was allowed to cool down to ca. 42°C. The overlay was prepared in a Falcon tube in conditions of sterility by mixing sterile LB medium (20 mL) containing agar (0.12 g) at ca. 42°C, with 20 mg/mL solution of X-gal (333 μL), 50 μg/mL solution of kanamycin (20 μL) and 1 × 10^8 CFU/mL of the *S. typhimurium* strain (100 μL) that harbours reporter transcriptional *lacZ*-fusions to either the archetypal PhoP-activated gene *virK* (14028 *virK::lacZ)* or to a gene activated by the OmpR–EnvZ two-component system, *tppB* (14028 *tppB*::Mud*I*). Final concentrations were: agar (0.6 % w/v), kanamycin (0.05 μg/mL), X-gal (333 μg/mL) and 5 × 10^5 ~~UFC~~/mL of the corresponding *S. typhimurium* strain.

Thin-layer chromatography and bioautography

Samples were loaded (100 μg/spot unless otherwise noted) onto silica-gel plates (10 cm × 10 cm) and subjected either to bioautography without prior development or developed in dichloromethane:methanol (94:6) and then subjected to bioautography. For the bioautography, the warm (ca. 42°C) staining solution (14 mL) was distributed over the TLC plate. After cooling and solidification, the plate was incubated at 37°C for 16 h. Clear spots (representing areas exhibiting inhibition of the PhoP–PhoQ system activity) were observed against a sky-blue background.

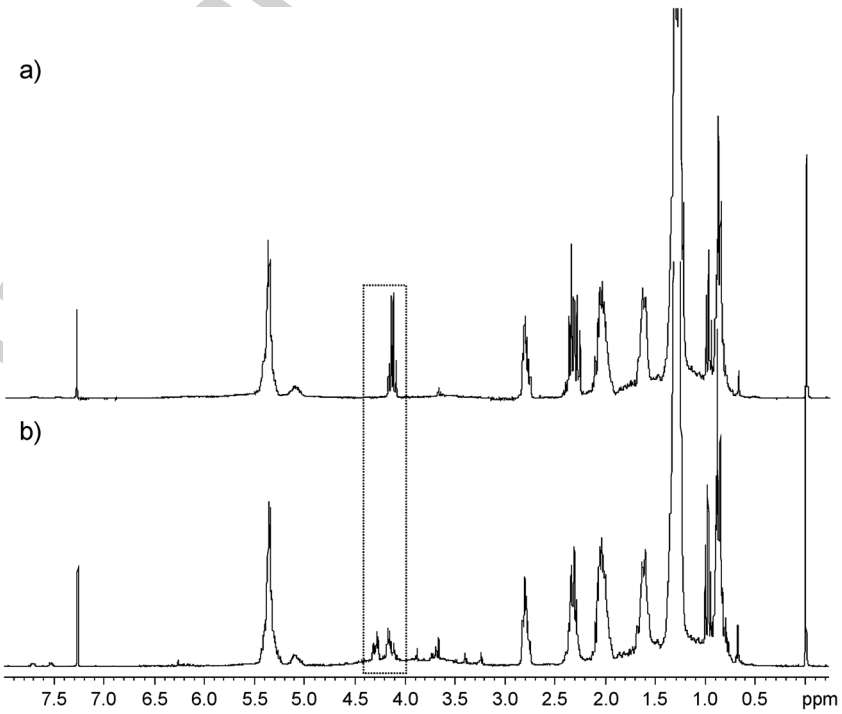

Figure 4. ^{1}H-NMR spectra of the unmodified hexane fraction and hydrolysed hexane fraction of *L. amplexicaule*. (a) Comparison of the ^{1}H-NMR spectrum of the hexane fraction of *L. amplexicaule* and (b) the spectrum of this hexane fraction treated with KOH in refluxing EtOH.

Results and discussion

Eight mixtures were screened by agar-overlay bioautography. Four mixtures were crude methanol extracts *U. urens*, *L. amplexicaule*, *B. rapa* and *S. rhombifolia*. Four other mixtures were modified extracts prepared by hydrolysis of the crude extracts with KOH in refluxing ethanol. Each mixture was spotted onto a TLC plate and analysed for activity without development of the plate.

Inhibitory activity was detected by overlaying the plate with an aqueous solution of soft agar containing X-gal and a homogeneous suspension of the *S. typhimurium* strain that harbours a reporter transcriptional *lacZ*-fusion to an archetypal PhoP-activated gene *virK*. Compounds that down-regulate the PhoP–PhoQ system will decrease the expression of the *lacZ* reporter and, therefore, production of β-galactosidase.

When cleaved by β-galactosidase the colourless substrate X-gal yields galactose and 5-bromo-4-chloro-3-hydroxyindole that spontaneously dimerises to produce the blue product 5,5′-dibromo-4,4′-dichloro-indigo (Fig. 1). Consequently, extracts that F1 contain an inhibitor of the PhoP–PhoQ system will produce a clear halo (intact X-gal) over a sky-blue background.

Using this method, inhibitory activities by the hydrolysed extracts of *L. amplexicaule* L., *S. rhombifolia* and *U. urens* were observed. These positive extracts were then subjected to TLC separation and re-assayed. Under these conditions, the three

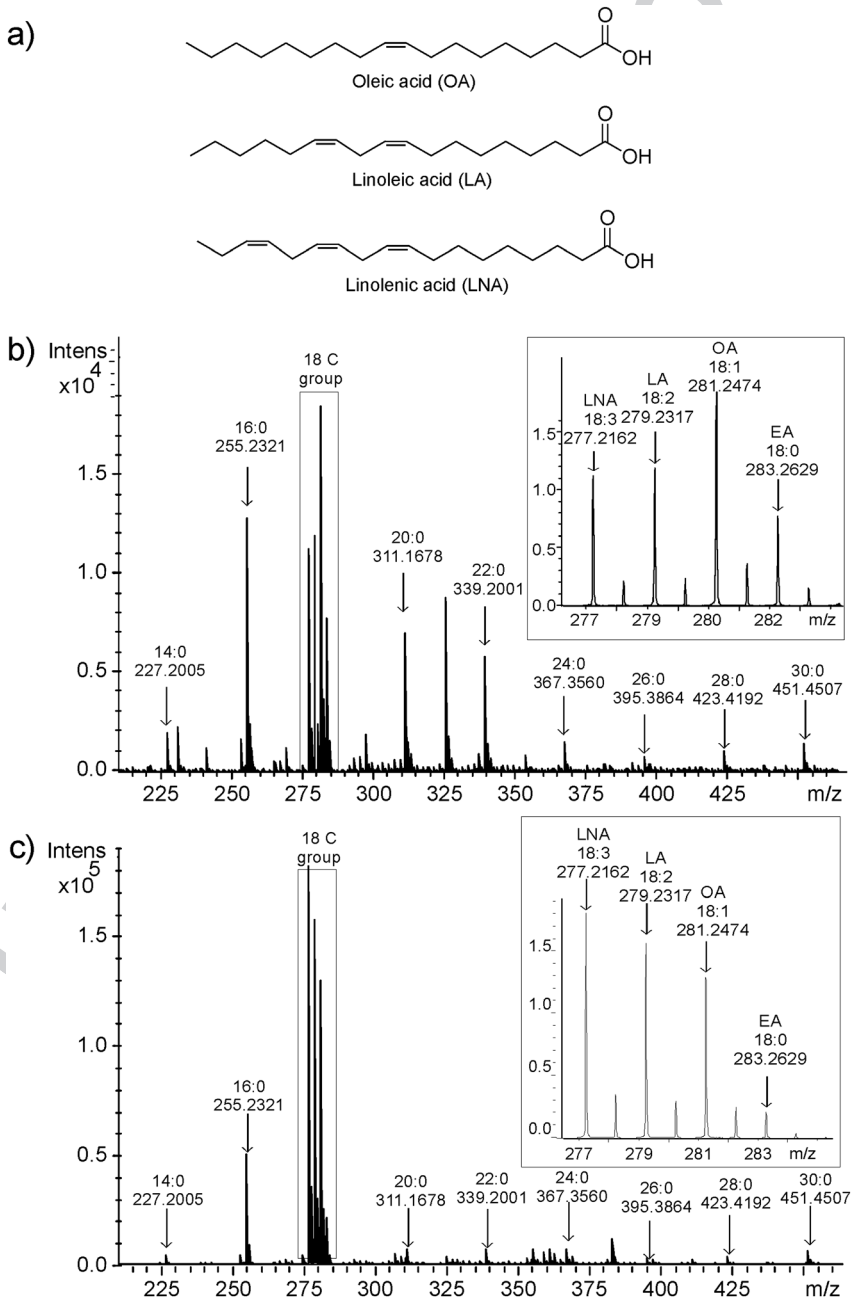

Figure 5. Mass spectra of the unmodified hexane fraction and hydrolysed hexane fraction of *L. amplexicaule*. (a) Structures of oleic (OA), linoleic (LA) and linolenic acid (LNA). (b) Comparison of mass spectrum of the hexane fraction of *L. amplexicaule* and (c) the mass spectrum of this hexane fraction treated with KOH in refluxing EtOH. The inset shows the peaks corresponding to linoleic, linolenic, oleic and stearic acids.

active hydrolysed extracts showed one inhibition spot at the same migration distance. The hydrolysed extract of *L. amplexicaule* showed the highest inhibition: 20 mg of this extract produced a clear inhibition spot, whereas no inhibition was observed for the same amount of unmodified crude extract (Fig. 2a). In order to confirm the specificity of the effect on the PhoP–PhoQ system, bioautography analysis was also carried out with a *S. typhimurium* strain that harbours a reporter transcriptional *lacZ*-fusion to the PhoP-unrelated gene *tppB* observing that the *L. amplexicaule* hydrolysed extract does not produce any inhibition halo (Fig. 2b).

The TLC analysis of the crude *L. amplexicaule* extract with spray reagents such us phosphomolybdic acid, ninhydrin and natural product reagent/PEG showed the presence of a complex mixture of compounds. In an attempt to decrease the complexity of the mixture, the crude *L. amplexicaule* extract was partitioned into hexane, dichloromethane and water fractions, and the hexane and dichloromethane fractions were modified through alkaline treatment. Although bioautography analysis showed that the bioactivity stayed in both fractions, the hydrolysed hexane fraction (HHF) of *L. amplexicaule* showed the highest inhibition (Fig. 3).

The presence of unsaturated fatty acids can be confirmed using ^{1}H-NMR spectroscopy (Knothe and Kenar, 2004). Thus, characteristic multiplet signals at about δ 5.4, 2.8 and 2.0 are assigned to olefinic, bis-allylic and allylic protons respectively. The protons on carbons α to the carbonyl resonate at about δ 2.4; the β carbon protons at about δ 1.6; other methylene protons about δ 1.3; and about δ 1.0 and 0.9 for the terminal methyl of linolenic acid and oleic or linoleic acids respectively. Based upon these characteristic NMR resonances, the ^{1}H-NMR spectrum of the hydrolysed hexane fraction suggests the presence of unsaturated fatty acids (Fig. 4). The ^{1}H-NMR spectrum of the unmodified hexane fraction is very similar but shows multiplets between 4.00 and 4.40 ppm characteristic of methylene hydrogen atoms of esterified glycerol (Lie Ken Jie and Lam, 1995), whereas in the spectrum of the hydrolysed hexane fraction the quartet at 4.1 ppm indicates the ethyl esters (Fig. 4). Overall, evaluation of these two spectra suggests that the change in bioactivity is due to the conversion of glycerol esters of fatty acids into ethyl esters or the free acids.

Figure 6. Bioautography of stearic acid (EA), oleic acid (OA), linoleic acid (LA), linolenic acid (LnA), palmitic acid (PA) and palmitoleic acid (PoA). Fifty micrograms of the samples were spotted on a TLC plate that was developed in DCM:MeOH (94:6). After development the solvent was removed and the plate was revealed by bioautography with a *S. typhimurium* strain that harbours a reporter transcriptional *lacZ*-fusion to a PhoP-activated gene *virK* as described in the Methods section.

Comparison of the mass spectra of the hydrolysed hexane fraction and the unmodified hexane fraction of *L. amplexicaule* indicates a tenfold increase in concentration of a series of free fatty acids due to the reaction. The major increase in concentration was observed for three unsaturated fatty acids, that is, linoleic, linolenic and oleic acids (Fig. 5). The measured accurate masses of the M − H ions of these fatty acids in the hydrolysed hexane fraction are: 277.21726 (linolenic acid, calculated *m/z* for $C_{18}H_{29}O_2$ 277.21730; 0.1 ppm error), 279.23294 (linoleic acid, calculated *m/z* for $C_{18}H_{31}O_2$ 279.23295; 0.1 ppm error) and 281.24857 (oleic acid, calculated *m/z* for $C_{18}H_{33}O_2$ 281.24860; 0.1 ppm error).

Pure samples of palmitic acid, palmitoleic acid, linolenic acid, linoleic acid, oleic acid and stearic acid were analysed by bioautography. The results show that free fatty acids down-regulate the PhoP-dependent gene and that the presence of double bonds apparently improves the activity: although oleic acid did not show a clear activity, the best results were observed for linoleic, linolenic and palmitoleic acids (Fig. 6). Similar to the previous observations with the modified extracts, the bioautography analysis shows that these unsaturated fatty acids do not down-regulate the PhoP-unrelated gene *tppB*.

Minimum inhibitory concentration (MIC) assays were carried out for palmitoleic acid, linoleic acid and linolenic acid in order to exclude bactericidal activity and to define subinhibitory concentrations for subsequent experiments. Inhibition on the expression was also obtained when the action of linoleic and linolenic fatty acids were assayed using well-known PhoP-regulated genes other than *virK*, indicating that the effect was global on the PhoP–PhoQ controlled regulon (Viarengo *et al*., 2013).

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Research Article

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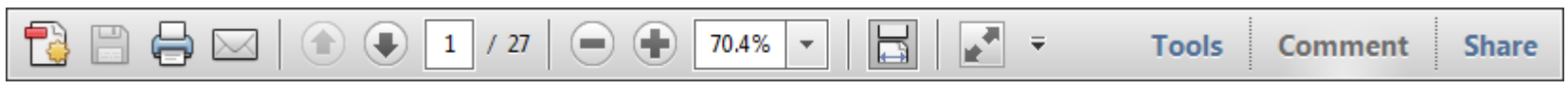

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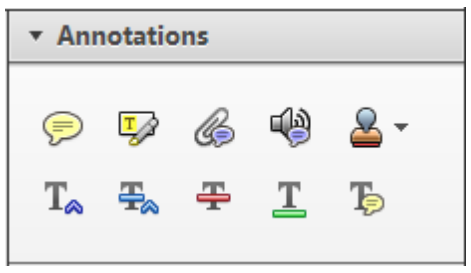

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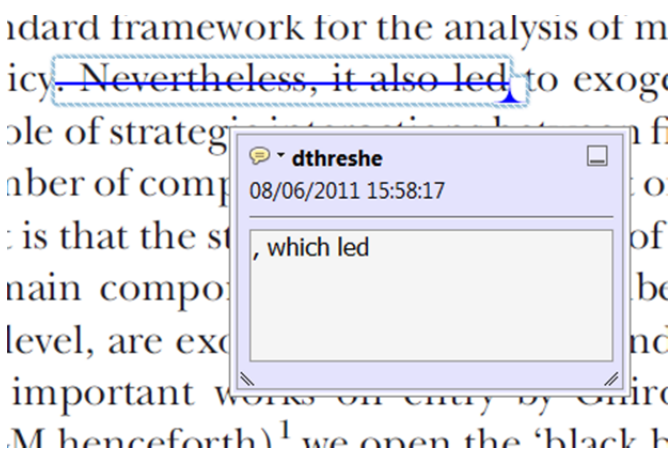
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

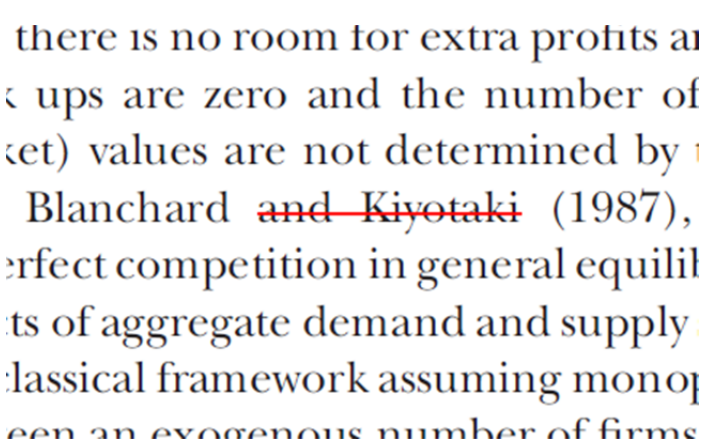


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

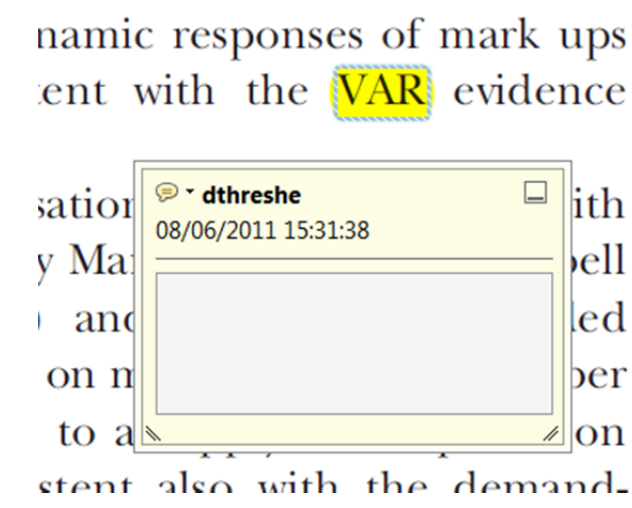


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

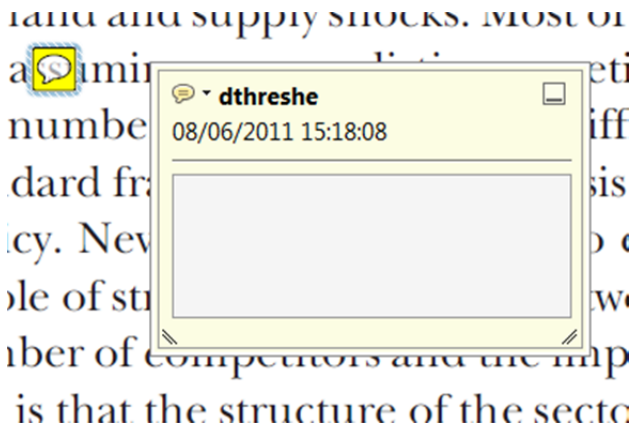


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



WILEY-BLACKWELL

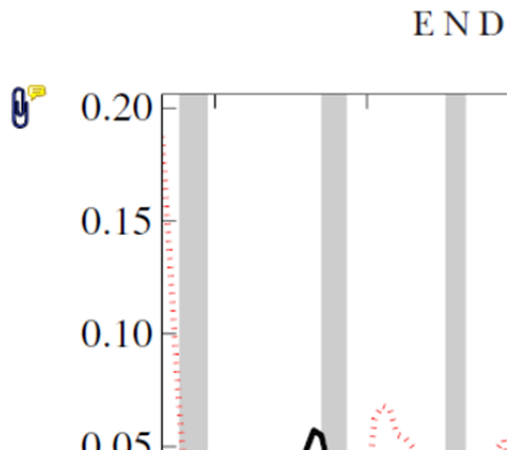
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

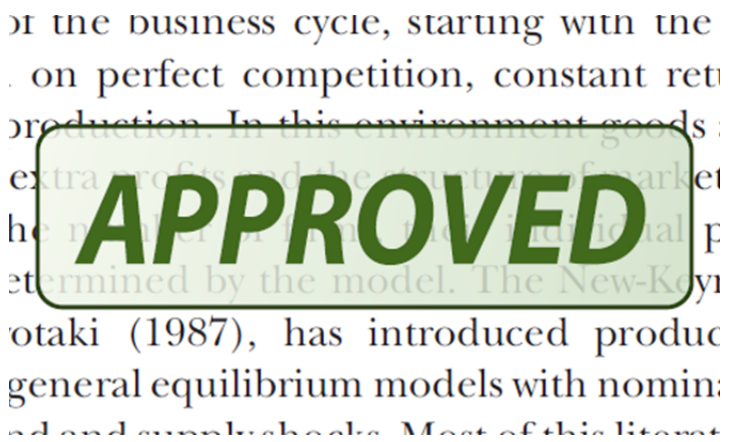


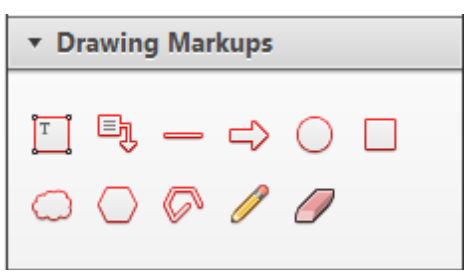
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



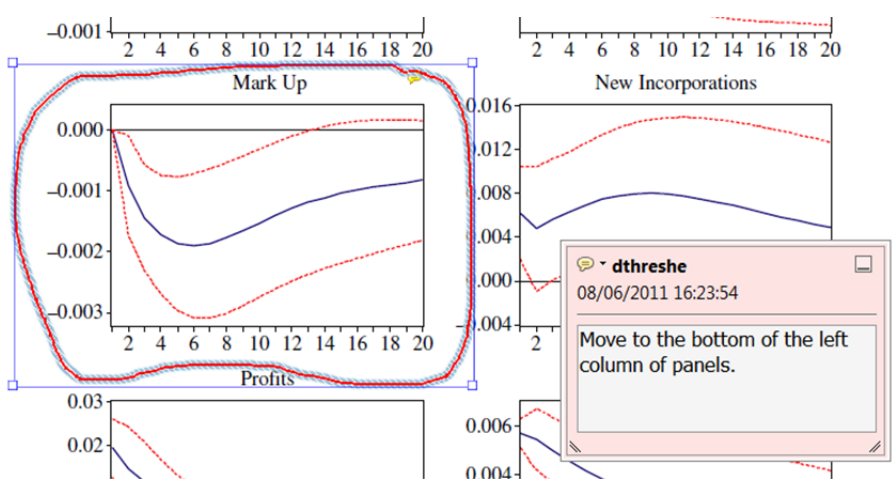


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: