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Thin Layer Chromatography-Autography-High Resolution Mass Spectrometry Analysis: Accelerating the Identification of Acetylcholinesterase Inhibitors

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ABSTRACT:

Introduction – The prevailing treatment for Alzheimer's disease is the use of acetylcholinesterase (AChE) inhibitors. Natural extracts are the principal source of AChE's inhibitors. However, their chemical complexity demands for simple, selective and rapid assays.

Objective – To develop a strategy for identification of AChE inhibitors present in mixtures employing high resolution mass spectrometry (HRMS) and thin layer chromatography (TLC)-biological staining.

Methodology – The strategy uses an autographic assay based on the α-naphthyl acetate – fast blue B system for the detection of AChE activity. The immobilisation of AChE in agar allowed the extraction of the compounds for analysis by HRMS. Three TLC experiments employing different solvent systems were used in parallel and the mass spectra of the compounds extracted from the inhibition halos, were compared. The analysis was performed under MatLab environment.

Results – The strategy was used to detect the presence of physostigmine in an extract of *Brassica rapa* L. spiked with the inhibitor. Similarly, caffeine was straightforwardly spotted as responsible for the inhibitory properties of an extract of *Ilex paraguariensis* Saint-Hilaire. Comparison of the HRMS profiles lead to the facile identification of the $[M+H]^+$ and $[M+Na]^+$ of the compounds responsible for the inhibition.

Conclusion – The proposed methodology, coupling TLC-AChE autography-HRMS, illustrates the feasibility of assigning molecular formulas of active compounds present in complex mixtures directly from autography. The new AChE agar-immobilised assay presented a more homogenous colour and a better definition than direct spraying methods, reducing the cost of the assay and improving its sensitivity. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: Acetylcholinesterase; bioautography; HRMS; thin layer chromatography

Introduction

The substantial increase in the ageing population has produced a concomitant increase in age-related brain disorders. Alzheimer's disease (AD) is one of the most common brain disorders, with an estimated 35 million people affected worldwide (Mandal, 2012). Characterised by a progressive cognitive decline, the prevailing treatment strategy for AD has been the use of acetylcholinesterase (AChE) inhibitors, whose basis is the cholinergic hypothesis (Terry and Buccafusco, 2003). Natural sources, particularly plants, have been the principal source of AChE's inhibitors (Mukherjee *et al.*, 2007). The alkaloids physostigmine (derived from *Physostigma venenosum* Balf.), galantamine (isolated from *Galanthus nivalis* L. and *Leucojum vernum* L.) and huperzine A (identified from *Huperzia* species) are the most renowned examples (Buccafusco, 2004).

Natural extracts are complex mixtures and their screening for a biological activity is not trivial; therefore there is a constant demand for simple, selective and rapid assays. Several tests have been developed for the screening of AChE inhibitors. Methods based on thin layer chromatography (TLC)-autography continue to play a key role, giving quick access to information concerning both the activity and the location on a TLC sheet of the active constituents of complex matrixes. There are two autographic assays most employed for the screening of AChE inhibitors. The first was developed by Rhee *et al.* (2001) and is based in Ellman *et al.*'s (1961) that uses acetylthiocholine iodide (ATCI) as the substrate and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as the visualising reagent. The second was developed by Hostettmann's group (Marston *et al.*, 2002) and employs α-naphthyl acetate (α-NAc) as the substrate and Fast Blue B (FBB) as revealing reagent. More recently, efforts have been made to develop new TLC assays by modifying conditions of reported substrate/detection systems (Mroczek, 2009; Yang *et al.*, 2009) or by employing alternative substrate/detection systems (Yang *et al.*, 2011).

Once an AChE inhibitor is located on the TLC sheet the isolation process begins, guided by analytic and spectroscopic tools to elucidate the structure responsible for the detected activity. Of the numerous types of analytical techniques used in many phases of drug discovery, mass spectrometry (MS) has become

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irreplaceable because of its high sensitivity, speed, selectivity, versatility, and ease of automation (Bart Emary and Zhang, 2013). High resolution mass spectrometry (HRMS) has become, with the advent of "omics" sciences, something more than a tool for accurate molecular weights.

MS has been coupled to TLC to provide the molecular formula of a compound with a given chromatographic behaviour (Van Berkel *et al*., 2005). This strategy can be guided using TLC-bioautography, as in the workflow recently introduced by the Morlock group, where estrogenic compounds detected in a bioautographic assay are extracted from a parallel high performance thin layer chromatography (HPTLC) plate for MS analysis (Morlock and Klingelhöfer, 2014; Klingelhöfer and Morlock, 2014). Similarly, Adhami *et al*. (2013) identified bioactive compounds present in galbanum resin by extracting samples from HPTLC plates and subjecting them to MS and NMR analysis. In these reports samples are separated by TLC and extracted for further analysis by MS, and a parallel TLC-autography is performed to know which spot has to be extracted. MS analysis of samples extracted directly from the TLC-autography has been more elusive; during the publication process of this manuscript Morlock and coworkers reported the first example. The report uses a direct bioautography with *Aliivibrio fischeri*, and the authors state that the MS analysis of samples extracted from this type of direct bioautography is restricted because the loading of ions on the plate, coming from buffer or the assay medium, is too high for rational MS recordings (Taha *et al*., 2015).

Independently of its coupling with TLC, MS has been employed to analyse natural products extracted from agar matrixes. Desorption/ionisation MS has been used for profiling and classification of microorganisms by measuring mass spectra from agar plates (Braga *et al*., 2013). Also, imaging mass spectrometry (IMS) has been applied recently to microbial natural product research revealing both phenotype and relevant and irrelevant chemotypes of microorganisms grown in agar media (Shih *et al*., 2014). Since different TLC autography assays have been developed using an agar overlay (Ramallo *et al*., 2006; Salazar *et al*., 2014; García and Furlan, 2015; Salazar and Furlan, 2007), such assay format could be useful for the direct coupling between bioactivity and MS analysis.

In this article, we report the development of the BIOMSID (bioautography coupled to mass spectrometry for the identification of compounds) technique. It consists of an agar gel immobilised AChE TLC-bioassay to spot inhibitors present in complex matrixes, such as natural extracts, coupled to HRMS analysis to obtain the molecular formula of the inhibitor (Figure 1).

A main advantage of the direct extraction of samples from the TLC-bioassay inhibition halo is that the bioactive compound can be followed and extracted from TLC plates developed under different conditions, collecting HRMS data that allows the identification of signals corresponding to the bioactive component of a complex mixture, even when standard low-resolution TLC is used.

Chromatographic sheets were spotted with the extracts and they were developed in three different mobile phase systems. After, the AChE autographic assay was performed samples were taken from the inhibition zone and from the coloured matrix (control), and subjected to HRMS analysis. Automated comparison of the obtained HRMS profiles using a specially designed MatLab algorithm cleans up the mass spectra from the matrix components and from inactive compounds present in the extract with similar chromatographic behaviour, leading to the unequivocal identification of the molecular weights of the compound responsible of the inhibition in each case.

The performance of the strategy is demonstrated by linking the AChE inhibitory activity observed in a *Brassica rapa* L. crude extract spiked with 0.1% of the known inhibitor physostigmine, and the AChE inhibitory activity observed in a crude extract of *Ilex paraguariensis* Saint-Hilaire to the presence of the naturally occurring alkaloid caffeine (Heck and De Mejia, 2007).

The proposed methodology complements the traditional bioguided isolation of active compounds. It provides information about the actives in early steps of the fractionation process facilitating dereplication of known actives and helping with the structural elucidation of unknown actives.

Experimental

Chemicals and materials

AChE from *Electrophorus electricus* (EC 3.1.1.7; Sigma product No. C3389), FBB, α-naphthol, α-NAc, ATCI, caffeine and physostigmine were purchased from Sigma (St Louis, MO, USA). Aluminium TLC sheets coated with Silica gel 60 F_{254} were purchased from Merck (Darmstadt, Germany). Tris-hydrochloride (Tris–HCl) was purchased from Anedra (San Fernando, Argentina). Agar was purchased from Britania (Bs. As., Argentina). DTNB was purchased from Aldrich (Milwaukee, WI, USA).

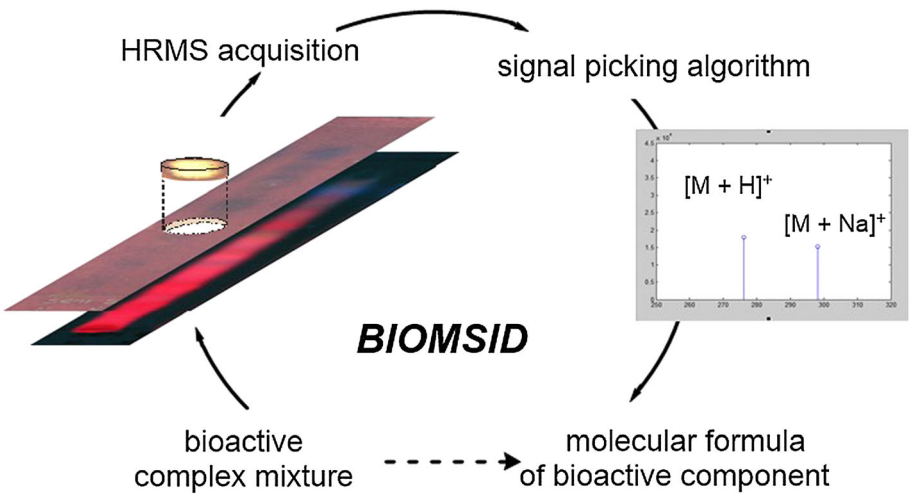

Figure 1. Process for the identification of the molecular weight of an active compound from an autographic assay through BIOMSID.

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Plant material

The methanol extract of *Ilex paraguariensis* Saint-Hilaire was prepared from commercial milled dried leaves. The 1000 g of plant material was refluxed in methanol (1 × 3500 mL, 90 min). Upon completion, the plant material was filtered and the extract was concentrated to dryness (25.62% final yield). The methanol extract of *Brassica rapa* L. was prepared from the whole plant. The plant material was collected in Pergamino, Buenos Aires, and identified by Dr Oscar Micheloni (National University of Northwest of Buenos Aires, Argentina). Voucher specimens were deposited at the Herbarium of the National University of Rosario. Dried and milled vegetal material (820 g) was refluxed in methanol (3 × 4 L, 45 min). Upon completion, the plant material was filtered and the extract was concentrated to dryness (12.70% final yield).

Solutions

Tris–HCl buffer (50 mM) was prepared in distilled water adjusted to pH 8.00 with HCl 0.1 N. α-NAc solution (2.5 mg/mL) was prepared, freshly before use, in ethanol. FBB solution (2.5 mg/mL) was prepared, freshly before use, in distilled water. α-NAc/FBB solution was prepared, freshly before use, by mixing of α-NAc (2.5 mg/mL) and FBB (2.5 mg/mL) in a final concentration relationship of 4:1 (FBB:α-NAc). AChE solution 30 U/mL was prepared in Tris–HCl buffer (pH 8.00) and stored at −20°C. α-naphthol solution (1.7 mg/mL) was prepared in water with 1% ethanol.

AChE autographic assay: "g" protocol

Agar was dissolved at 80°C in Tris–HCl buffer (50 mM, pH = 8.0). The solution was allowed to cool down to 50°C and AChE solution (30 U/mL) was added. The obtained solution was mixed by inversion. Final concentrations: agar (1% P/V), Tris–HCl buffer (47.8 mM), and AChE (1.3 U/mL). A 80 cm^2 TLC plate was sprayed with the α-NAc/FBB solution. Then the plate was dried under air current at room temperature, and 13.6 mL of AChE agar solution were distributed over the TLC plate. Once gelled, the plate was incubated at 37°C for 20 min in a closed and humid Petri dish in the dark.

AChE false positive autographic assay

Agar was dissolved at 80°C in Tris–HCl buffer (50 mM, pH = 8.0). The solution was allowed to cool down to 50°C and α-naphthol solution (1.7 mg/mL) was added. The obtained solution was mixed by inversion. Final concentrations: agar (1 % w/v), Tris–HCl buffer (47.8 mM), and α-naphthol (0.075 mg/mL). A 80 cm^2 TLC plate was sprayed with α-NAc/FBB solution. The plate was dried under air current at room temperature and then, 13.6 mL of agar-α-naphthol solution were distributed over it. The autography was analysed immediately after it was gelled.

Tested samples and chromatography separations

The stock solutions of physostigmine and spiked natural extracts were prepared in dichloromethane and methanol, respectively. For the determination of detection limits for physostigmine, solutions from 1 mg/mL to 10^{-7} mg/mL were prepared and 10 μL of each were spotted on a Silica gel TLC plate. For physostigmine determination in crude extracts, a spiked *Brassica rapa* extract was produced by adding 250 μL of methanol solution of physostigmine (0.1 mg/mL) to 625 μL of methanol solution of *B. rapa* crude extract (40 mg /mL). Final concentrations were 28.60 mg/mL (containing 0.1% w/w of physostigmine). TLC plates were spotted with 8.75 μL of spiked extracts, and developed with the following mobile phases: dichloromethane:methanol (90:10), ethyl acetate:methanol:water (50:50:20) and toluene:chloroform:ethanol (28.5:57:14.5). For caffeine identification TLC plates were spotted with 15 μL of a 40 mg/mL solution of *Ilex paraguariensis* methanol extract. The TLC plates were developed with: dichloromethane: methanol (90:10), ethyl acetate:methanol:water (100:13.50:10) and toluene: chloroform:ethanol (28.5:57:14.5).

Sample preparation and HRMS analysis

Circular portions of the agar layer were removed from the TLC-autography assay using a glass tube (internal diameter 1 cm) coupled to a pipette bulb. Samples were taken from inhibition zones and from zones located away from the TLC-lanes where the extracts were eluted (background samples). Each gel portion was extracted with dichloromethane (2 × 1 mL) and with ethanol (2 × 1 mL). Solvents were evaporated and the remaining sample was dissolved in 3 mL of methanol. The resulting solution was infused directly into the electrospray ionisation (ESI) chamber at a rate of 180 μL/min during 1 min for HRMS analysis. Mass spectra were recorded on a Bruker micrOTOF-Q II spectrometer (Bruker-Daltonics). MS parameters: source type, ESI; ion polarity, positive; 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 radio frequency, 150.0 Vpp. The spectra were visualised, baseline corrected (Flatness = 0.95) and exported in ASCII format, with Data Analysis Version 4.0 SP1. The mass list, conformed by *m/z* and respective intensity values, were generated with Peak finder Apex algorithm (Data Analysis Version 4.0 SP1) with the following parameters: peak width (full width at half maximum, FWHM) = 0.04, signal-to-noise ratio (S/N) threshold = 6.5, Relative intensity threshold (Base peak) = 0, Absolute intensity threshold = 0.

Results and discussion

We describe a methodology for the tentative identification of AChE inhibitors present in complex mixtures employing HRMS. The proposed methodology combines standard TLC, *in situ* measurement of inhibitory properties of the chromatographed mixture, and HRMS analysis of the compounds present in the inhibition zones.

A crude extract is analysed by TLC-autography in a bioassay where the enzyme is gel immobilised. This assay format allows direct extraction of the active compounds from inhibition zones of the plate for HRMS analysis: once an inhibition zone is detected on the autographic assay, the compounds are recovered from the gel and subjected to HRMS analysis.

Because of the limited resolution of standard TLC and the high complexity of crude plant extracts, inactive compounds are co-extracted with the active compound hampering its straightforward identification.

In order to solve this problem, two or three solvent systems were used in parallel and the mass spectra of the compounds extracted from the inhibition zones, were compared using a MatLab algorithm to identify the signals, i.e. the molecular formula, corresponding to the active compound.

Assay development

The available autographic assays to detect AChE inhibitors are methods in which enzyme and reagents are sprayed directly on the TLC plate surface (Rhee *et al.*, 2001; Marston *et al.*, 2002). In order to optimise the proposed extraction of active molecules from the inhibition halos, an autographic assay on agar layer was developed. During the setting up of the autography, eight modified protocols were applied using α-NAc as substrate and FBB as detection reagent. These protocols differed essentially in the mode of application of enzyme, substrate and detection agent over the TLC sheet.

Initially, gel stability problems arose due to the low water-solubility of the substrate α-NAc, and the consequent use of ethanol as solvent. Both dipping of a gelled plate in an ethanol solution ("a" and "b" protocols, see Supporting Information) and spraying the gel with ethanol solution ("c" protocol, see Supporting Information) produced gel disruption. However, applying the substrate and the detection agent separately at different stages ("d", "e" and "f" protocols, see Supporting Information) generated reddish rather

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than violet autography background. This may have been caused by the inappropriate final ratio [α-NAc]/[FBB] achieved within the gel [the optimal was reported as 1:4 (Marston *et al*., 2002)].

The best results were obtained using "g" protocol (see Experimental section) that produced violet gel colouration and allowed detection of 1 μg of physostigmine producing a well-defined colourless inhibition zone. Briefly, the TLC was sprayed with the solution α-NAc/FBB (1:4) and left to dry. Then, an AChE solution in molten agar was deposited over the previously sprayed TLC and the whole system was incubated during 20 min at 37°C. This protocol was also evaluated with ATCI as substrate and DTNB as detection agent. In this experiment, AChE and DTNB were solubilised in molten agar solution, and distributed on the TLC sheet. Once the agar layer solidified, the plate was immersed in a solution of the substrate ATCI (see Supporting Information). Although colour development was evident, the definition and contrast of the inhibition zone produced by 1 μg of the known inhibitor physostigmine spotted on the plate was poor, decreasing the sensitivity of the assay.

In order to determine the sensitivity of the assay using the system α-NAc/FBB, decreasing amounts of physostigmine were spotted on a TLC plate (Figure 2). Enzyme inhibition was detected at quantities of inhibitor that are below 1×10^{-3} μg making this test more sensitive than some of the reported methods (detection limit 1×10^{-2} μg) (Rhee *et al*., 2001; Marston *et al*., 2002).

Additionally, AChE immobilisation on agar led to other interesting characteristics compared to spray based methods. Firstly, it is easier to know the concentration of enzyme per square centimetre of TLC plate. In enzymatic reactions, when the v_{max} is reached, kinetics becomes independent of substrate concentration, only depending on enzyme concentration. Homogeneous enzyme distribution ensures uniform background coloration over the plate, an important factor for semi-quantification of compounds using image analysis strategies. Secondly, the use of gel avoids the run off of spots usually observed when aqueous solutions or suspensions are sprayed over TLC plates. Finally, according to our experience (Ramallo *et al*., 2006; García and Furlan, 2015; Salazar and Furlan, 2007) the presence of the gel delays drying of the plate and the concurrent enzyme inactivation.

Since the system α-NAc/FBB may produce false positives due to the reaction between the staining chemicals and components from the extract (Yang *et al*., 2009), a non-enzymatic assay on agar layer was developed. In this test, α-naphthol was immobilised on an agar layer over a thin-layer chromatogram previously sprayed with a solution of α-NAc and FBB. In this way, a violet coloration was generated across the plate. Colourless zones indicate the presence of compounds that react either with α-naphthol or with FBB. AChE inhibitors can be distinguished from false positives by comparison between the control and the enzymatic assay, before further analysis.

Molecular formula determination

In order to determine the molecular formula of an AChE inhibitor present in a complex mixture of compounds, it is necessary to identify the signal or the signals that belong to this compound in the mass spectrum of a sample extracted from an inhibition zone.

The obtained mass spectra usually include a number of signals from ions coming from buffer, substrate, revealing agents, and other impurities. These signals can be cleaned out by comparing the spectrum obtained from the halo with a spectrum obtained from gel extracted from an extract-free TLC area.

Additionally, the low resolution of standard TLC and the high complexity of the natural crude extracts may hamper this identification because of co-extraction of inactive compounds together with the inhibitor. This fact increases the complexity of the obtained mass spectra and consequently its analysis, in particular, when the active compound: (a) is present in a very low concentration in the extract, (b) is difficult to ionise by electrospray, and/or (c) co-exists in the extract with inactive compounds of similar chromatographic behaviour.

In order to solve this problem, two or three solvent systems were used in parallel and the mass spectra of the compounds extracted from the inhibition zone in each of the three TLC plates were compared with each other and with background samples. Our expectation was that the composition of the mixture of compounds, co-extracted with the active compound from the inhibition zone in different chromatographic systems, would differ from each other. Consequently, only the signals that are common to all spectra from inhibition zones samples and that are absent in background samples belong to ions produced by the active compound.

The analysis was performed under MatLab environment, a program that allows calculations on large data sets. The design of tailored routines increases the versatility of the program compared to HRMS-signal processing programs. An algorithm was written based on two parameters: % filter and tolerance.

The parameter % filter defines the threshold intensity above which a signal was considered significant. It is calculated as a percentage of the intensity of a selected *m/z* signal that is present in all the spectra. Selection of an appropriate value for this parameter is essential to reduce the complexity of the mass spectra without losing the important signals: a high % filter value decreases the number of signals to be analysed reducing errors in the calculus caused by noise; however, it also increases the chances of losing the signals of interest (from de-active compound) when their intensity is low.

The parameter tolerance contemplates the error of the equipment. This parameter defines a range wherein different *m/z* values are considered as equals. For example, for a signal with 276.1707 *m/z*, when a tolerance value of 0.0002 is used, all signals with *m/z* values comprised in the range 276.1705–276.1709 will be

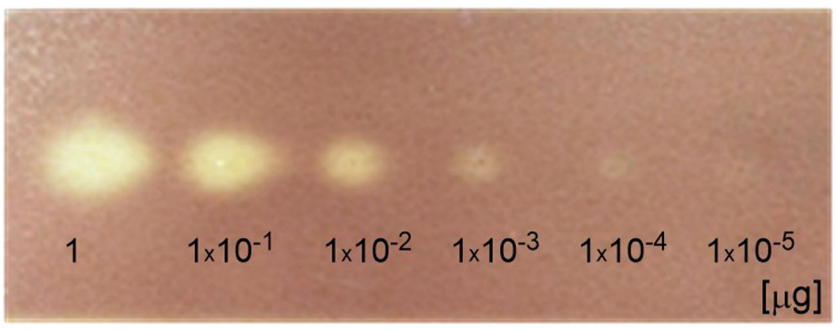

Figure 2. Autography assay showing the detection limit of AChE autography assay for physostigmine.

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considered as equivalent. During spectra comparison, this parameter defines which signals are actually present in the different samples; consequently, it is involved in the decision of whether a signal is associated with the active compound or not.

This algorithm starts cutting off, with the same % filter value, the mass spectrum taken from a sample of the inhibition zone and the spectrum from the background, both obtained from the same TLC-autography (TLC number one). Then the filtered mass spectrum of background is subtracted from the filtered mass spectrum of the inhibition zone. This step eliminates signals that are not related to the inhibition (i.e. buffer, substrate, revealing agents, and other impurities). The same filtering and subtracting processes are applied to the mass spectra of samples extracted from a second and a third TLC-autography assay spotted with the same active extract but eluted with different mobile phases (TLC number two and three). Finally, the algorithm compares the three background subtracted spectra of the inhibition zones and searches for common signals. It was expected that those common signals would be linked to the structure of the active compound. The whole process is depicted in Figure 3.

In order to test the proposed strategy on a supervised system, the *Brassica rapa* L. methanol extract (inactive against AChE) was spiked with 0.1% w/w of the known inhibitor physostigmine (Figure 4) (Bracesco *et al.*, 2011).

Three TLC layers, spotted with the spiked extract, were chromatographed using dichloromethane:methanol (90:10), toluene:chloroform:ethanol (28.5:57:14.5), and ethyl acetate:methanol:water (50:50:20), respectively. Each thin-layer chromatogram was examined for AChE activity and then equal circular samples of the gel were taken. Two samples were taken from each TLC plate: one from the inhibition zone and one from the background. The samples were extracted with dichloromethane and ethanol, the solvents were evaporated and the extracts were dissolved in methanol for HRMS analysis. Since chemical

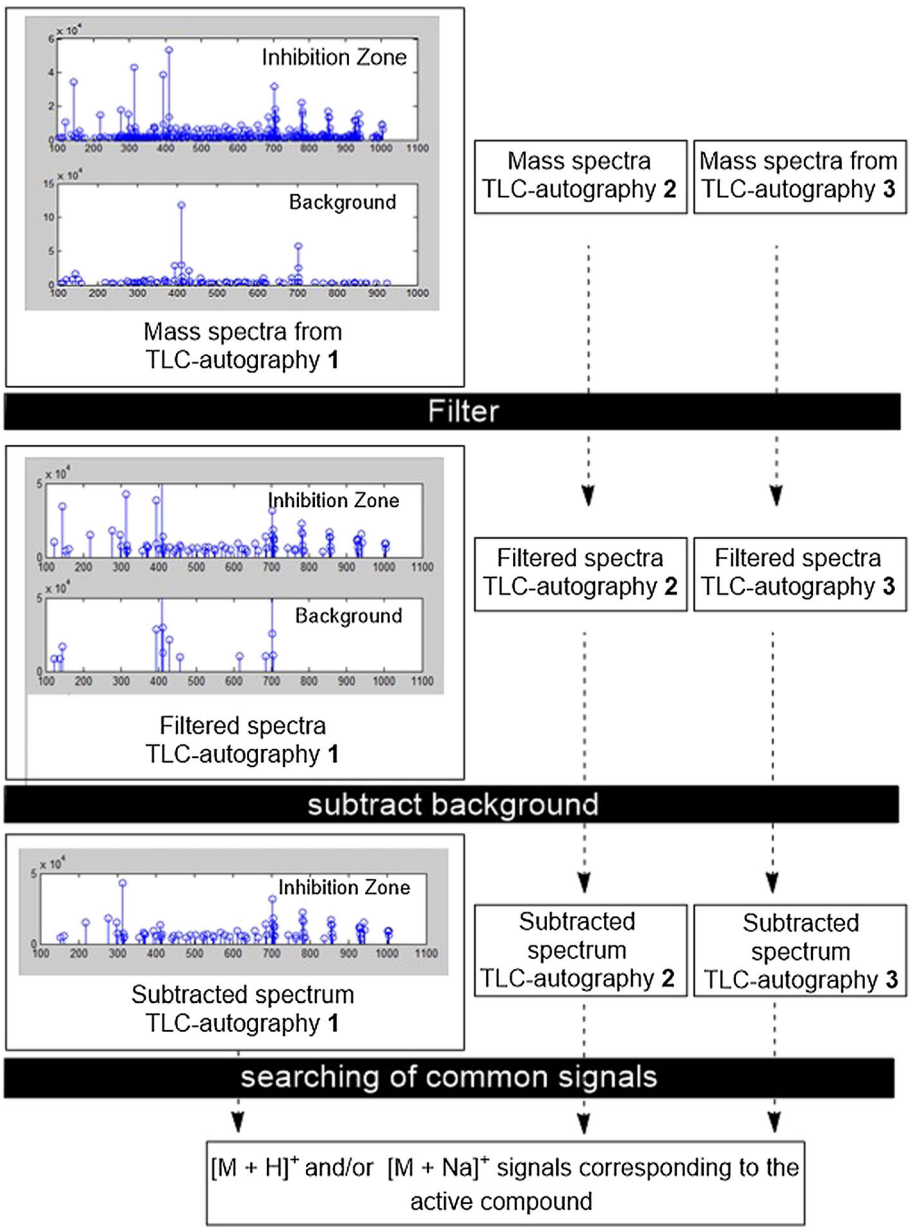

Figure 3. Scheme showing the followed steps for the identification of the $[M+H]^+$ and/or $[M+Na]^+$ of an active compound.

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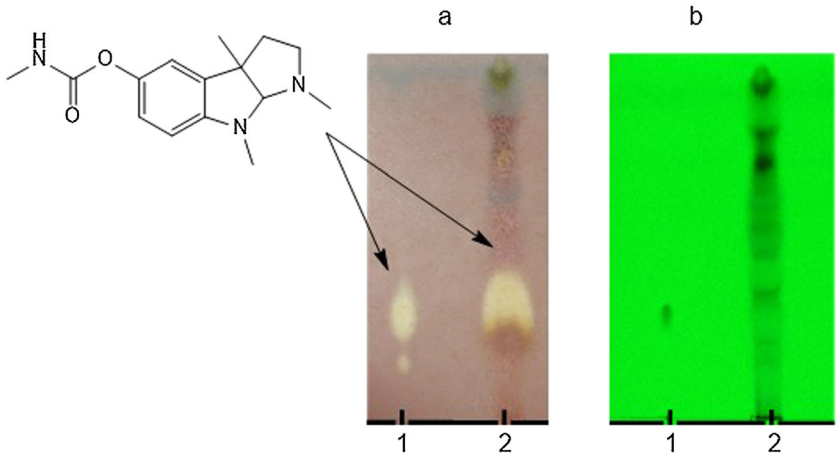

Figure 4. (a) Autographic assay showing inhibitory AChE activity. (b) TLC under $UV_{254\ nm}$ light before autographic assay. Lane 1: physostigmine, 0.25 μg applied. Lane 2: 0.1% physostigmine spiked *Brassica rapa* methanol extract, 250.25 μg applied.

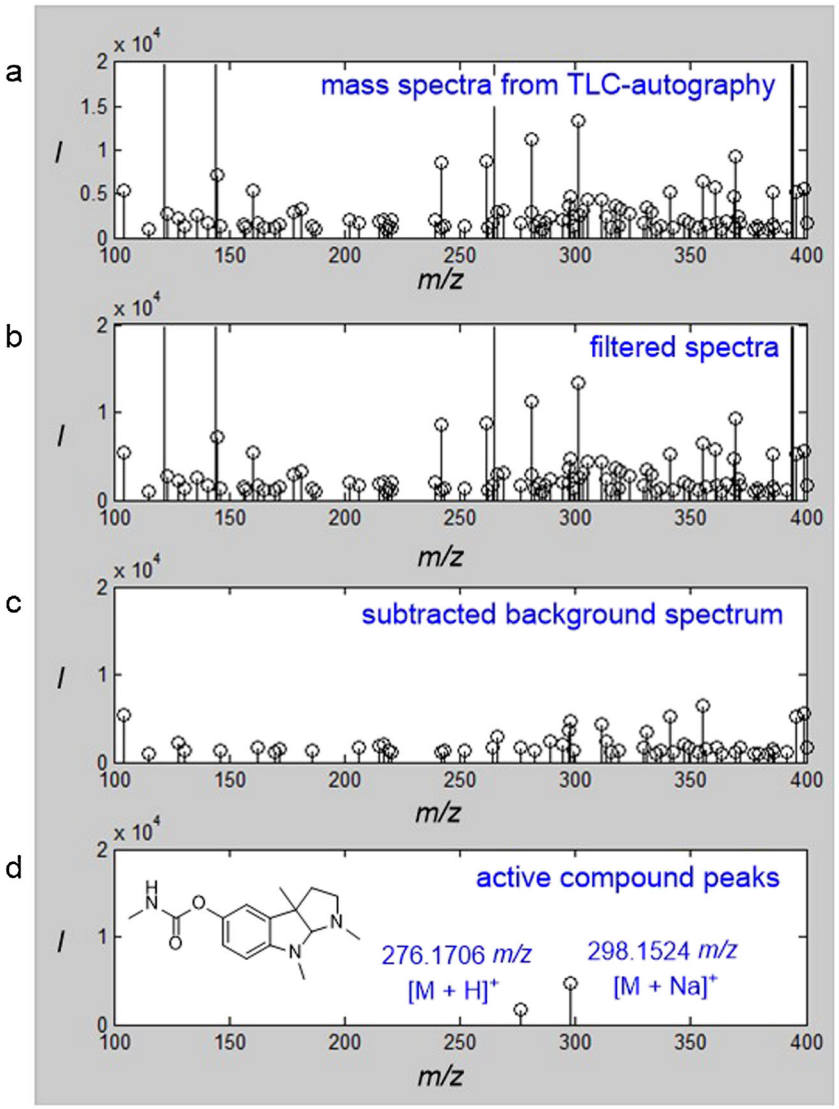

Figure 5. MatLab output of the different processing stages of the mass spectra obtained from 0.1% physostigmine spiked *Brassica rapa* methanol extract. (a) Raw mass spectra obtained from TLC-autography halo. (b) Mass spectra after filtering with 1% of tris signal intensity. (c) Filtered mass spectra after subtracting the background. (d) Common signals corresponding to the active compound.

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composition varied across the different samples the intensity of the buffer tris signal, 122.0818 *m/z* was used to set the parameter % filter value. The buffer tris is used for pH regulation in the biological assay so it is expected to be present in identical concentration all over the gel (i.e. in all gel samples).

Spectra obtained from the inhibition zone resulted in a matrix containing approximately 500 signals. After filtering at 1% of the intensity value of the tris buffer the number of signals of each matrix was reduced 50%. Subtraction of the spectra from background was carried out using a tolerance value of 0.0005 (error = 1.66 ppm).

This value allows molecular formula validation for HRMS with an error that is below the generally accepted value for molecules with molecular weight of 300 or higher. This value could be considered the average molecular weight for natural products (Feher and Schmidt, 2002). After background subtraction, each spectrum was reduced to a matrix of approximately 160 signals. Presumably, they include signals of the active compound and signals of co-eluting inactive components of the mixture. On the last step the background-subtracted matrixes of the halo's samples from the three TLCs were compared looking for all *m/z* signals that they have in common.

For *Brassica rapa* L. methanol extract spiked with physostigmine (0.1% w/w), the MatLab algorithm identified only two signals, both related with the active compound (Figure 5). The most intense signal with 298.1532 *m/z* (*I* = 3883) was validated for $C_{15}H_{21}N_3NaO_2$ $[M+Na]^+$ (Calculated 298.1526, error = −1.9 ppm). A second signal with 276.1703 *m/z* (*I* = 1720) was validated for $C_{15}H_{22}N_3O_2$ $[M+H]^+$ (Calculated 276.1710, error = 1.5 ppm).

This approach made possible the identification of the *m/z* signals corresponding to an active compound present in 0.1 % w/w in an extract employing three autography assays, developed over standard TLC plates, and their respective direct infusion mass spectra.

It is worth mentioning that the intensity of the $[M+H]^+$ signal, with *m/z* = 276.1703, was very low (*I* = 1720). Although it only represented around the 1.3% of the base peak and nearly 60% of signals of the spectra had higher intensity values (Figure 6), this signal was correctly assigned to the active compound.

The methodology was also applied to analyse the methanol extract of *Ilex paraguariensis* Saint-Hilaire. The dried and minced leaves of this species are widely used in South America (Argentina, Brazil, Chile, Paraguay and Uruguay) to prepare a popular tea beverage called "*mate*". There are several publications describing the health benefits of *mate* including memory enhancement properties and stimulation of the central nervous system (Heck and De Mejia, 2007). Several phytochemicals have been identified in *Ilex paraguariensis* including polyphenols (chlorogenic acid), xanthines (theophylline, theobromine, etc.), flavonoids (quercetin, kaemferol), vitamins (A, E, C, B_1 and B_{12}), minerals (P, Fe, and Ca), tannins, and triterpenic saponins derived from ursolic acid (Bracesco *et al.*, 2011).

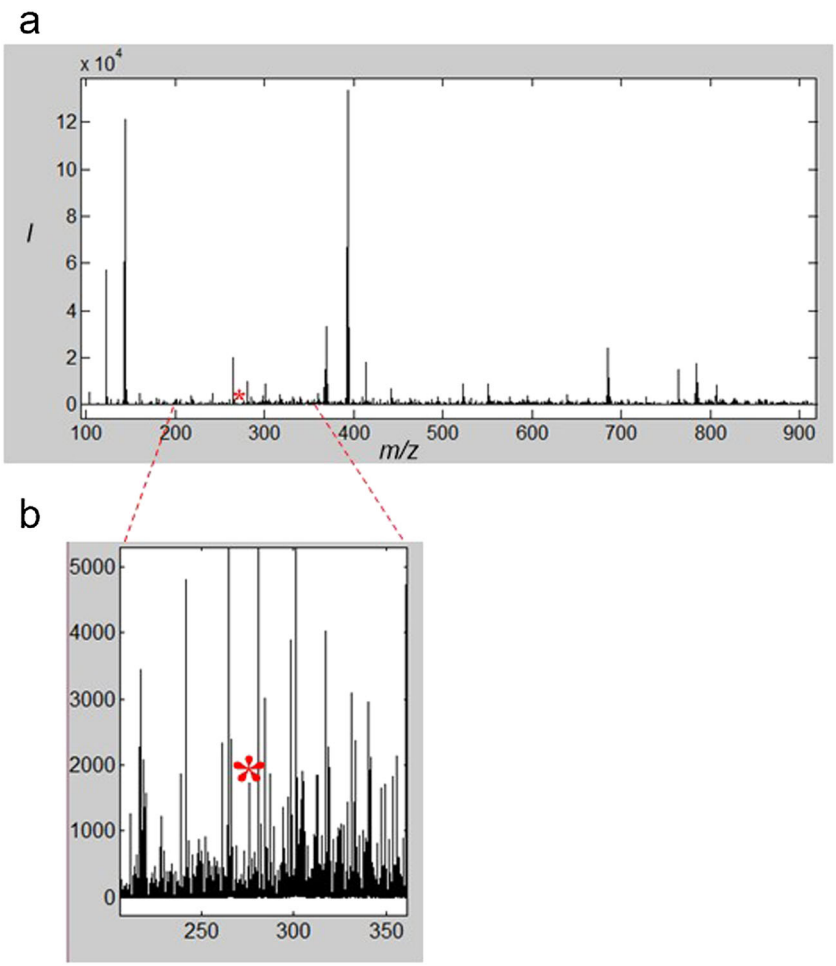

Figure 6. (a) Mass spectrum of the sample obtained from the halo generated by physostigmine in a 0.1% physostigmine spiked *Brassica rapa* methanol extract. (b) Enlargement of the *m/z* and intensity axes of the mass spectrum. The signal with *m/z* = 276.1710 corresponding to the $[M+H]^+$ ion of physostigmine is marked with an asterisk.

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Three TLC sheets were spotted with methanol extract of *Ilex paraguariensis* and developed in the solvent systems dichloromethane:methanol (90:10), ethyl acetate:methanol:water (100:13.50:10) or toluene:cloroform:ethanol (28.5:57:14.5). The TLC-AChE autography-HRMS procedure was applied observing one inhibition zone which was linked to two signals in the mass spectra with 195.0878 *m/z* and 217.0700 *m/z*, respectively. These two signals could correspond to $[M+H]^+$ and $[M+Na]^+$ ions for the molecular formula $C_8H_{10}N_4O_2$ (error = −0.1 ppm and −0.4 ppm, respectively) from caffeine, an alkaloid constituent of the plant. It has been reported that caffeine is present in 1% to 2% of dry weight of *Ilex paraguariensis* (Heck and De Mejia, 2007), and that it is an AChE inhibitor with a K_i value of 175 ± 9 μmol/L (Pohanka and Dobes, 2013).

The link between the presence of caffeine and the inhibition produced by the *Ilex paraguariensis* methanol extract was corroborated by TLC-autography with pure caffeine: one inhibition zone with the same RF that the inhibition zone observed in the extract was observed in the caffeine lane (Figure 7).

The proposed methodology BIOMSID was useful for the rapid detection of physostigmine as the AChE inhibitor in a crude extract of *Brassica rapa* spiked with 0.1% w/w of this known inhibitor. Additionally the methodology allowed the rapid detection of caffeine as the AChE inhibitor in an *Ilex paraguariensis* extract. Development of a simple assay and strategic calculations facilitate the determination of molecular formulae of the compounds that are responsible for the bioactivity observed in a complex mixture, even when they are minor constituents and/or cannot be visualised under UV light or with chemical reagents normally used in TLC analysis.

In addition, the developed assay for AChE in agar gel, showed some interesting features such as good sensitivity, uniform background coloration and good contrast of inhibition spots.

Although experiments were carried out only for one bioassay, the same strategy could in principle be applied to TLC-autographic assays with other gel entrapped enzymes such as xanthine oxidase (Ramallo *et al*., 2006), β-glucosidase (Salazar and Furlan, 2007) or Tyrosinase (García and Furlan, 2015). It could also be applied to agar overlay bioautographic assays to detect antifungal compounds (Saxena *et al*., 1995) or inhibitors of the *Salmonella* PhoP-PhoQ Regulatory System (Salazar *et al*., 2014).

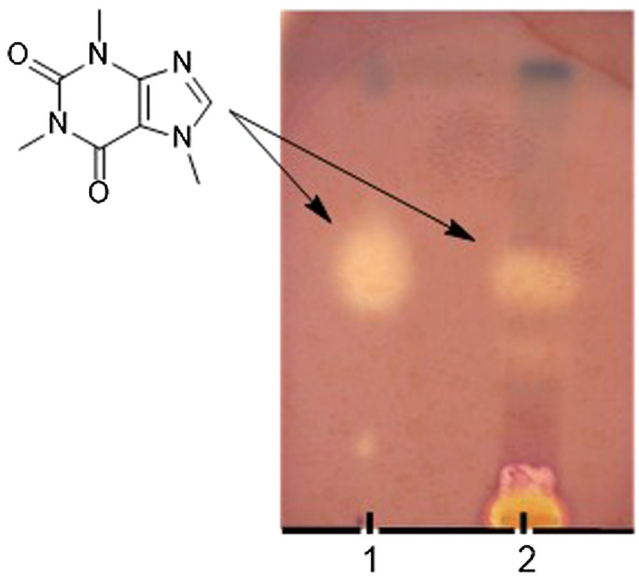

Figure 7. Autographic assay showing inhibitory AChE activity in *Ilex paraguariensis*. Lane 1: caffeine, 10 μg applied. Lane 2: *I. paraguariensis* methanol extract, 600 μg applied. Phase mobile: dichloromethane: methanol (90:10).

Apart from plant extracts, BIOMSID could be useful for the identification of bioactive compounds present in other complex mixtures such as chemically engineered extracts (Lopez *et al*., 2007; Ramallo *et al*., 2011) or, with further development, for the effect-directed analysis (Buchinger *et al*., 2013; Morlock and Klingelhöfer, 2014) of different complex matrixes.

BIOMSID, is the first example of coupling TLC-autography-HRMS in which the link between bioactivity and molecular formula is established by direct extraction and HRMS analysis of the components present in the bioactivity zone. It can be a complement of a bioguided fractionation that provides information about the molecular formula of the active compound at initial steps. Such information can be useful: (a) for the early dereplication of known compounds, (b) as a MS tag that facilitates following the active compound during fractionation, and (c) to complement NMR data for structure elucidation of elusive bioactive compounds.

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Supporting information

Supporting information can be found in the on-line version of this article.

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