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KINETIC MODEL DEVELOPMENT ON SOURCE - SEPARATED ORGANIC WASTE FOR ETHANOL PRODUCTION BY S. CEREVISIAE STRAIN

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Abstract: Ethanol production from organic fraction of municipal solid waste with inclusion of construction/demolition waste can be an effective waste management strategy to overcome the growing problems with landfill space and dependency on conventional fuels. The main challenge in ethanol conversion is the high cost of processing in which pre-treatment, enzymatic hydrolysis and fermentation are the major steps. This study investigates impact of several key parameters, namely: pH, temperature, adsorption capacity, cellulose hydrolysis rate, cell mass, enzyme and substrate loading doses on ethanol yield. The pre-treatment incorporates pre-processing and enzymatic hydrolysis steps through the use of a thermal screw press (TSP) and cellulose-organic-solvent based lignocellulosic fractionation (COSLIF) on the source-separated organic (SSO) waste to liberate fermentable sugars. Enzymatic hydrolysis experiments were featured with the addition of a commercially available enzyme complex, Accellerase 1500, to mediate the process and increase sugar yields. A kinetic model that uses a semi-mechanistic rate equation for cellulose hydrolysis was adapted and modified to accommodate batch simultaneous saccharification and co-fermentation (SSCF) process on pre-treated SSO waste by yeast, Saccharomyces cerevisiae DA2416. New experimentally defined SSO parameters have been fitted into a kinetic model to evaluate the sugar and ethanol yields. It was found that the model was capable of predicting ethanol productions with diminutive variance from experiments with substrate concentrations between 10 g/L and 50 g/L. Model predictions from experimental data deviated significantly with substrate loading rate from 60 g/L and higher. Fermentation results demonstrated that S. cerevisiae DA2416 produced ethanol in the range of 35 - 50 g/L, with ethanol yield of 0.48 - 0.50 g of ethanol/g sugar, in 5 days with 96% cellulose conversion. This study provides important insights for investigation on the use of SSO waste for ethanol production by S. cerevisiae DA2416. Furthermore, the model was proven to be a useful tool to facilitate future process optimization for up-scale bioreactors.

Keywords: Biomass, Organic Waste, Ethanol, Kinetic Modeling

Introduction

For many years, the main source of fuel for human society has come from fossil resources, which are not infinitive. Lignocellulosic biomass, on the other hand is a promising alternative to fossil fuels and it is the only foreseeable sustainable source of organic fuels and materials available to humanity (Shao, 2007). Lignocellulosic biomass such as the organic portion of solid waste is particularly attractive because of low cost and considerable availability. As estimated in (Ragauskas et al., 2006 and Zhang et al., 2006) it has a yearly supply of approximately 200 billion metric tons worldwide. However, the current cost of conversion creates a hold-up for commercial applications (Houghton et al., 2006). Among the strategies to reduce the processing costs are pre-treatment and usage of all fermentable sugars present in biomass with technologies available in today's market. Four approaches for cellulosic biomass processing featuring enzymatic hydrolysis have been reported: separate hydrolysis and

fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidating bioprocessing (CBP) (Shao, 2007). There are four biologically mediated events in each approach: cellulase production, cellulose hydrolysis, pentose and hexose fermentations (Department of Energy, USA, 1998, Lynd, et al., 2002).

SHF and SSF approaches are featured in many experimental designs for immediate implementation, while SSCF and CBP reqiure more research from deep-rooted process development (Shao, 2007). It has been reported (Department of Energy, USA, 1998) that a major disadvantage of SHF was inhibition of cellulose hydrolysis by glucose. They were unable to obtain glucose concentrations higher than 5.5% using SHF. The SSF approach, featuring enzymatic hydrolysis and fermentation of hexose in one integrated step, considerably increases inhibition to cellulase by cellulose hydrolysis products (Zhang et al., 2009). The SSCF process is similar to SSF except that hexose and pentose fermentations occur in one step. Unlike SHF and SSF, the SSCF process offers potential for more streamlined processing and a lower capital cost (Lynd et al., 2002; Xiao et al., 2004).

The SSCF approach was chosen in this work. SSCF has become more attractive with the emergence of new microorganisms that produce ethanol at a high yield from both glucose and xylose and reduce inhibition hydrolysis by xylose (Kim and Lee, 2005).

The CBP approach has a similar prospective but requires a higher temperatute for the enzmatic hydrolysis reaction than SSCF. Moreover, CBP uses native Clostridium thermocellum which produces ethanol with significant amounts of acetic acid and cannot utilize xylose (Wyman, 1999).

Normally the process for ethanol production from lignocellulosic biomass is initiated with physiochemical pre-treatment to increase the exposure of substrate to enzymatic hydrolysis followed by the biological conversion of resulting sugars to ethanol by a chosen fermenting strain. A recombinant strain of S. cerevisiae DA2416, which is capable of fermenting both glucose and xylose to produce ethanol at high yield, was used in this work. The S. cerevisiae DA2416 strain has by-passed problems with glucose repression, by taking advantage of xylose utilization pathways. As a result, key fermentation parameters (ethanol yield and inhibition) can be improved further for greater results in the fermentation phase on SSO waste.

Lignocellulosic biomass, such as pre-processed SSO waste, was used in this work as a feedstock for all enzymatic hydrolysis and fermentation processes. Due to its compositional analysis and relatively easy pre-treatment, SSO was chosen as a potential substrate for future industrial applications.

An existing kinetic model was adopted from (Zhang, 2008) and modified in this work to predict batch SSCF on SSO waste by glucose and xylose utilizing strain S. cerevisiae DA2416. This model accounts for cellulose and hemicellulose enzymatic hydrolysis and competitive uptake of glucose and xylose. There are only a few published studies on the conversion of cellulose and hemicellulose via SSCF (McMillan et al., 1999; Teixeira et al., 2000; Kim and Lee, 2005) and only few kinetic models have been proposed in the literature (Shao, 2007, Zhang, 2008). A kinetic model development used in this work was based on a semi-mechanic rate equation for cellulose hydrolysis as initially proposed by (South et al., 1995) and further modified by (Shao, 2007) and (Zhang, 2008) to accommodate cellulose and hemicellulose hydrolysis. The parameters presented in (Shao, 2007) and (Zhang, 2008) were based on data for paper sludge only. Therefore, new values of interest such as adsorption capacity, enzymatic hydrolysis constant, ethanol inhibition and ethanol yield were recalculated to accommodate batch mode SSCF, particularly on SSO waste.

The overall goal of this work is to better comprehend the process of converting SSO waste via SSCF approach to produce ethanol. Specific objectives include: 1) revise batch SSCF, specifically for the SSO waste by means of adaptation and further amendment of an existing kinetic model; 2) examine the impact of major variables involved in the performance of SSCF on SSO waste (temperature, pH, adsorption capacity, enzymatic hydrolysis rate

constant, ethanol inhibition and ethanol yield); 3) experimentally obtain new parameters for a tailored kinetic model to predict batch SSCF on SSO waste and compare results of model prediction and experiments.

Materials and Methods

The S. cerevisiae DA2416 recombinant strain used in this study was kindly provided by Dr. Yong-Su Jin from the Department of Food Science and Human Nutrition, University of Illinois, USA. It was kept at -80oC in 30% (v/v) glycerol for storage. The enzyme complex Accellerase 1500 used in the hydrolysis experiments was a gift of Sigma Aldrich Corp., USA.

The SSO waste utilized in this work was initially pre-processed mechanically, under high temperature (120oC) and pressure (over 50 bars) with a thermal screw press to form a dry stable mass. SSO samples were prepared as a heterogeneous substrate by blending demolished construction waste, approximately 20% in form of woodchips and organic green bin waste as in (Bekmuradov et al., 2014a). Optimum Waste Recycling Systems, Toronto, Canada, supplied the biomass feedstock used in this work (Optimum waste and Recycling System, 2010). Prior to testing, the SSO waste was oven dried at 45oC-50oC for 48 hours.

The COSLIF method, which uses cellulose solvent (phosphoric acid) and organic-solvent (ethanol), was applied to the SSO waste followed by enzymatic hydrolysis and fermentation processes (Zhang et al., 2007; Sathitsuksanoh et al., 2009; Rollin et al., 2011). Five grams of dry lignocellulose was placed in a 250mL centrifuge bottle and then mixed with 40mL of 85% concentrated phosphoric acid using a glass rod. The solid/liquid slurry was placed in a bench-top shaking incubator at 150rpm and 50oC ± 0.2oC for 2 hours. One hundred mL of ethanol was then added and mixed well. After centrifugation at 7000rpm at room temperature for 15 minutes, the supernatant was decanted. The solid pellet was then re-suspended with 200mL of ethanol and centrifuged. The supernatant again was decanted. Next, the solid pellet was re-suspended with 200mL of distilled water and centrifuged two times and stored in a freezer for a short period of time.

Enzymatic hydrolysis experiments were carried out with the addition of commercially available enzyme, Accellerase 1500. After thawing, the treated solid pellet containing amorphous cellulose was neutralized to pH 4.8-5.0 by NH4OH. The SSO samples were then brought to 50oC before adding 30 FPU/ g glucan of Accellerase 1500. Both the pH value and temperature described were the optimum conditions for the Accellerase 1500 enzyme to mediate hydrolysis and release as many fermentable sugars as possible (Dowe and McMillan, 2008). The hydrolysis experiment was conducted in the shaking incubator (MAXQ4450). The incubator was set at 250rpm to keep solids in constant suspension with the temperature of 50°C for 72 hours. Samples were taken and measured for sugar content at specified times: 0, 12, 24, 48 and 72 hours. The relevant composition of the SSO was the same as reported in (Bekmuradov et al., 2014b).

Protein content of the SSO substrate was measured by Lowry modified method (Thermo Fisher Scientific Inc., 2011). Adsorption of cellulases onto SSO substrate was done by mixing them in an incubator shaker at 100rpm in an Innova-40 shaker, at a temperature of 25oC, in 10mL glass tubes, under controlled pH and concentration of cellulases. Centrifugation of the whole reaction tube followed an incubation period. Then unbound cellulases present in the supernatant were decanted off. The amount of cellulase adsorbed onto a solid substrate was determined as the difference between the total amount of cellulase initially applied [Einit] and the amount of free cellulase in the solution [Enon ads]. The amount of free cellulases in the solution was measured by “in situ” and rapid UV spectrophotometer technique (Liu et al., 2011; Wang et al., 2012). The technique determines free cellulase concentration in the solid SSO substrate suspension from the second derivative of the absorption spectra at 750 nm with respect to wavelength through calibration. Each data point in the plots was an average of 5 replicates.

The carbohydrate content of SSO was determined via quantitative saccharification (QS) method based on 2 hours incubation in 72wt% H2SO4 at 30oC (Ruiz and Ehrman, 1996; McMillan et al., 1999; Moxley and Zhang, 2007). The cell mass was determined by counting colony forming units on agar plates as described in (Zhang et al., 2009).

Following enzymatic hydrolysis, batch soluble sugar fermentation was carried out to evaluate ethanol yields from SSO samples as a result of conversion using recombinant strain - S. cerevisiae DA2416. Soluble sugar batch fermentation was performed in 250mL serum bottles with 100mL working volume. Temperature was maintained at 30oC and pH was controlled at 6.0 by 1M potassium hydroxide (KOH) as suggested by a previous study (Mohageghi et al., 2004). Compositional analysis of the samples for ethanol concentrations was carried out at 0, 12, 24 and 48 hours by high performance liquid chromatography (HPLC).

The kinetic model adapted in this study uses a semi-mechanistic rate equation for cellulose hydrolysis as proposed by (South et al., 1995) and further modified as in (Zhang et al., 2009). The parameters presented in Zhang's kinetic model were based on cellulose and hemicellulose hydrolysis for pre-treated paper sludge. In this study adsorption parameters were recalculated based on overall carbohydrate content of pre-treated SSO waste. The binding capacity or specific capacity of the carbohydrate component for cellulase of SSO samples was obtained using Langmuir isotherms. The cellulose hydrolysis rate constant was in the range of 0.662 to 0.725. The remaining cellulose hydrolysis parameters were as reported by (Zhang, 2008). Experimental data on glucose and xylose consumptions and growth parameters were fitted using the non-linear function of Polymath 5.1 (Polymath Software). All other parameters were dynamically fitted with the curve fitting function in the Berkeley Madonna computer program with a fourth-order Runge-Kutta algorithm. Runs were performed on a standard laptop.

A sensitivity analysis was carried out based on the least square method (Stigler, 1986; Bretscher, 1995). It was performed to test the impact of the value of several important constants on the model prediction of ethanol production if the constant had changed to a value ±10% from its experimentally measured value. The analysis was performed to determine the difference between experimental data and modified kinetic model predictive ability.

Results and Discussion

Due to its prospect for commercial application, SSO waste was chosen as the substrate to evaluate the values on sugar and ethanol yields by fermentation using S. cerevisiae DA2416 strain.

The SSO composition was previously analyzed (Mirzajani, 2009; Ehsanipour, 2010; Bekmuradov et al., 2014a) and is shown in Table 1:

Table 1 Composition of the SSO on dry weight.

	Percentage
Glucose	31%
Xylose	19%
Other sugars	7%
Extractives	16%
Lignin	27%
Total	100%

Approximately, more than half of the original sample was composed of moisture. Essential polymeric sugars in the oven dried SSO samples included: 31% glucose, 19% xylose, 7% of other sugars, 16% extractives and 27% of lignin (Mirzajani, 2009). These homogeneous samples had a pH range of 5.2-5.5 and consisted of around 80% food waste and approximately 20% wood chips (Douglas fir type). The SSO samples pre-treated by concentrated

phosphoric acid (85% w/w) and ethanol (95% v/v) were hydrolyzed and glucan digestibility was found to be 72% after 24 hours and 90% after 72 hours. The high glucan digestibility was achieved for the COSLIF-pre-treated SSO with addition of 30 FPU/ g glucan of Accellerase 1500 (Bekmuradov et al., 2014b).

Recognizing that cellulase mixtures contain a mixture of cellulase and hemicellulase portions which bind to cellulose and hemicellulose, adsorption capacity constant "σ" was recalculated using the modified method of Lowry and resultantly were in the range of 0.264 to 0.280. Similarly, the binding capacity CS was determined as between 0.442 g protein/g carbohydrate and 0.466 g protein/g carbohydrate using Langmuir isotherms. And cellulose hydrolysis rate constant – k (1/h) had a range values of 0.662 and 0.725. The remaining cellulose enzymatic hydrolysis parameters were adapted (Zhang, 2008). Adsorption of Accellerase 1500 cellulase to the SSO waste samples was evaluated after hydrolysis was allowed to proceed for specified time, (6, 12, 24, 36, 48, 60 and 72 hours) resulting in various values for fractional conversion up to 85% as shown in Figure 1.

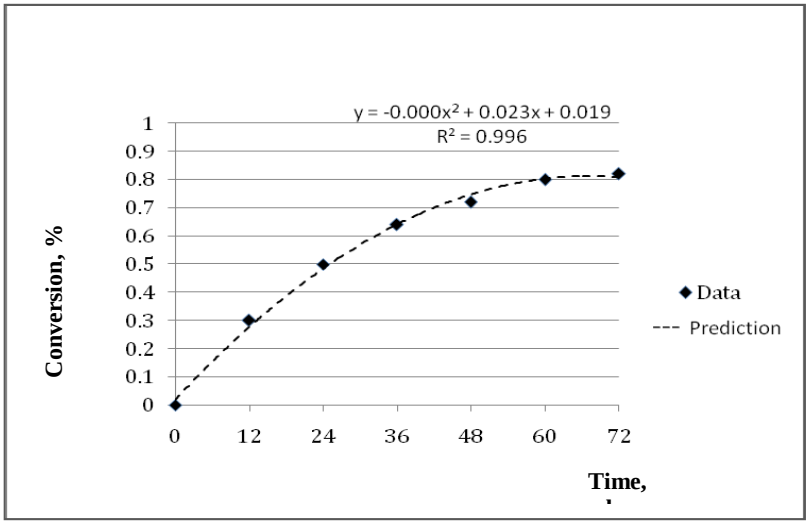

Figure 1 Conversion data for pre-treated SSO samples using Berkeley Madonna software

The predictions were confirmed by experimental data obtained during the addition of Accellerase 1500 enzyme complex to SSO samples throughout the course of hydrolysis. The predictions over the time of reaction are almost identical. The good fit obtained in this study suggests that for the SSO samples it seems reasonable to assume a constant adsorption capacity normalized to the amount of cellulose remaining and there is no reason to hypothesize adsorption affinity as a function of a conversion.

Adsorption parameters KS and σS (Table 2) were then fit to the data of all conversions by minimizing the sum of squares for the predicted and observed data. The new adsorption parameters with conversion data were used to fit the parameters k, c and e in the cellulose rate equation of (South et al., 1995). The values of parameters are presentedin Table 2:

Table 2 Parameter values for SSCF of the SSO

K_S	0,442 - 0,466	This work
σ_S	0,264 - 0,280	This work
K	0,662 - 0,725	This work
E	0,510 - 0,516	This work

C	0	Shao, 2007

With parameter values in hand for adsorption, hydrolysis and fermentation, a pre-existing kinetic model for SSCF was chosen and modified to account a newly defined SSO feedstock constant. In view of this, we selected a simple correlation model reported in the following equation (1) from (Zhang, 2008):

$$r_{Xn} = \frac{XI}{GI} \times r_{Gn} \qquad \text{Equation (1)}$$

where: r_{Gn} and r_{Xn} - formation of glucan and xylan respectively;
GI and *XI* – initial glucan and xylan concentrations.

Equation (1) above describes the correlation of glucan and xylan hydrolysis and is derived from the simple relationship of X1=X2 in which X1 and X2 are the conversion of glucan and xylan respectively. To understand the enzyme hydrolysis performance with the S. cerevisiae DA2416 strain, the percentage of glucan and xylan conversion was calculated. The average glucan conversion to monomer sugars was 96%, and the average xylan conversion to monomer sugars was 94% at 30oC. However, we observed a slightly higher residual xylose accumulation than residual accumulation of glucose during experimental tests. The slower consumption rate of xylose than glucose was consistent with the fermentation of soluble sugars in other studies as well utilizing S. cerevisiae (Kuyper *et al.*, 2005; Zhang and Lynd, 2010, Thermo Fisher Scientific Inc., 2011).

Before SSCF runs, Accellerase 1500 enzyme were added and mixed with yeast inoculum into a vessel at optimum value of 30FPU to increase the sugar's accessibility to cells during the mass transfer limited period. As a result, high glucan digestibility (>90%) was achieved. Batch soluble sugar fermentation experiments were carried out to find the fermentation related constants, exclusively ethanol inhibition and yield in SSCF kinetic model by performance of recombinant strain - S. cerevisiae DA2416. In S. cerevisiae strains, there are a large number of genes encoding hexose transporters (Reifenberger *et al*., 1997), which are also believed to be function with low affinity xylose transporters in recombinant xylose utilizing S. cerevisiae (Sedlak and Ho, 2004). Based on this examination, a competitive substrate inhibition model for growth in glucose and xylose by S. cerevisiae DA2416 was chosen to capture the growth kinetics. Inhibition of growth and fermentation has been described using different equations in the literature, including exponential inhibition, linear inhibition, and linear inhibition beyond threshold (van Uden, 1989). Among them, a threshold linear inhibition model was chosen because it fit best with the data. A threshold linear inhibition model equation (2) as described elsewhere (South *et al*., 1995) accounts for glucose fermentation with an additional term representing sugar uptake from xylose and inhibition from ethanol:

$$\mu_{Gl} = \left[\frac{X \times \mu_{Gl}^{Max} \times G_l}{(K_{Gl} + Gl + I_1 \times X_l)}\right] \times \left(1 - \frac{Et\square}{Et\square_{Gl}^{Max}}\right) f^1 \qquad \text{Equation (2)}$$

where: μ_{Gl}^{Max} and $Et\square_{Gl}^{Max}$ - maximum specific growth rate and

maximum ethanol concentration for growth on glucose respectively;

G_l, X_l , $Et\square$ - concentration of glucose, xylose and ethanol respectively;

K_{Gl} , I_1 , f^1 - related constants.

The rate of formation of xylose was described by an approach similar to that used for glucose formation shown in equation (3) from (South *et al*., 1995):

$$\mu_{Xl} = \left[\frac{X \times \mu_{Xl}^{Max} \times (Xl - XlT)}{K_{Xl} \times X + Xl + I_2 \times Gl}\right] \times (1 - \frac{Et\square}{Et\square_{Xl}^{Max}}) \qquad \text{Equation (3)}$$

where: μ_{Xl}^{Max} and $Et\square_{Xl}^{Max}$ - maximum specific growth rate and maximum ethanol concentration for growth on xylose respectively;

K_{Xl} , I_2 - related constants;

XlT - threshold concentration.

Values for inhibition factors I1 and I2 in this work were found to be 0.108 and 6.032 respectively, indicating that the inhibition of xylose utilization by glucose is more than 50 times stronger than the inhibition of glucose utilization by xylose. In order to test the SSCF performance on SSO feedstock with newly redefined constants, batch fermentations were carried out in a separate series of experimental evaluation at different initial substrate concentrations of 10g/L, 50g/L, 60 g/L and 100g/L with the enzyme loading of 30FPU cellulase. The model accurately predicts the sugar and ethanol concentration along with cell mass concentration for substrate concentration ranging from 10g/L and 50g/L (Figures 2-3), but not for the 60 g/L and higher.

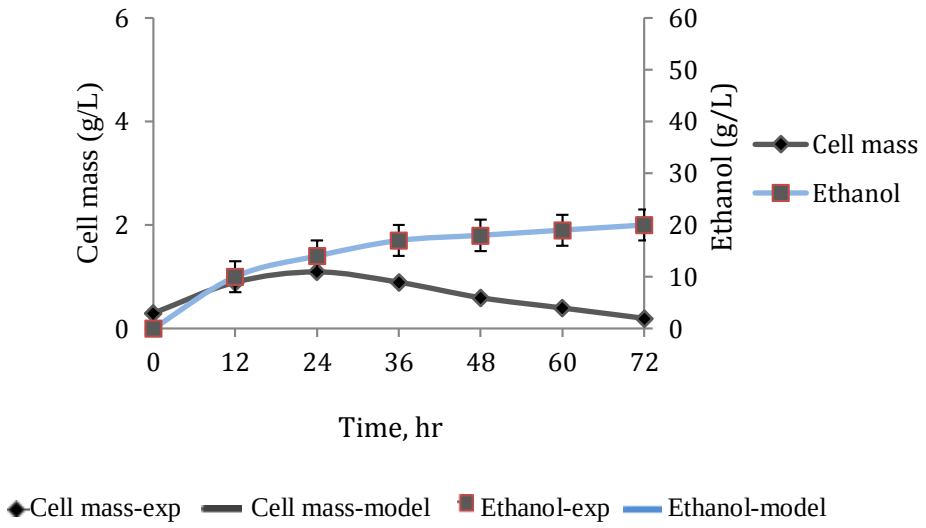

Figure 2 Experimental data and kinetic model prediction for SSO samples (substrate concentration 10g/L)

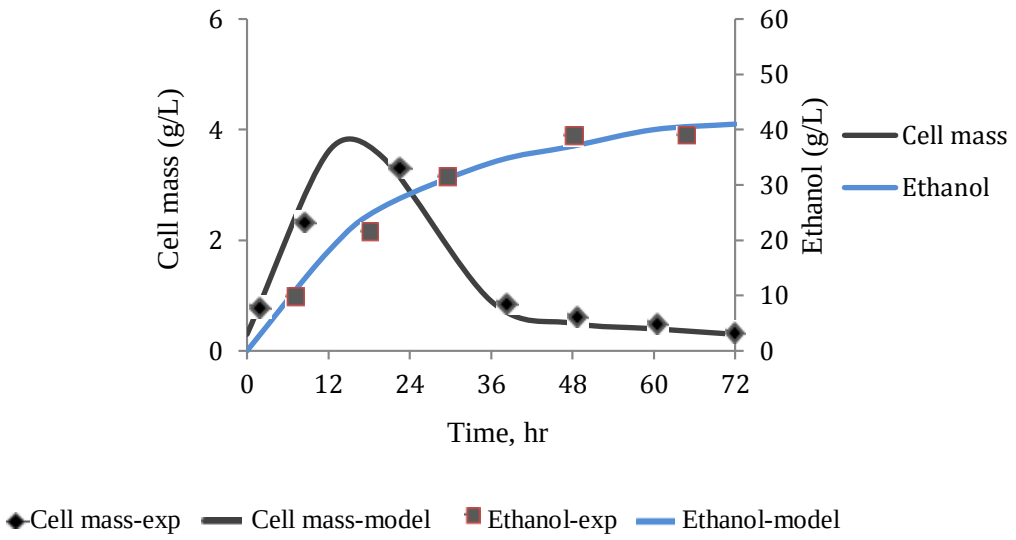

Figure 3 Experimental data and kinetic model prediction for SSO samples (substrate concentration 50g/L)

In higher substrate concentration mode at 60g/L, reaction was deviated from experimental values as shown in Figure 4 suggesting that ethanol inhibition is not a factor causing this discrepancy.

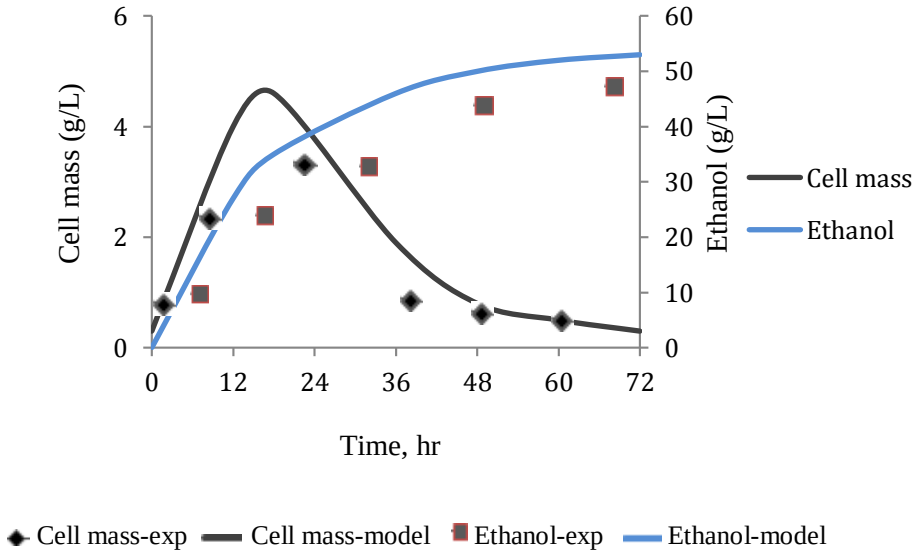

Figure 4 Experimental data and kinetic model prediction for SSO samples (substrate concentration at 60g/L).

We believe that there are other reasons attributed to it for example, enzyme adsorption by lignin and/or obstruction of lignin on the surface of cellulose to the point that enzymes are not able to access cellulose (Collins, 2007).

Another set of experiments was conducted to assess the contribution of ethanol inhibition to the loss of cell viability. We expected good cell viability at lower SSO sample concentrations if inhibition was a major factor for cells lost. It was noticed in experiments that ethanol concentration was a major factor of declining cell viability, but at the same time, one or more factors other than ethanol inhibition that are not yet determined may contribute to the loss viability in SSCF using S. cerevisiae DA2416.

According to the sensitivity analysis conducted in this study a few parameters had the highest response values and exhibited the greatest influence on ethanol yields. Among them is a cellulose adsorption constant, enzymatic hydrolysis rate; ethanol yields from glucose and xylose; and ethanol tolerance. Table 3 presents the results of sensitivity analysis.

Table 3 Sensitivity analysis of kinetic parameters for SSO used in this work

10% increase		10% decrease	
Constants	S^*	Constants	S^*
Enzymatic hydrolysis constants			
K	1.26	K	0.32
K_S	0.44	K_S	0.34
K_{SP}	0.35	K_{SP}	0.24
M	0.21	M	0.32
K_C	0.03	K_C	0.03
Microbial growth related constants			
$Y_{Eth/Gl}$	5.42	$Y_{Eth/Gl}$	0.46
$Y_{Eth/Xl}$	0.53	$Y_{Eth/Xl}$	0.29
f_1	0.32	f_1	0.3
I_1	0.022	I_1	0.022
I_2	0.021	I_2	0.021
$Et\square_{Gl}^{Max}$	0.36	$Et\square_{Gl}^{Max}$	0.32
$Et\square_{Xl}^{Max}$	0.24	$Et\square_{Xl}^{Max}$	0.48

The sum of variance between the measured ethanol and the ethanol predicted by the model were calculated. Single parameters were varied to ±10% of the experimental values and responses were calculated as absolute values based on the least square method (Bretscher, 1995). As seen from Table 3, cellulase adsorption constant CS had the highest response value followed by the cellulose enzymatic hydrolysis constant k among enzymatic hydrolysis constants. Evidently, a 10% decrease of CS is 5 times more sensitive than a 10% increase, demonstrating that increase in enzyme loading will be more effective than increase in substrate concentration for ethanol yield.

Among microbial growth related constants, the ethanol yield from glucose and xylose exhibited the highest sensitivity, with ethanol tolerant related constants showing moderate sensitivity.

In summary, a comparison of performance of different S. cerevisiae strain parameters is presented in Table 4 below:

Table 4 Comparison performance of different S. cerevisiae strains

	DA2416	RWB222*	D5A*
g ethanol/g sugar consumed	0.50	0.42	0.44
Final glucan conversion	0.96	0.93	0.94
final xylan conversion	0.94	0.95	0.93
ethanol yield, %	114	105	100

DA2416 – this work
RWB222* and D5A* - data from (Zhang and Lynd, 2010)

Experimental results demonstrated that S. cerevisiae DA2416 produced about 50g/L ethanol with an ethanol yield of 0.50g of ethanol/g potential sugar fed for SSO in less than 5 days with 96% cellulose conversion. All strains exhibited almost the same value of glucan and xylan conversion. The total ethanol yield on sugar consumed was higher for DA2416 strains. As a result, we came to the conclusion that S. cerevisiae DA2416 was more tolerant to inhibitors than other two strains. Different substrates should be tested to validate the DA2416 strain in the future.

Conclusions

The SSO waste samples utilized in this research were pre-processed by the TSP and further used as substrates for all enzymatic hydrolysis and fermentation processes.

COSLIF pre-treatments were applied for cellulose extraction from processed SSO waste. Results indicated that a kinetic model with integrated values of experimentally defined SSO feedstock constants were able to predict ethanol yield accurately with diminutive variance from experiments. We examined that the cellulose adsorption constant, ethanol tolerance and ethanol yield played very important roles in the fermentation process. We also learned that a good fermenting strain should have the ability to withstand ethanol toxicity and common inhibitors. The discrepancy between experiments and model predictions, particularly at high substrate concentrations needs to be examined more comprehensively.

This study demonstrated and affirmed that S. cerevisiae DA2416 is a promising strain for SSO substrate in SSCF. In the future, the kinetic model used should be expanded to introduce the inference of lignin in lignocellulosic biomass.

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DETERMINATION OF LUNG SOUND AS NORMAL OR ABNORMAL, USING A STATISTICAL TECHNIQUE

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Abstract: In this study the authors investigate a possibility of objectively differentiating a lung sound as normal or abnormal using a statistical technique. For the study, breath sounds were recorded from 30 nonsmoking, healthy subjects and 7 subjects with respiratory disorders whose external physical symptoms were not shown, using an electronic stethoscope. A 4th order Butterworth bandpass filter removed environment sounds and an Adaptive filter using Least Mean Square algorithm cancelled other body sounds from the recorded sound to obtain only the lung sound. After amplifying a lung sound signal up to the initial recorded amplitude, signal was compared with a standard normal and a standard abnormal lung sound. The comparison was done by calculating the Mahalanobis Distance mean values. The Mahalanobis distance mean values obtained from subjects with respiratory disorders showed considerable deviations from the specific range of values obtained by subjects with normal lung sounds concluding this method is capable of distinguishing between normal and abnormal lung sounds and could developed to noninvasively determine the progress of patients with respiratory disorders.

Keywords: Adaptive Noise Cancellation; Least Mean Square Algorithm; Lung Sound Analysis; Mahanaobis Distance.

Introduction

Normal lung sounds are generated either by rapid fluctuations of gas pressure or oscillations of solid tissues while abnormal lung sounds occur due to deformation or obstruction of respiratory track. Normal lung sounds can be categorized to tracheal, bronchial, broncho-vesicular and vesicular (Kandaswamy *et al*, 2004). They are concentrated in the frequency range of 50Hz- 250Hz (Sovijarvi *et al*, 2000, Vannuccini *et al*, 2000). Abnormal lung sounds can also be categorized to continuous and discontinuous. Continuous abnormal lung sounds can be further classified as wheeze, rhonchus and stridor while discontinuous abnormal lung sound as crackles (Bouzakine *et al*, 2005). Abnormal lung sounds are concentrated in the range of 150Hz-2500Hz (Sovijarvi *et al*, 2000). The normal and abnormal lung sounds are categorized based on pitch, intensity, location and inspiratory to expiratory duration.

Normal and abnormal lung sounds are analysed to determine pulmonary disorders. Wheeze sound indicates that the person is susceptible to asthma or pneumonia and rhonchus sound indicates chronic obstructive pulmonary disorder or bronchitis while crackles indicate pneumonia, pulmonary fibrosis or congestive heart failure (Chowdhury and Majumder, 1982). Auscultation is the traditional method of determining pulmonary disorders while other noninvasive methods such as pulmonary function test, respiratory inductance plethysmograph, and phonopneumography techniques are also used. But invasive methods such as computerized tomography (CT) scan, chest X ray and bronchoscopy are

also used to diagnose the pulmonary disorders. The existing non-invasive diagnostic procedures are mostly subjective while invasive diagnostic procedures are expensive, time consuming and some are harmful. Therefore with the development of automation, many researchers are conducting researches to automatically detect pulmonary disorders non-invasively. For that as the initial stage, researchers have investigated computerized methods to classify a lung sound as normal or abnormal and have developed methods to identify some types of lung sounds such as wheezes, crackles and rhonchus automatically.

The analyses of lung sounds by computerized methods have been done using statistical and machine learning techniques. Statistical methods such as higher order crossing discrimination analysis, analysis of variance and machine learning techniques such as artificial neural network (Guler *et al*, 2005), hidden Markov model (Matsunaga *et al*, 2009), fuzzy analysis (Zolnoori *et al*, 2012), autoregressive model (Mendez *et al*, 2008) and mel frequency cepstrum coefficients (Chang *et al*, 2010) were used in lung sound analysis.

In this paper, the aim is to explore whether the normal and abnormal lung sound patterns could be compared using Mahalanobis distance statistical method and thereby to determine whether a person's lung sound is normal. This is also a validation of the use of Mahalanobis distance to analyse lung sounds.

Theoretical Background of Mahalanobis distance

The Mahalanobis distance is one of the most common measures in multivariate statistics. It can be used to determine whether a sample is an outlier or whether a sample has a similarity with another group or not (McLachlan, 1999). The mathematical definition of Mahalanobis distance is given by equation (1).

$$D(X,Y) = \sqrt{(X-Y)^T * S^{-1} * (X-Y)} \quad (1)$$

Where D is the Mahalanobis distance matrix of data points(X) of the sample matrix to the data points in parent matrix. Y is the mean of the parent matrix. S^(-1) is the inverse of covariance matrix of parent matrix and T is the transpose (Mahalanobis, 1936). In Mahalanobis distance comparison, if two similar matrixes are compared, the mean value of Mahalanobis distance is equals to 1.

Methodology

Data Acquisition

This study was performed on 30 (15 male and 15 female) nonsmoking healthy subjects who lived a minimum of 4 years in Rathmalana area in Sri Lanka with no known lung, heart or renal diseases and 7 subjects with respiratory disorders in the same area, falling to the age group 19-35 whose having a Body Mass Index (BMI) in the range of 18.5-24.9. Having taken the consent of the participants to the study, each was given a questionnaire to be filled. Then, one person at a time was taken into a room with less interference of background noise but, not sound proofed where, he/she was asked to sit on a stool and relax for 5 minutes. The breath sound at the posterior lower region of the right lung was directly recorded on to the MATLAB software by the electronic stethoscope model "Spirit CK-E600". The subject was asked to hold the breath for the first 10 seconds of the recording and then to take

deep breaths in next 50 seconds. The characteristics of 7 subjects with respiratory disorders are as shown in Table 1.

Table 1 The characteristics of 7 subjects with respiratory disorders.

Female		Male	
Index	Description	Index	Description
A	Has had wheezing condition within one year before the recording of breath sound, no external physical symptoms shown.	D	Has had wheezing condition within one year before the recording of breath sound, no external physical symptoms shown.
B	Has had wheezing condition within the one year before the recording of breath sound, no external physical symptoms shown.	E	Has had wheezing condition within one year before the recording of breath sound, no external physical symptoms shown.
C	Has had wheezing condition within one year before the recording of breath sound, no external physical symptoms shown.	F	Had detected low lung volume by a pulmonary function test within one year before the recording of breath sound.
		G	Had detected low lung volume by a pulmonary function test within two years before the recording of breath sound.

The acquired breath sounds which are seen as in Figure 1 were arranged in 16 bit Mono audio format with sampling frequency of 44100Hz and stored in .wav file format.

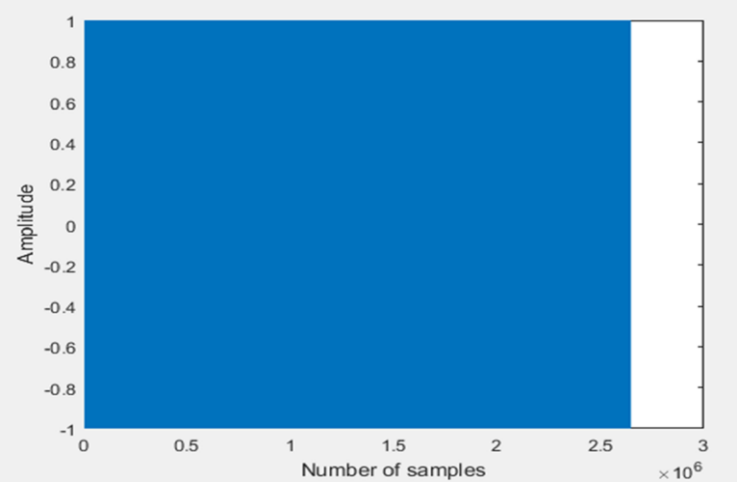

Figure 1 Plotted breath sound after capturing through electronic stethoscope on to MATLAB. The signal is not seen due to external background noise.

Background noise filtering

Since there was a high amount of noise in the recorded signal, a Butterworth bandpass 4th order filter with a cutoff frequency at 100Hz and 400 Hz was designed on MATLAB. Then, the recorded signal is filtered to remove environment noise as seen in Figure 2. Each sound file recorded from the electronic stethoscope was filtered.

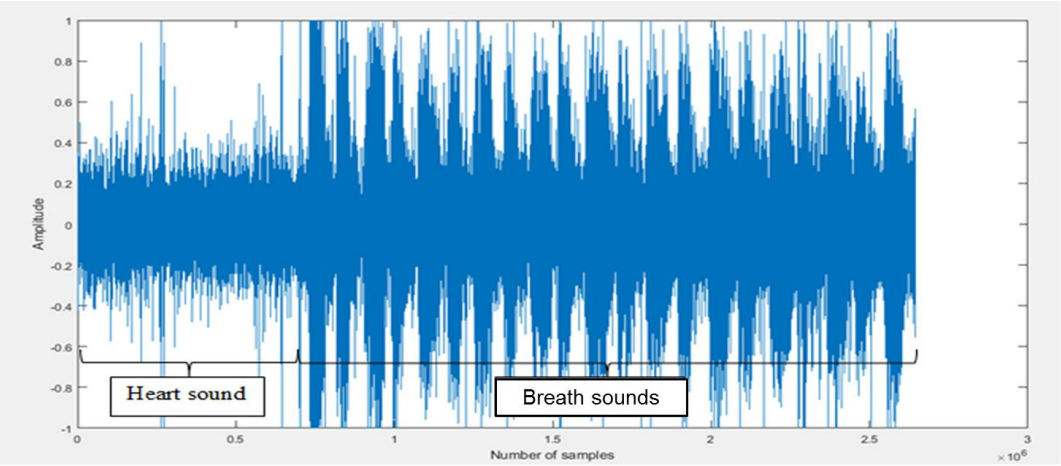

Figure 2 Environment noise filtered waveform of the recorded sound, clearly indicating the heart and breath sounds components. It is seen that the heart and other body sounds have overlapped with lung sounds in the breath sound regions.

Extraction of lung sounds

It can be seen in Figure 2 that mainly heart sounds interfere with lung sounds because the heart sound frequencies which is in the range of 20H- 150Hz overlaps with lung sound frequencies (Hadjileontiadis and Panas, 1997). Therefore a Least Mean Square Adaptive filter was used to acquire only the lung sound (Sathesh and Muniraj, 2012). First the recorded portion of the heart sound of the filtered recording where, the subject was asked to hold the breath (the heart sound) was selected manually, while listening to the audio files and observing the wave patterns of both acquired and standard heart and lung sounds. Then, it was aligned with a selected breath cycle of the above filtered recording where the subject was asked to breath heavily (the breath sound). After that, these two signals were fed into the least mean square adaptive filter designed on MATLAB with a step size of 0.0001 and filter length of 1 where, the output obtained was observed to be a cycle containing only the lung sound as seen in Figure 4. Similarly, 3 consecutive inspiration- expiration cycles of each filtered breath sound were run through the Least Mean Square adaptive filter. The Figure 3 demonstrates the operation of the Adaptive filter (Potdar *et al*, 2015).

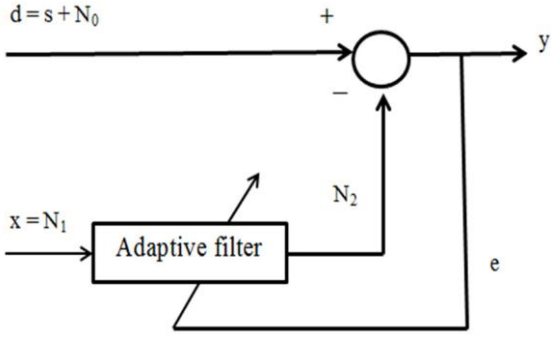

Figure 3 Diagram of Least Mean Square adaptive filter where d= breath sound, s= lung sound, N_0=heart sound and noise, x= heart sound and noise, N_2= adaptive filtered output, y=lung sound and e= error.

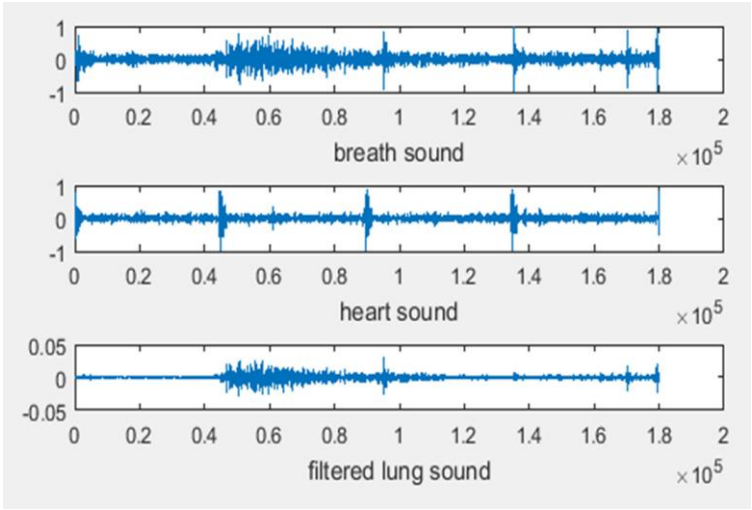

Figure 4 The external noise filtered breath sound, the heart sound and the adaptive filtered lung sound waveforms.

Next the power of the adaptive filtered lung sound is restored to the power of the filtered breath sound to eliminate the power losses of the lung sound signal due to adaptive filtering.

Comparison

The recorded normal lung sounds were compared with the standard normal lung sound using Mahalanobis distance method and obtained a mean value for each normal lung sound and plotted all the mean values in a graph. Then the normal lung sounds were compared with standard abnormal lung sound and plotted the mean values. The standard lung sounds were collected from the lung sound library in R.A.L.E.® Repository. The Figure 5 and Figure 6 shows the standard normal lung sound and standard abnormal lung sound respectively. Then a range was formed using the plotted values where 90% of the values are to be in the range. Next the recorded abnormal lung sounds were compared with standard normal lung sound and also with standard abnormal lung sound and plotted all the mean values in two separate graphs as previously. Then it was observed whether the values obtained for the abnormal lung sounds are in the formed range or whether they have deviated from the range.

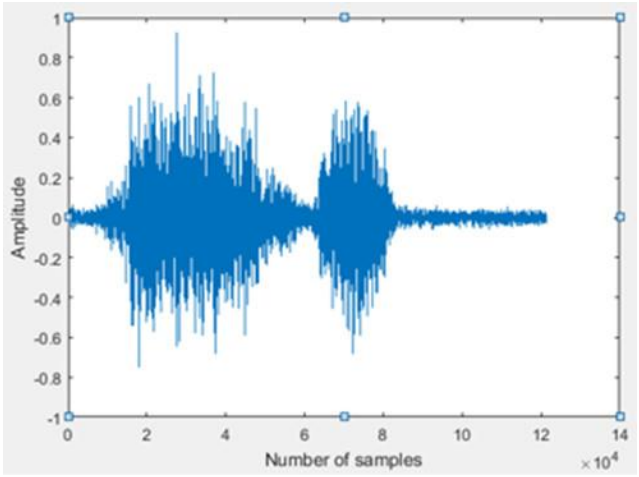

Figure 5 Standard normal lung sound which shows inspiration and expiration.

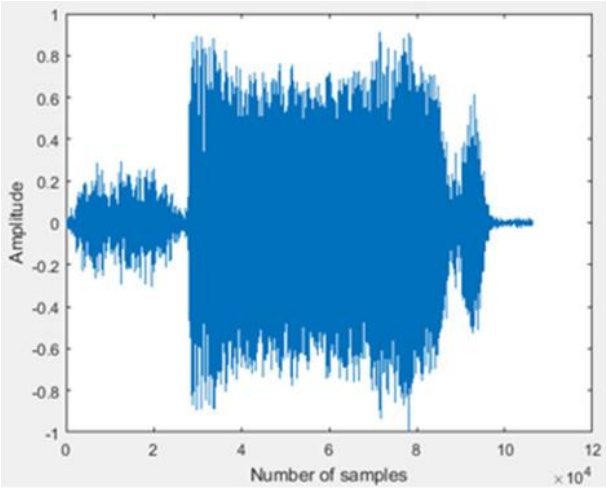

Figure 6 Standard abnormal lung sound which is a wheeze sound.

Results and Discussion

The Mahalanobis distance mean values of each normal lung sound when compared with standard normal lung sound are as shown in Figure 7 and when compared with standard abnormal lung sound are as shown in Figure 8.

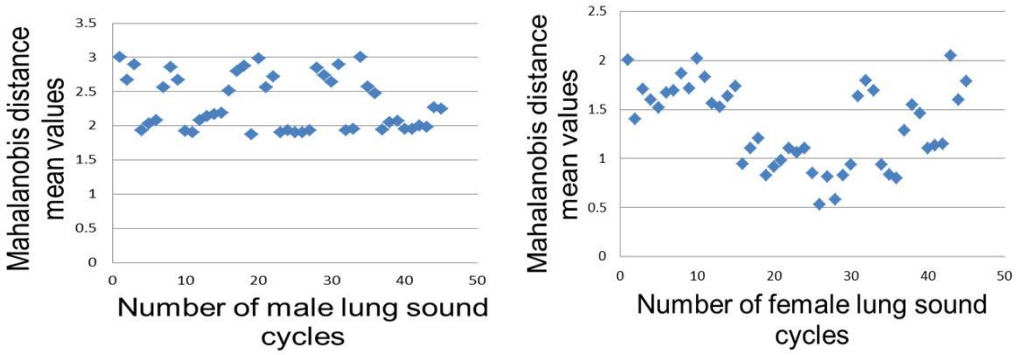

Figure 7 Mahalanobis distance mean values of each normal lung sound in healthy males and females when compared with standard normal lung sound.

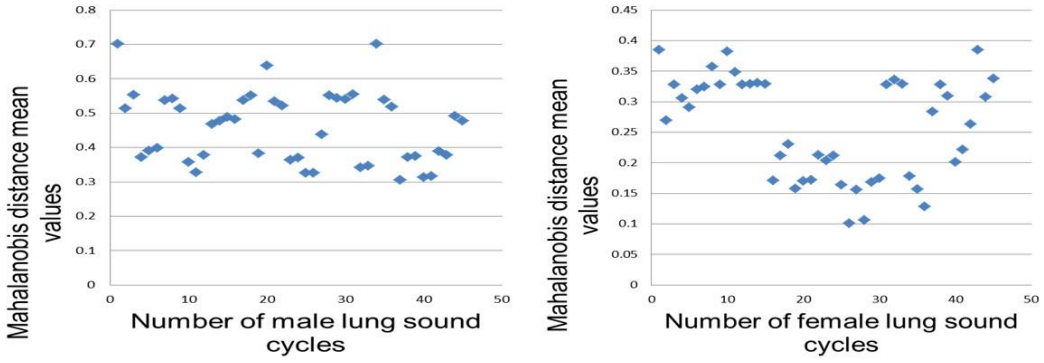

Figure 8 Mahalanobis distance mean values of each normal lung sound in healthy males and females when compared with standard abnormal lung sound.

It was observed that the values obtained for males are higher than for females and that the values are in a specific range. It was also observed that the values obtained for normal lung sounds when compared with standard normal lung sound are much closer to 1 than the values obtained for normal lung sounds when compared with standard abnormal lung sound, indicating that the normal lung sounds are much similar to standard normal lung sound.The plotted graphs were used to form ranges as shown in Table 2.

Table 2 Ranges formed using Mahalanobis distance mean values obtained for normal lung sounds.

	Male		Female	
	Standard normal lung sound	Standard abnormal lung sound	Standard normal lung sound	Standard abnormal lung sound
Upper boundary	3	0.59	1.9	0.35
Lower boundary	1.8	0.3	0.7	0.13

The Table 2 explains for an example that if a lung sound of a healthy male compared with standard normal lung sound, the Mahalanobis distance mean value should be in the range of 1.8 to 3.

The abnormal lung sounds which were compared with standard normal lung sound and standard abnormal lung sound are shown in Figure 9 and Figure 10 respectively.

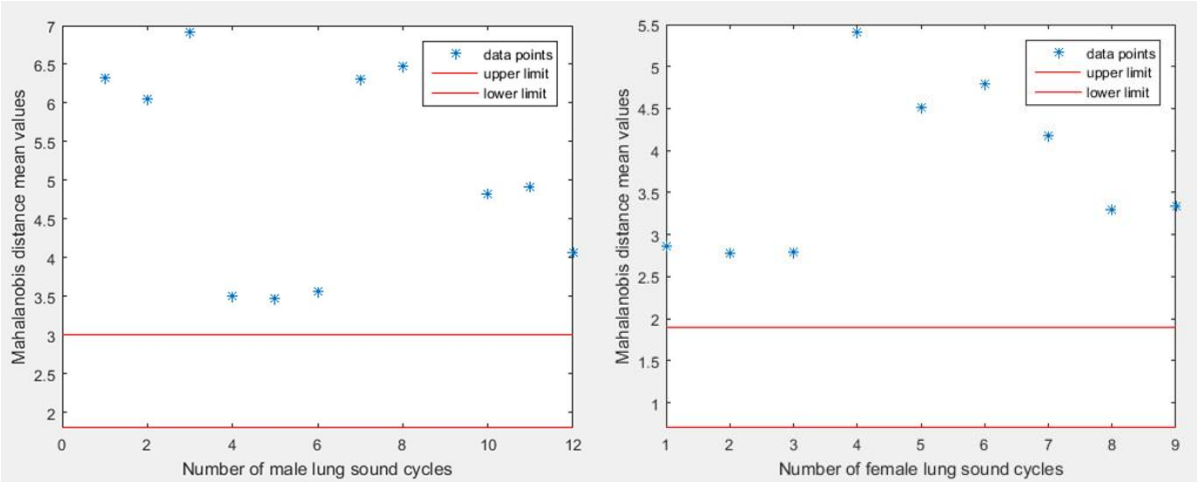

Figure 9 The data points indicate Mahalanobis distance mean values of abnormal lung sounds in males and females with respiratory disorders when compared with standard normal lung sound. Data points have deviated from the formed range which is shown by upper and lower limit.

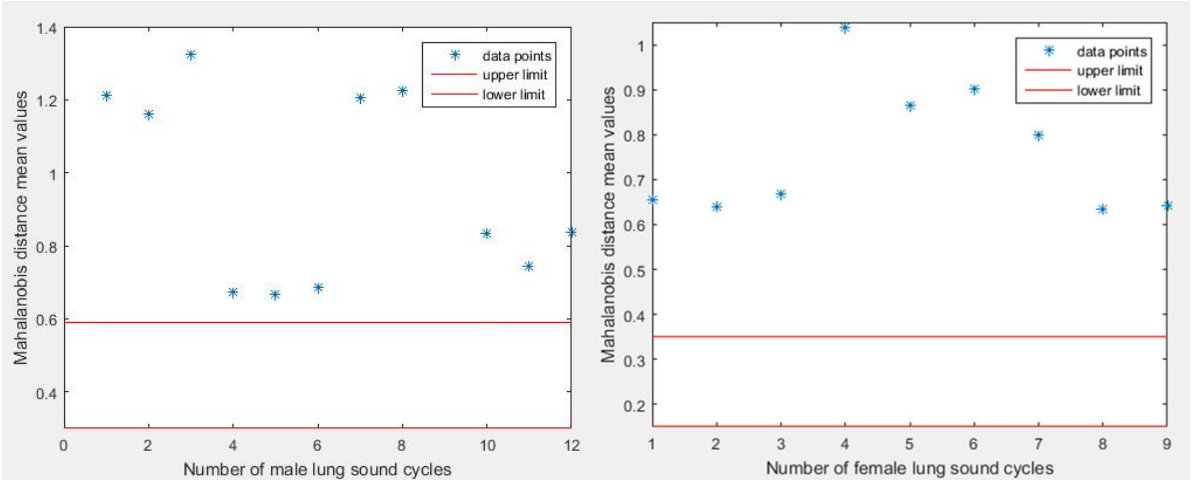

Figure 10 Data points indicates Mahalanobis distance mean values of abnormal lung sounds in males and females with respiratory disorders when compared with standard abnormal lung sound. Data points have deviated from the formed range which is shown by upper and lower limit.

It was observed that the Mahalanobis distance mean values obtained for abnormal lung sounds have deviated from the formed ranges when compared with standard normal lung sound and with standard abnormal lung sound. It was also observed that the values obtained for abnormal lung sounds when compared with standard abnormal lung sound are much closer to 1, indicating that the abnormal lung sounds are much similar to standard abnormal lung sound.

Conclusion

In this paper, a methodology has been implemented to compare normal and abnormal lung sounds and thereby to detect whether a person's lung sound is normal. It was seen that by using the Mahalanobis

distance method wheeze sounds and sounds due to low lung volume can be detected as abnormal lung sounds when compared with the ranges formed using normal lung sounds and therefore with reference to the results obtained, there is feasibility in distinguishing between normal and abnormal lung sounds of individuals using Mahalanobis distance method. This study can be further carried out to the other age groups with a higher sample size as well. Also, this study can be further developed by using subjects with vast variety of respiratory disorders and this research can be conducted for all other positions of the lung.

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IDENTIFICATION OF SPRING WHEAT GERMPLASM RESISTANT TO POLLUTION OF COPPER AND LEAD FOR THE DEVELOPMENT OF ECOLOGICALLY CLEAN TECHNOLOGY

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Abstract:
The creation and usage of technologically resistant breeds of agricultural plants in production is an effective way of solving the problem of soil contamination by heavy metals. At the first stage of this process it's necessary to study the gene pool of cultural and wild-growing plants and to allocate forms which accumulate the minimum quantity of pollutants in a commodity part of a harvest. Objects of an experiment are various spring wheat genotypes from a collection of East Kazakhstan agricultural scientific research institute. During the research growth indicators were identified, the index of tolerance of plants was calculated, as well as the regularity of accumulation and distribution of heavy metals on spring wheat bodies in the conditions of the increased contamination of the environment have been studied. Screening of spring wheat breeds in the laboratory conditions allowed to identify resistant and sensitive genotypes. By indicators of sprouts and roots' growth, the genotype GEK 2082/1 was the most resistant to the influence of lead. The genotype Lutescens 718 – is the least resistant genotype. By indicators of sprouts and roots' growth, the genotype GEK 2077/1 was the most resistant genotype to the influence of copper. The least resistant genotype is GEK 2033/5.

Keywords: Heavy Metals, Wheat, Growth Parameters, Resistant Genotypes

Introduction

One of the most important environmental problems is the contamination of soils by heavy metals (HM). It has an impact on almost all biosphere components.

The main sources of heavy metals contamination of Kazakhstan's topsoil are the enterprises of ferrous and nonferrous metallurgy, mining and processing industry, thermal power stations, transport et al. [1]. That is why contamination of soils by heavy metals has become one of the most important environmental problems in the country, especially in large metropolitan cities and industrial centers. In the industrial regions of the country there are significant foci of anthropogenic pollution of the topsoil.

In addition to such factors as type of soil; concentration; form of the location of HM; soil pH and its granulometric composition; content of organic substances, cation absorption capacity in soil; presence of technogenic sources of ecosystem pollution, the level of accumulation of heavy metals by plants also depends on their genetic and species features [2].

The study of the features of wheat resistance to heavy metals and identification of germplasm, which has resistance to their impact, is an important step towards the formation and usage of breeds which are resistant to anthropogenic impact in production. For this reason, it is necessary to study the gene pool of cultivated plants and identify the forms that accumulate the minimum amount of pollutants.

The aim of our study is to identify the genetic resistance potential of spring wheat to copper and lead in order to identify metal-resistant forms and donors for selection for metal resistance.

Methods

The objects of research are: the genotypes of spring wheat GEK 2077/11, GEK 2033/5, Lutescens 718, GEK 2071/8, GEK 2082/1. For the experiment, these plant samples were taken from the collection of the East Kazakhstan agricultural scientific research institute.

Wheat genotypes grew on a nutrient mix that contained 0.1 mM of CaSO4 and Cu ions at 400 mg/l concentration (as a CuSO4 salt) or Pb at 400 mg/l concentration (as a PbSO4 salt) under model contamination conditions for 14 days. Samples were grown in water medium at a t-20^{0}C during the day and 16^{0}C at night, with 10-hour photoperiod, light intensity - 5 thousand lx, humidity - 60%.
Measurement of growth indicators was carried out according to generally accepted methods. The tolerance index or the Wilkins coefficient was calculated [3].

The method of atomic absorption spectrophotometry was used to determine the concentration of heavy metals [4].

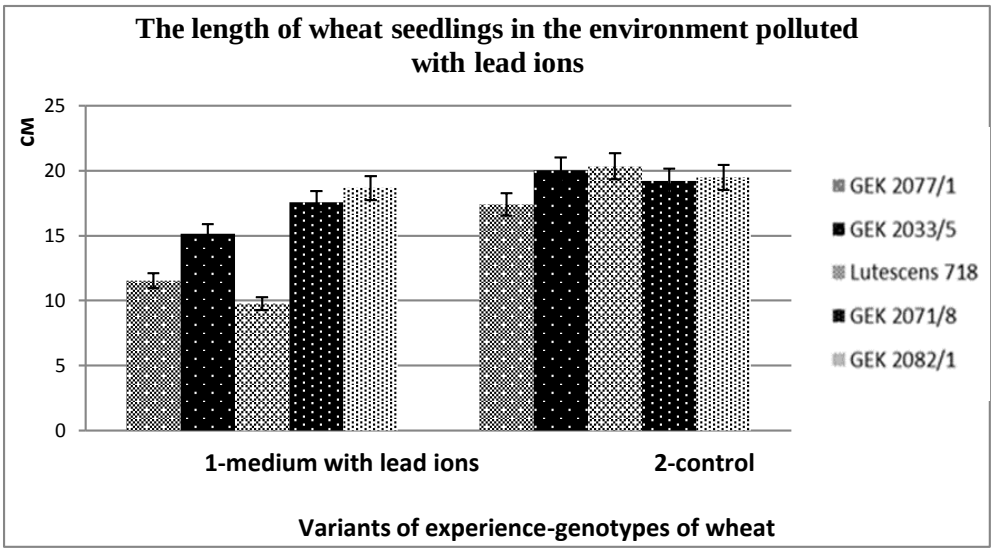
Results

A study of the genotypic specificity of wheat at resistance to the impact of heavy metals was carried out for copper and lead, the most priority pollutants in the East Kazakhstan region. Since the direct negative effects of heavy metals on plants is manifested in a suppression of the growth and development (attenuation of growth of shoots and roots), indexes of growth processes of wheat genotypes were investigated. This allowed to identify specific features of toxicity of certain metals depending on different genotypic differences of wheat plants.

Our studies of the influence of lead on the growth parameters of wheat germs in laboratory conditions showed that the ions of copper suppress the plant growth (Figure 1).

During the study of genotypes from the collection of the East Kazakhstan agricultural scientific research institute (EKASRI) it was found out that according to the results of study of the above-ground organs' growth with a high concentration of lead in the growth medium, the genotypes can be arranged as follows: GEK 2082/1 > GEK 2071/8 > GEK 2033/5 > GEK 2077/1 > Lutescens 718 (Figure 2).

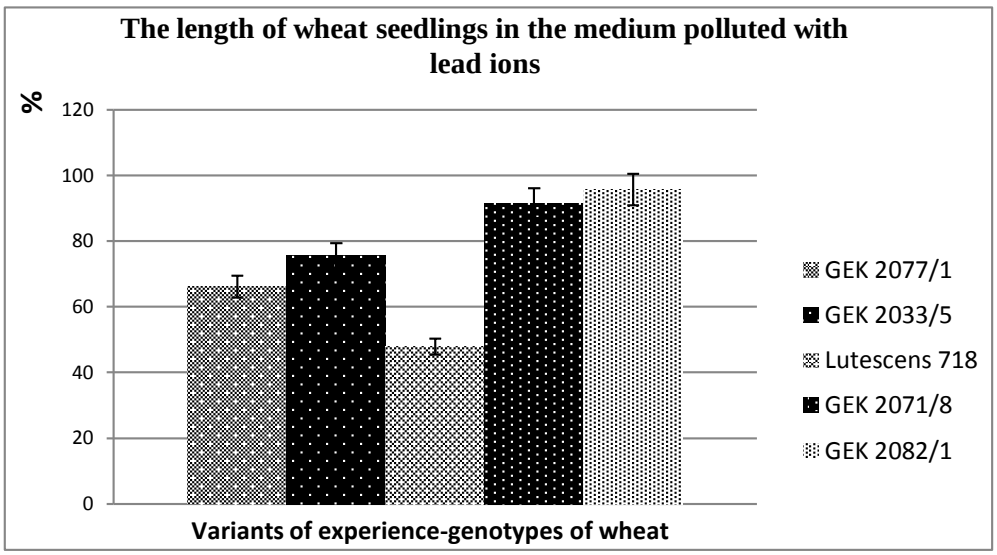
Figure 1 – The influence of the presence of ions of lead in the growth medium on the growth of seedlings of different spring wheat genotypes



The most resistant to the adverse effect of lead ions, on indicators of growth of above-ground organs, are genotypes GEK 2082/1 and GEK 2071/8. In genotypes of GEK 2082/1 and GEK 2071/8, the growth of the aerial organs is inhibited to a lesser degree than in the other genotypes when the lead salt is introduced into the growing medium. The

suppression of growth seedlings of these genotypes is 4,2 and 8,5 percent, respectively, compared to control (Figure 2).

Figure 2 - Reduction in the length of seedlings of different wheat genotypes (in percents to control) under conditions of contamination of the medium with lead ions



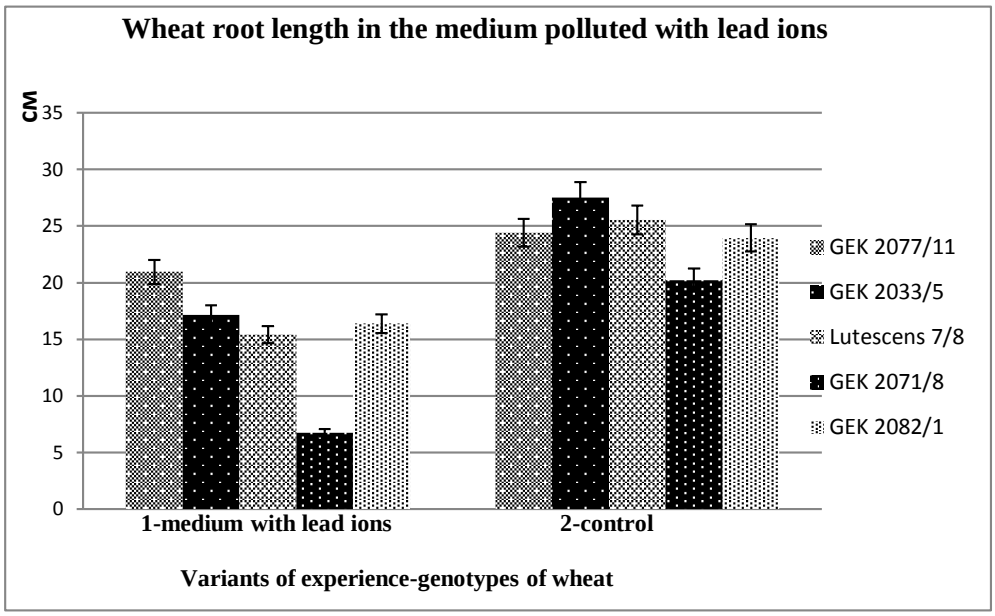
The average level of resistance to negative influence of ions of lead are in genotypes GEK 2033/5 and GEK 2077/1. The suppression of seedling growth of these genotypes, compared with control - 24.5 and 33.8 percent, respectively in the growth medium polluted by lead salt.

According to the growth of seedlings, the most vulnerable to adverse effect of lead ions is the spring wheat variety Lutescens 718. The growth of seedlings of this spring wheat variety is suppressed in comparison with the control by 52.1 percent (Figure 3).

Thus, according to the growth parameters of the above-ground organs, the genotypes GEK 2082/1 and GEK 2071/8 proved to be the most resistant to unfavorable influence of lead ions, the Lutecens 718 spring wheat variety was the most unstable to the adverse effect of lead ions.

Our studies of the effect of lead on the growth parameters of wheat roots in the laboratory conditions showed that lead ions inhibit the growth of plant roots. Herewith, root growth suppression is more significant than the suppression of seedling growth (Figures 3 and 1).

Figure 3 – The influence of the presence of lead ions in the medium of growth on root growth of different genotypes of wheat

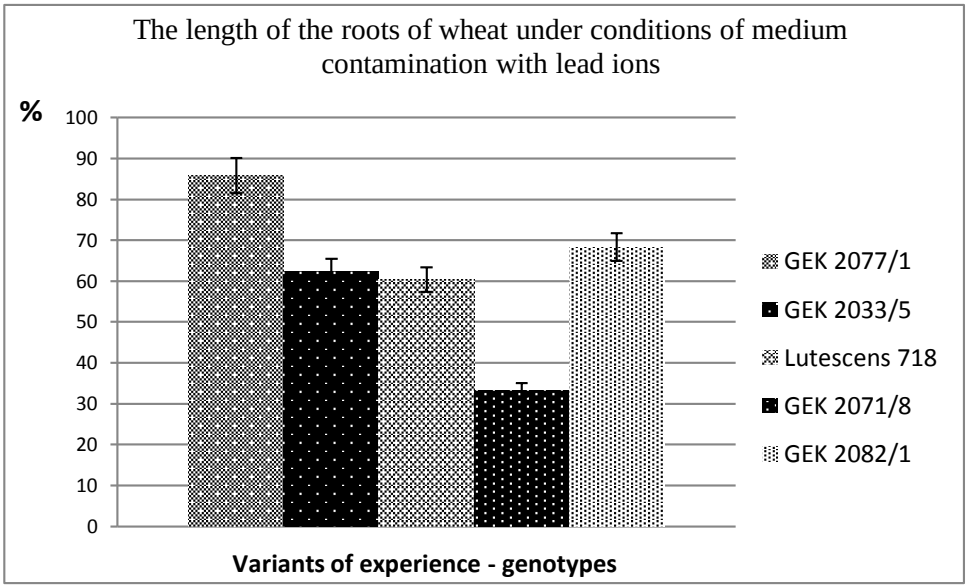


According to root growth at the time of introduction of the lead salt into the feeding medium, the genotypes can be arranged as follows: GVK 2077/1 > GVK 2082/1 > GVK 2033/1 > Lutescens 718 > GVK 2071/8 (Figure 4).
The root system of GEK 2077/1 genotype proved to
be the most resistant to the adverse effect of lead ions.

This spring wheat genotype's root growth is inhibited to much lesser extent than other genotypes' with a high concentration of lead ions in the medium of growth. Suppression of root growth in comparison with control is 14.2 percent.

It was found that the wheat genotypes GEK 2082/1, GEK 2033/1 and Lutescens 718 have an average level of root resistance to adverse effects of lead. In the meduim contaminated with lead ions, the root growth inhibition in these genotypes is 39.7, 37.6 and 31.6 percent, respectively, compared to the control.
The roots of wheat genotype GEK 2071/8 turned out to be the most unstable to unfavorable impact of lead ions. The root growth inhibition in comparison with control occurs at 66.6 percent (Figure 4).

Figure 4 – Decreasing of root length of different wheat genotypes (in% to control) under conditions of contamination of the medium with lead ions.



The Wilkins coefficient was also determined, which shows plant tolerance to heavy metals and is calculated by the formula $I_t=I_{me}/I_c$, where I_{me} is the root increment in the solution with the studied metal, and I_c is the root increment in the solution without metal (Table 1).

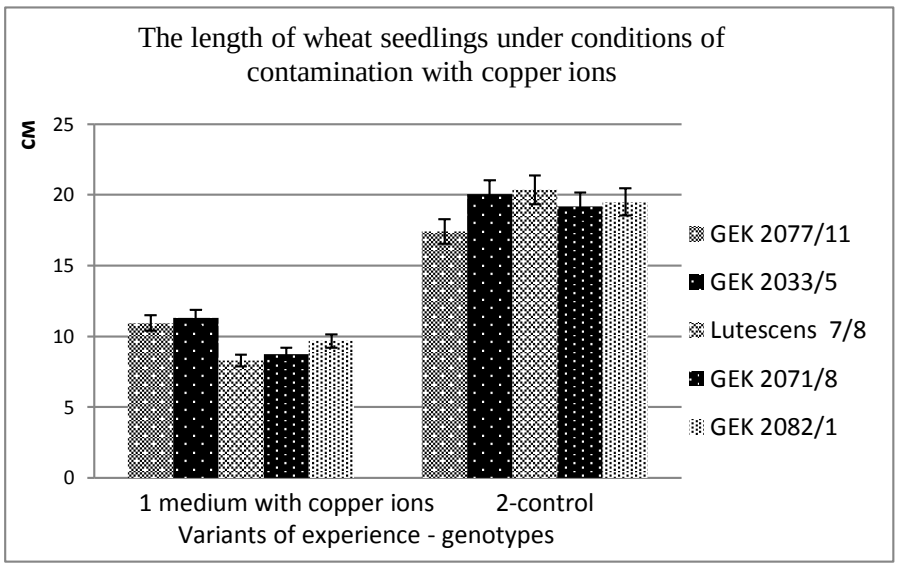
Table 1 - Wilkins coefficient or tolerance index of roots of wheat seedlings in conditions of environment of growth contaminated by lead ions

Wheat genotypes	GEK 2077/1	GEK 2033/5	Lutescens 718	GEK 2082/1	GEK 2071/8
I_{me},	20,95	17,16	15,41	16,38	6,75
I_c	24,40	27,52	25,54	23,95	20,23
I_t	0,86	0,62	0,60	0,68	0,33

The highest Wilkins coefficient or tolerance index at a high concentration of lead ions in the growth medium has GEK 2077/1 genotype, the average one - GEK 2082/1genotypes, GEK 2033/5 and Lutescens 718, the lowest - GEK 2071/8 (Table 1).
According to the results of study of the root growth in contaminated by copper ions medium and the tolerance index, the genotype GEK 2077/1 can be identified as a genotype with the most resistant root system to adverse effect of lead ions.
The average level of root resistance to adverse effects of lead, both on the growth of roots with contamination of the medium by copper ions, and on the tolerance index was revealed in wheat genotypes of GEK 2082/1, GEK 2033/1 and Lutescens 718.
The most unstable to the adverse effect of lead ions on the basis of the results of the ascertainment of both indicators were the roots of plants of genotype GEK 2071/8.
Thus, according to the results of study of the root growth the GEK 2077/1 genotype proved to be the most resistant to the action of lead ions, the genotype

of GEK 2071/8 was the most unstable to unfavorable action of this metal. According to the results of study of the above-ground organs growth, the Lutecens 718 spring wheat sort is most unstable to the adverse effect of lead ions, GEK 2082/1 and GEK 2071/8 genotypes turned out to be the most resistant to lead ions. Our studies of the effect of copper on the growth parameters of wheat seedlings under the conditions of the model experiment have shown that copper ions suppress plant growth (Figure 5).

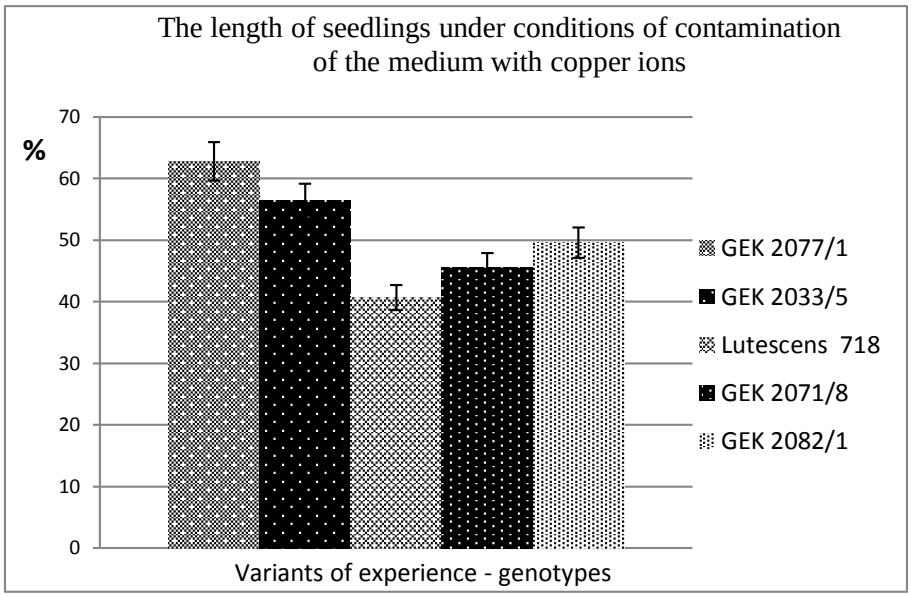
Figure 5 - Influence of the presence of lead ions on the growth of seedlings of different wheat varieties in the medium of growth



Wherein suppression of growth of spring wheat seedlings under conditions of contamination of the medium with copper ions is more significant than the suppression of seedling growth under conditions of contamination with lead ions (Figures 5 and 1).

When studying the effect of copper on the growth parameters of seedlings of different genotypes from the EKASRI collection, it was shown that according to the growth of the above-ground organs with high concentration of copper in the growth medium, the genotypes can be arranged as follows: GEK 2077/1 and GEK 2033/5> GEK 2082/1> GEK 2071/8> Lutescens 718 (Figure 6).

Figure 6 – The reduction of the length of seedlings of different wheat genotypes (in% to control) under conditions of contamination of the medium with copper ions



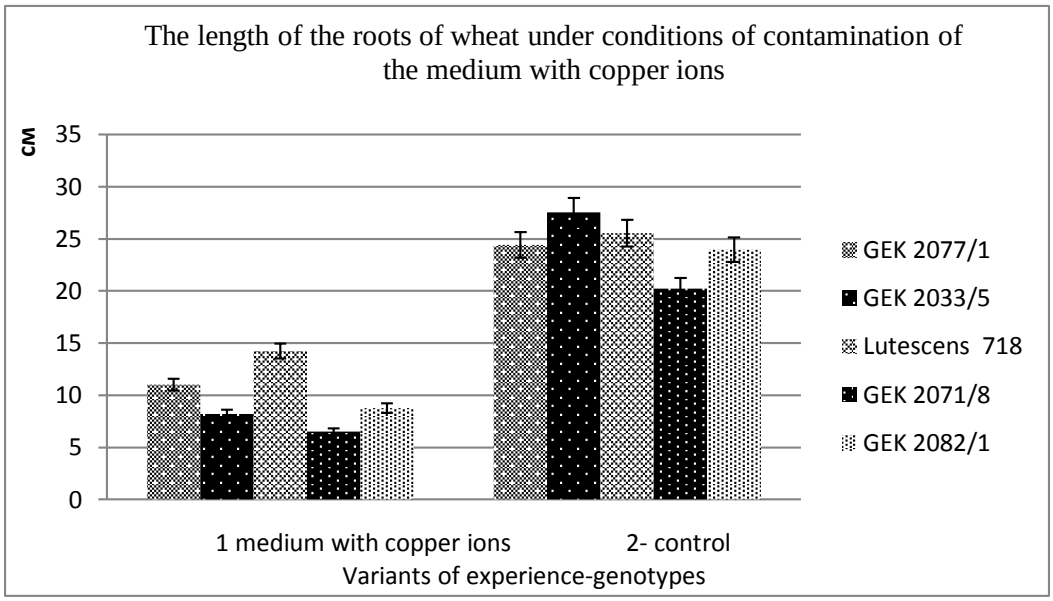
The genotypes of spring wheat GEK 2077/1 and GEK 2033/5 proved to be the most resistant to unfavorable influence of copper ions, according to the results of study of growth indices of above-ground organs. The suppression of seedlings growth during cultivation in an medium contaminated with copper ions compared to the control occurs at 37.2 and 43.2 percent, respectively.

The growth of the above-ground organs of the genotypes of spring wheat GEK 2082/1 and GEK 2071/8 is inhibited in an average degree in comparison with other genotypes at the time of addition of copper salt into the growth medium.

Suppression of growth of seedlings of these genotypes in comparison with the control occurs at 54.4 and 50.4 percent, respectively.

The most unstable to unfavorable action of copper were the above-ground organs of the spring wheat variety Lutescens 718. Suppression of growth of seedlings in comparison with the control occurs at 59.3 percent (Figure 6).
Our studies of the copper impact on the growth parameters of the roots of wheat in laboratory conditions showed that copper ions inhibit root growth. Furthermore, the root growth suppression is more significant than suppression of seedling growth (Figures 7 and 5).

Figure 7 – The influence of the presence of copper ions in the growth medium on root growth of different wheat genotypes



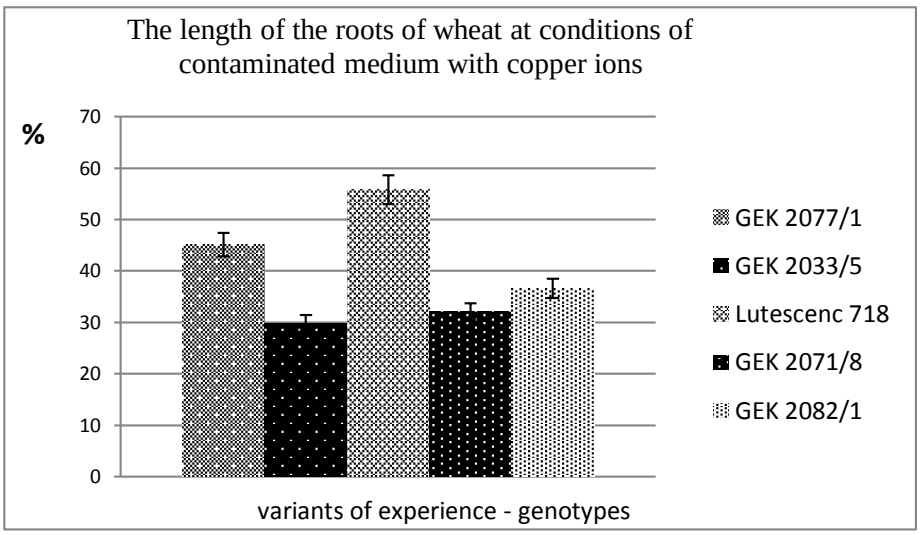
Studies of the effect of copper on plant growth parameters of genotypes from the EKASRI collection have shown that genotypes can be arranged according to the study of root growth at a high concentration of copper ions as follows: Lutescens 718> GEK 2077/1> GEK 2082/1> GEK 2071/1> GEK 2033 / 5 (Figure 8).

The root system proved to be the most resistant to the unfavorable effect of copper in the genotypes of spring wheat Lutescens 718 and GEK 2077/1.. When copper is entered into the growing medium, root growth is less inhibited in these genotypes than in other genotypes. The inhibition of root growth occurs 44.2 and 54.9 percent compared to control, respectively (Figure 8).

The genotype of spring wheat GEK 2082/1 has the average level of resistance of the roots in the conditions of contamination of the growing medium by copper ions. The suppression of root growth occurs at 63.4 percent compared to control, respectively.

The roots of the spring wheat genotypes GEK 2071/8 and GEK 2033/5 were the most unstable to the adverse effects of copper. The suppression of root growth occurs 67.9 and 70.1 percent respectively compared to the control in medium contaminated by copper ions (Figure 8).

Figure 8 – Reduction of root length of different spring wheat genotypes (in percents to control) in conditions of medium contaminated with copper ions



The Wilkins coefficient was also determined in the case of studying the influence of copper on wheat growth parameters, as well as in the case of the study of the responses of wheat plants of different genotypes to the adverse effect of lead ions in the growing medium (table 2).

Table 2 – The Wilkins coefficient or tolerance index of wheat roots in terms of contamination by copper ions

Wheat genotypes	GEK 2077/1	GEK 2077/1	GEK 2033/5	Lutescens 718	GEK 2071/8	GEK 2082/1	GEK 2071/8
I_{me},	11,01	11,01	8,23	14,25	6,50	8,77	6,75
I_c	24,40	24,40	27,52	25,54	20,23	23,95	20,23
I_t	0,45	0,45	0,30	0,56	0,32	0,37	0,33

At a high copper concentration Wilkins coefficient or tolerance index is the highest at Lutescens 718, average is in GEK 2077/1, GEK 2082/1 and least is in GEK 2033/5 and GEK 2071/8 (Table 2).

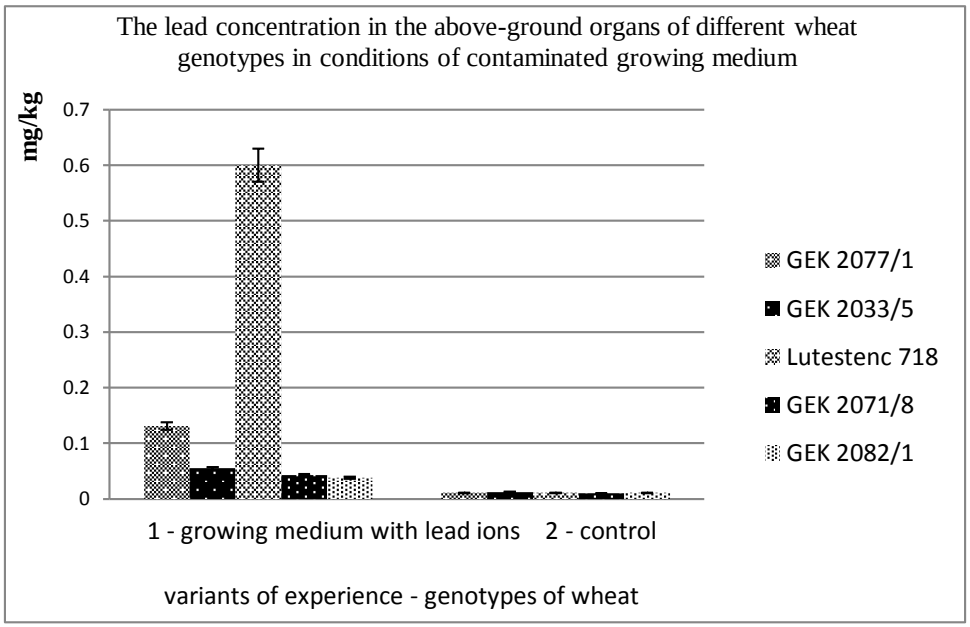
According to the study of root growth and the tolerance index, genotypes Lutescens 718 and GEK 2077/1 have the most resistant root system to the adverse effects of copper ions.

The average level of resistance of the roots to the unfavorable effect of copper, both on the growth of roots with contamination of the medium by copper ions, and the tolerance index was revealed in wheat genotypes of GEK 2082/1.

According to the results of the determination of both indicators, the roots of the genotypes of GEK 2033/5 and GEK 2071/8 turned out to be the most unstable to the adverse effect of copper ions.

Thus, the genotypes of spring wheat Lutescens 718 and GEK 2077/1 proved to be the most resistant according to root growth, the genotypes of GEK 2033/5 and GEK 2071/8 proved to be the most unstable to the adverse effect of copper. According to the growth of the above-ground organs, the genotypes of spring wheat GEK 2077/1 and GEK 2033/5 proved to be the most resistant to the action of copper, Lutescens 718 is the least resistant.

The plants of genotype GEK 2077/1 show root stability and stability of above-ground organs to unfavorable action of copper ions. The studying of the patterns of accumulation and distribution of copper and lead in the organs of spring wheat depending on the genotypic differences in the samples at the conditions of the model experiment was carried out.



The results of the determination of the lead content (at a dose of lead in the medium of 400 mg/l) showed that the genotypes GEK 2082/1 and GEK 2071/8 accumulated the smallest amount in above-ground organs. The seedlings of the spring wheat breed Lutescens 718 accumulate the largest amount of lead in the above-ground organs.

The remaining genotypes occupy an intermediate position between them - GEK 2077/1 and GEK 2033/5 (Figure 9). Thus, the GEK 2082/1 and GEK 2071/8 showed the greatest resistance of lead intake in the above-ground organs, the largest amount of lead in the above-ground organs is in genotype of spring wheat Lutescens 718 (Figure 9).

Figure 9 – The lead content in the above-ground organs of different wheat genotypes in conditions of contaminated growing medium

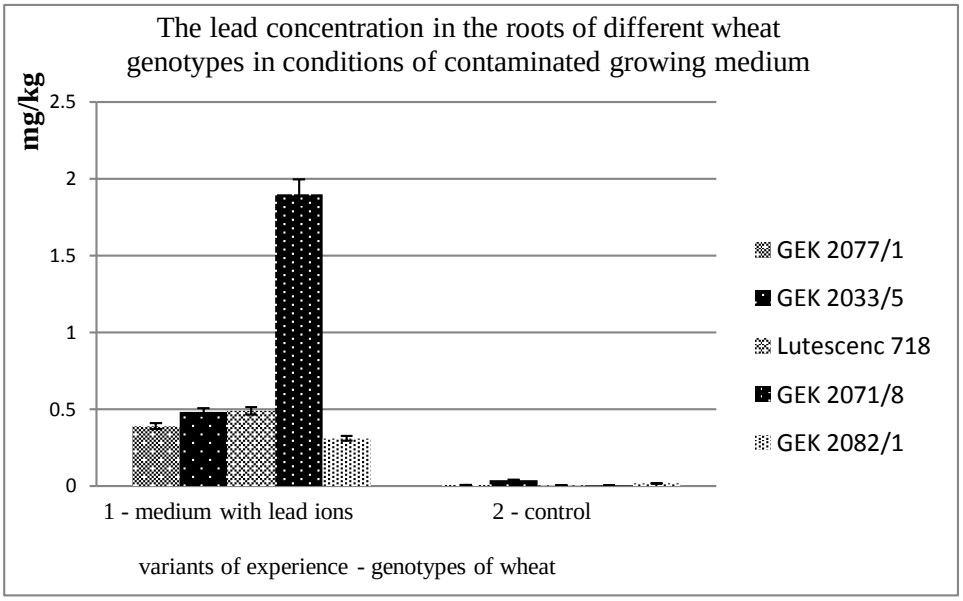
A study of the lead content, at a dose of lead in a medium of 400 mg/l, of different spring wheat

genotypes showed that GEK 2082/1 accumulated the least amount in the roots.
In these experimental conditions, the highest amount of lead accumulated in the roots of spring wheat GEK 2071/8. The remaining varieties occupy an intermediate position between them – a breed of spring wheat Lutescens 718 and genotypes of spring wheat GEK 2033/5 and GEK 2077/1.

Thus, the least accumulation of lead is observed in the roots of spring wheat genotype GVK 2082/1, the plants of spring wheat GEK 2071/8 accumulate the largest amount of lead in the roots (Figure 10).

According to the study results, genotype GEK 2082/1 can be distinguished as a genotype with the greatest root resistance to lead. The sprouts of spring wheat genotypes GEK 2082/1 and GEK 2071/8 have shown as genotypes resistant to translocation of lead into the above-ground organs.

Figure 10 – The lead content in the roots of different wheat genotypes in conditions of contaminated growing medium



The study of lead accumulation in roots and above-ground organs, of growth parameters of wheat germs of different genotypes made it possible to identify the most sensitive and resistant genotypes. The GEK 2082/1 was most resistant genotype to lead and the most sensitive was spring wheat variety the Lutecens 718.

The study of the copper content in the above-ground organs of different genotypes of spring wheat showed that the lowest accumulation of copper (its content of 400 mg/l in the medium) is observed in sprouts of genotype GEK 2077/1.

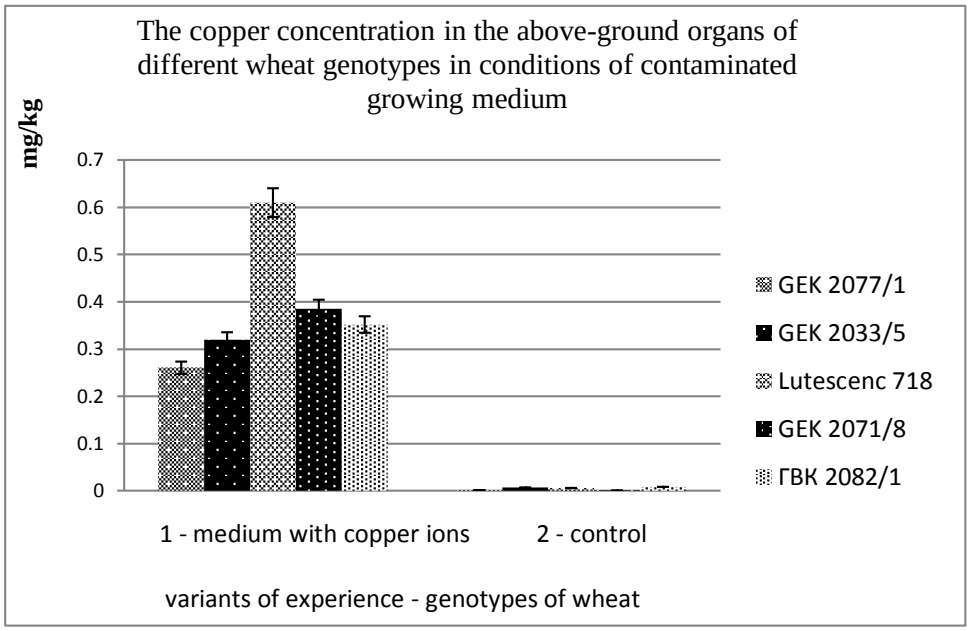
Genotypes of GEK 2071/8, GEK 2082/1 and GEK 2033/1 are characterized by an average accumulation of copper in the above-ground organs at these experimental conditions.

In the above-ground organs, the greatest content of copper is observed for breed of the Lutescens 718 (Figure 11). Thus, the greatest resistance to the entry of copper into the aerial organs was shown by the genotype of GEK 2077/1, the greatest accumulation of copper is observed in the seedlings of the breed Lutescens 718 and GEK 2071/8 (Figure 11).

The study of copper accumulation in roots of plant, (its content in the medium of 400 mg/l) showed that the least amount contained in seedling roots of spring wheat breed Lutescens 718.

Genotypes of GEK 2071/8, GEK 2077/1 and GEK 2082/1 are characterized by an average accumulation of copper in the roots of plants at these experimental conditions. The highest content of copper is observed in the roots of plants of genotype GEK 2033/5 (Figure 12).

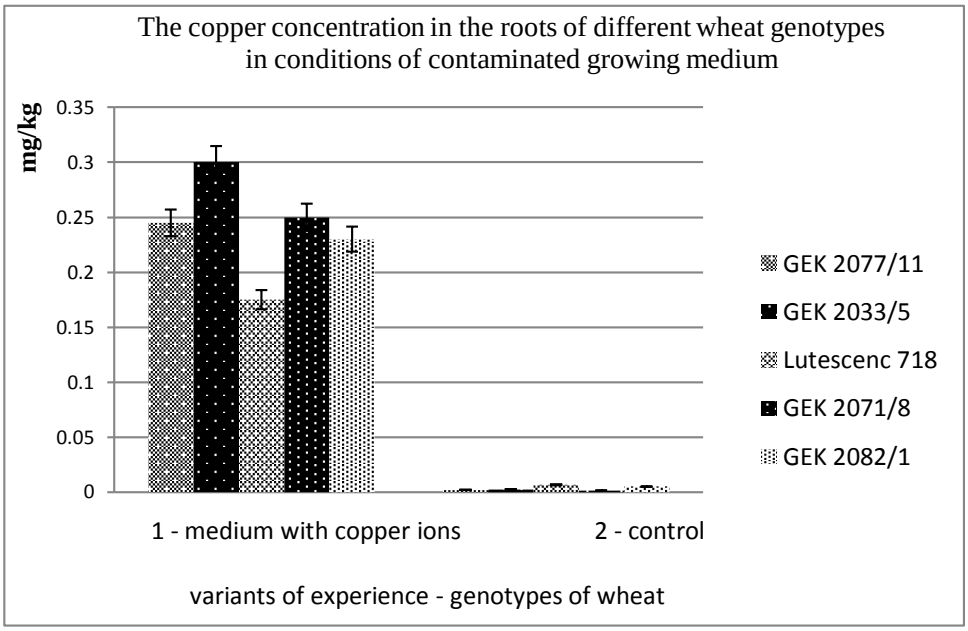
Figure 11 – The copper content in the above-ground organs of different wheat genotypes in conditions of contaminated growing medium



Thus, the smallest accumulation of copper is observed in the roots of the spring wheat breed Lutescens 718, the largest - in the roots of the genotype GVK 2033/5 (Figure 12).

The study of copper accumulation in the roots, above-ground organs and growth parameters allowed to reveal the most sensitive and stable genotypes. The most resistant to the action of copper was the genotype of spring wheat GVK 2077/1, the most unstable to the adverse effect of this metal was the breed of spring wheat Lutescens 718.

Figure 12 – The copper content in the roots of different wheat genotypes in conditions of contaminated growing medium



Conclusion

According to the research results, studied genotypes showed a genotypic specificity in relation to the adverse effects of lead and zinc. The above-ground organs and the root system have differential features of accumulation of heavy metals, which in the future may effect on their growth and development parameters. In the roots of the genotype of spring wheat GEK 2082/1 observed the smallest accumulation of lead, plants of the genotype Lutescens 718 accumulate the largest amount of lead in the roots. The genotypes of GEK 2082/1 and GEK 2071/8 showed the greatest resistance to the intake of lead in the above-ground organs, the genotype of spring wheat Lutecens 718 was the least resistant.

The lowest accumulation of copper is observed in the roots of the spring wheat variety Lutescens 718, the highest - in the roots of the genotype GEK 2033/5. Genotype GEK 2077/1 showed the greatest resistance to copper entering the above-ground organs, the greatest accumulation of copper was in sprouts of GVK 2071/8 and Lutescens 718

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STUDY ON THE BACTERIAL CELLULOSE PRODUCTION FROM FRUIT JUICES

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Abstract: Need for large quantity of bacterial cellulose (BC), used as a matrix for production of numerous materials with desirable properties, has increased in the fields of biomedicine and electronics. To achieve the goals further investigations are essential in order to understand the intracellular polymerisation reaction; to increase the biosynthesis rate and reduce cost of the overall production process. Carbon sources for the BC production are usually glucose, fructose, and sucrose, so juices from low grade fruits can successfully substitute the carbohydrates, vitamins, ascorbic acid, and proteins in the growth medium and can form low-cost substrates. We used strain *Gluconoacetobacter xylinus* CICC10529 to produce cellulose from watermelon and mandarin juices (70% v/v and 80% v/v) with or without yeast extract supplement. The liquid media (with working volumes 50 mL and 100 mL) made from fruit juices always contained $MgSO_4.7H_2O$ (1.5 w/v%), K_2HPO_4 (0.1 w/v%), as well as ethanol (1 v/v%). Two modes of operation: static biosynthesis in incubator and dynamic biosynthesis in orbital shaker (at 200 rpm) were conducted at 30°C. The production process was monitored during 7 to 10 days. Thermal properties of BC produced at different conditions were investigated through thermal gravimetric analysis and width of the cellulose fibrils/ribbons were compared via microscopic observations.

Keywords: Bacterial Cellulose, Fruit Juices, *Gluconoacetobacter xylinus*

Introduction

Bacterial cellulose (BC) secreted by the *Acetobacter* bacteria, so-called "vinegar plant", can be used as a matrix for production of materials with desirable properties. Cellulose produced by bacteria is a promising material for many applications, for example, it can be used for edible packing in the food industry, as wound dressing materials, artificial skin, and scaffolds in regenerative medicine, as electrically conductive paper, and as organic-inorganic hybrid for visible light transmission among others (Shah et al., 2013; Campano et al., 2016). Broad range of the above applications is determined by the particular properties of the BC, which does not have lignin and hemicellulose in comparison to the cellulose derived from plants. Having the identical molecular structure to that of plant cellulose, distinctive properties of BC are revealed in higher purity, higher crystallinity, higher degree of polymerisation, higher water absorption and retaining capacity, higher tensile strength, and strong biocompatibility. These properties are caused by extremely fine pure fibrils, which form a 3-dimentional network structure (Chen et al., 2010; Lee et al., 2014). The arrangements of fibrils and the conformation of the porous matrix are greatly dependent on several factors: the activity of producing organisms, composition of the culture media, variation in culture time and conditions, amount of the inoculum, and the carbon source (Tang, et al, 2010; Ul-Islam et al., 2016). Watanabe et al. (1998) obtained different structural features and properties of the BC produced under static and agitated culture conditions. The reported BC, which was produced in agitated culture had a lower degree of polymerisation and crystallinity compared with that produced under static conditions.

Kurosumi et al. (2009) studied production of BC varying carbon sources found in the fruit waste and juices. The reported yield of BC obtained from orange juice was 0.48 g DW BC from 79.5 g orange juice.

The aim of this work was to produce BC using natural complex substrates such as watermelon and mandarin juices at different concentrations. Static and dynamic modes of bacteria cultivations were applied in order to produce matrices with high yield and varied structure. Thermal properties of BC produced at different conditions were investigated through thermal gravimetric analysis (TGA) and thickness of the fibrils and ribbons produced were compared via microscopic observations.

The watermelon and mandarin juices were chosen for our investigations because of their different contents of carbohydrates, e.g., glucose, fructose, and sucrose, as well as different acidities of the above juices. Thus influence of the juice composition and its acidity on the BC production was also determined.

Materials and Methods

Cultivation of Bacteria

Gluconoacetobacter xylinus strain (CICC 10529) was purchased from China Centre of Industrial Culture Collection, Beijing. It was maintained on glucose agar containing all chemicals described in the CICC growth medium. Plated cultures was stored at 4°C in a refrigerator and sub-cultured every 2 months for inoculum development.

CICC growth medium contained: glucose - 20.0 g, yeast extract - 5.0 g, K_2HPO_4 - 1.0 g, $MgSO_4 .7H_2O$ - 15.0 g, and 5 ml of anhydrous ethanol, all components were dissolved in 1.0 L water purified by reverse osmosis.

Compositions of Media for BC production

1) Mixture of glucose, fructose, sucrose, yeast extract (FGS), citric acid, ethanol, and mineral salts was used with the following composition: glucose - 2 w/v %; fructose - 2.5 w/v %; sucrose - 5.5 w/v %; citric acid (CA) – 0.12 w/v %, and ethanol - 0.5 mL in 100 mL water at pH=5.5.

2) Sucrose-based medium containing yeast extract, ethanol and mineral salts as follows: sucrose - 5.0 w/v %, yeast extract - 0.5 w/v %, ethanol - 1 mL in 100 mL water, and mineral salts (SYE).

3) Fruit juices such as watermelon juice (50, 70 and 80 v/v %) and mandarin juice (70 and 80 v/v %) were studied for the production of BC. Both 80 v/v % juice containing media were supplemented only with ethanol, the other medium concentrations (50 v/v and 70 v/v %) contained also yeast extract.

Watermelon juice (WMJ) containing the above mentioned mineral salts in the identical quantities as in the growth medium included also the following components:

a) 50 v/v% juice, 0.5 yeast extract w/v%, and 0.5 ethanol v/v%

b) 70v/v% juice, 0.5 yeast extract w/v%, and 0.5 ethanol v/v%

c) 80 v/v% juice and 0.5 ethanol v/v% in water.

Pure watermelon juice had a pH=5.42.

Mandarin juice (MJ) was used for BC production, it contained the mineral salts reported and the following components:

a) 70% v/v juice, 0.5% yeast extract w/v, and 0.5% ethanol v/v

b) 80%v/v juice and 0.5% ethanol v/v in water.

Pure mandarin juice had a pH=3.75, it was adjusted to 5 for the biosynthesis process.

Optimal temperature for cultivation of the *Gluconoacetobacter xylinus* stain was 30°C. Two modes of operation were performed: static in the incubator and dynamic in the orbital shaker. Duration of each experimental run was 7 days. Static process was investigated in 500 mL Erlenmeyer flask and working volume of medium 100 mL. It was inoculated with a BC dose of polymer containing bacteria grown on the surface of the liquid medium in the inoculation tube with size (R=1cm) and dry weight (DW=0.02g).

Dynamic process was carried out in shaker at 200 rpm with inoculum volume 10 v/v % of liquid medium. Two volumes of media were used 50 mL in 250 ML Erlenmeyer flasks and 100 mL in 500 mL Erlenmeyer flasks.

Concentrations of bacteria were determined using Chinese version of the McFarland Standards (Pro-Lab Diagnostics) BW2705. Number of bacteria in the inoculation polymer dose was obtained by dissolving it in 10ml citrate buffer (pH=4.7) using commercial enzymes (Sigma Aldrich) cellulose (1mL) and glucosidase (1mL). The average total number was estimated via UV/VIS spectrophotometry and BW2705 calibration standard, it was in the range of 22-25*10^6 cells/mL.

Chemical Analytical Methods

Total reducing sugar concentrations in the media were analysed via colorimetric the dinitrosalicylic method (DNS) method. Digital refractometer (Ningbo, China) was used to measure sucrose in the fruit juices as Brix %.

Product Separation

Separation of the bacterial cellulose products was carried out by filtration. Samples were heated in 100 mL of 0.1M NaOH at 80°C for 20 min. Then using vacuum-pump, they were washed with purified water until neutral pH=7. Finally, BC was dried in the oven at 80°C for 5 hours and dry weight was measured until its constant value.

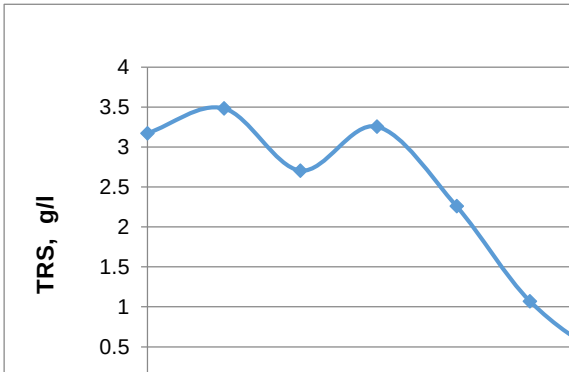
Thermal Gravimetric Analysis

TGA analyses of BC samples (approx. 10-15mg) were carried out under non-isothermal conditions or dynamic conditions in TG/DTA Apparatus (SII 6300 EXSTAR6000 Japan) with nitrogen flow rate 50 mL/min and heating rate 10°C/min. A temperature range from 50°C to 700°C was investigated.

Results and Discussion

Effect of Carbon Sources

Evaluation of the carbon source concentrations during the biosynthesis was carried out using DNS method and the results obtained are shown in Figures 2 and 3. Total reducing sugars in the synthetic media containing glucose, sucrose, and fructose were consumed following 6-7 days of the dynamic cultivation, whereas their depletion in the fruit juices (e.g., mandarin juice) was twice quicker as shown in Figure 3. This can be explained by the higher affinity of the bacteria to glucose and fructose containing media compare to the sucrose, which contents was also measured in the fruit juices. The fastest consumption of carbon source and BC production process was observed in the shaker (3 days), whereas slower biosynthesis was recorded in the incubator (4 days) at identical other conditions.



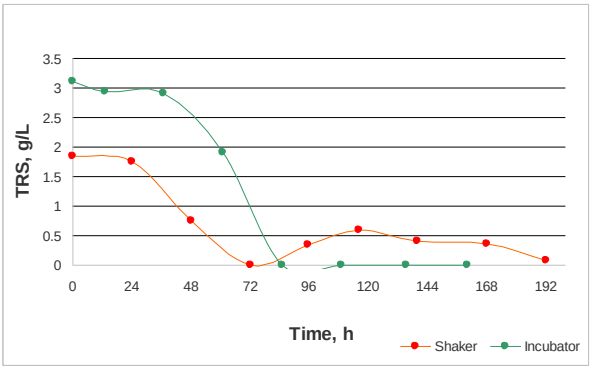


Figure 1. Variation of [TRS] in the FGS medium. Figure 2.Variation of [TRS] in 80 v/v % mandarin juice

The Gluconoacetobacter xylinus strain (CICC 10529) was able to produce BC from the fruit juices like watermelon and mandarin without any protein additions under the conditions described above. It was possible because watermelon and mandarin naturally contains nitrogen sources, which can enhance bacterial growth without supplements.

Yields and Productivities

The BC yield and productivity varied substantially at different conditions. As shown in Fig. 4, the highest yield based on dry weight (DW =16.4 g/L) was obtained from 70 v/v % watermelon juice supplemented with yeast extract during the dynamic cultivation, then 80 v/v % watermelon and 80 v/v % mandarin juices with ethanol additions gave yields approx. 12 and 11 g/L on DW basis, respectively. From the synthetic media high yield was produced using sucrose-based medium (about 14g/L of DW) during the dynamic biosynthesis.

The highest BC productivity was achieved during the dynamic biosynthesis in the media containing yeast extract (up to 3.0 g DW/L.d) and in 80% WMJ without yeast extract (YE) supplement (2.0 g DW/L.d). During the static biosynthesis, productivity of the BC varied from 0.80 to 1.10 g DW/L.d in the watermelon and mandarin juices, respectively.

Table 1. The productivity of the BC production via dynamic biosynthesis.

Medium used for dynamic BC production	Glucose,YE, EtOH	FGS,CA, EtOH	Sucrose, YE, EtOH	50%WNJ,YE, EtOH	70%WMJ,YE, EtOH	80%WMJ, EtOH
Productivity, g DW/ L.d	1.20	1.42	2.0	1.40-1.70	2.4-3.0	1.50 -2.0

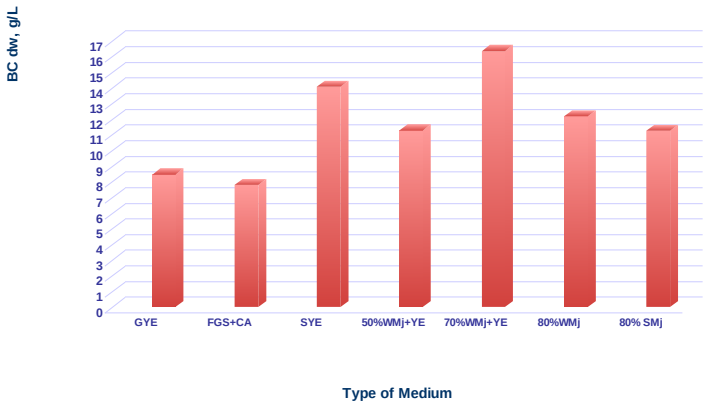


Figure 3. The yields of the BC produced via dynamic biosynthesis in different media.

Thermal Analysis

We studied thermal properties of various BC matrices using TGA analysis. Differences in the weight loss of the BC samples under non-isothermal conditions at the temperatures studied were recorded by the TG/DTA apparatus. The weight [(initial sample weight –incidental sample weight)/initial sample weight] in %) versus temperature were plotted for the dynamic and static modes of cultivation, they are depicted in Figures 4 and 5. Final amount of material detected at the temperature of 700°C was equal to 25% in the shaker and 15% in incubator. The thermal curves of the BC produced from the glucose-based synthetic medium and 80 v/v % watermelon juice followed similar paths at the same dynamic conditions. However, the BC produced in the static process performed differently. A well-pronounced step in the weight loss was observed at the inset temperature of 250 to outset temperature of 400°C, the weight loss was equal to about 50% of the BC. This dramatic change was not found during the dynamic cultivation where smooth weight loss was around 40% at the same temperatures. The BC produced from fruit juices exhibited even smaller changes in the weight (in the range of 15-30%) at the identical temperatures recorded. Final weight % of the samples measured at ~700oC was equal to 25% of their initial weight produced in the dynamic mode, and 15% - in the static mode. These events could be explains by the variation of BC morphology in the samples, e.g. different width and size of the fibrils/ribbons produced under different modes of the bacterial cultivations.

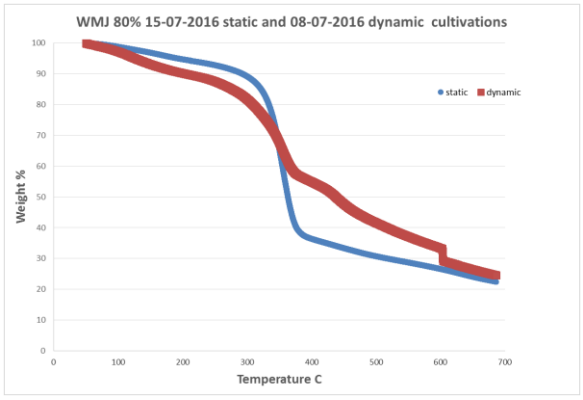


Figure 4. TGA of the WMJ samples produced in static dynamic modes.

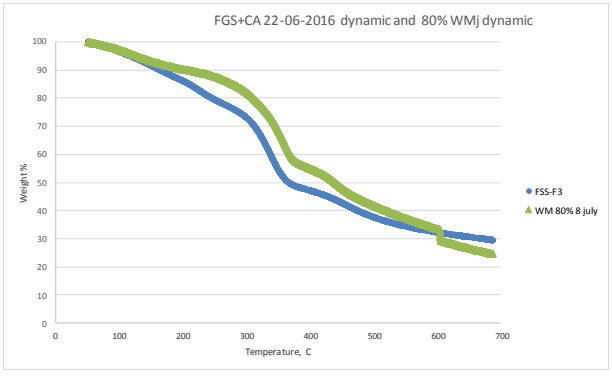


Figure 5. FGS and WMJ samples produced in the dynamic mode.

Static versus Dynamic Growth

Morphological changes of the BC fibrils produced statically and dynamically were reported previously by Watanabe et al. (2009). The authors examined scanning electron micrographs of BC and found that fibrils produced sin static culture were more highly extended and had larger width. Whereas, fibrils of the BC produced under agitated conditions were curved and entangled with each other resulting in a denser reticulated structure than those of the static BC. The width of the fibrils produced dynamically appeared smaller than that of the static fibrils. Our microscopic observations showed analogous results, which illustrates lower width of the dynamically produced BC fibrils, and respectively, ribbons formed from those fibrils compare with the statically produced structures. As shown in Figures 6 and 7, the average width of the ribbons (around 28 microns) was produced in the shaker from mandarin juice. In the same time ribbons with average width of approx. 40 microns were synthesized under the static conditions. SEM observations also showed denser morphological structure with smaller width of the BC fibrils produced in the dynamic mode than those produced in the static mode (Figures 8 and 9).

Figure 6. Size of the BC ribbons observed by the light microscopy (x400) in 80% SMJ of dynamic cultivation.

Figure 7. Size of the BC ribbons observed by the light microscopy (x400) in 80% SMJ in the static incubator.

Figure 6.

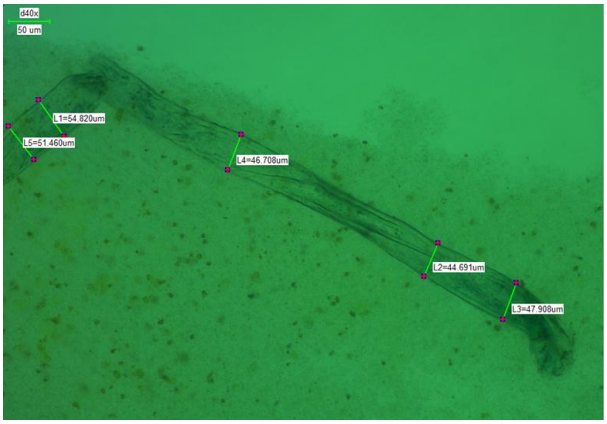


Figure 7.

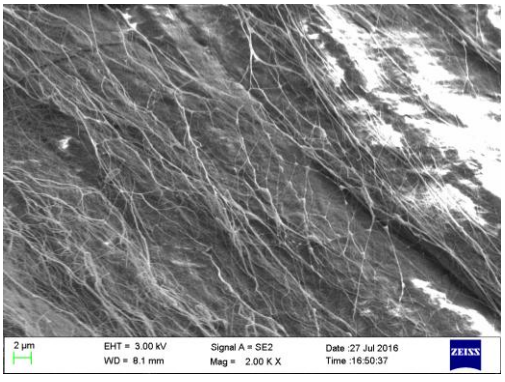


Figure 8. Sample of 80% WMJ- dynamic mode.

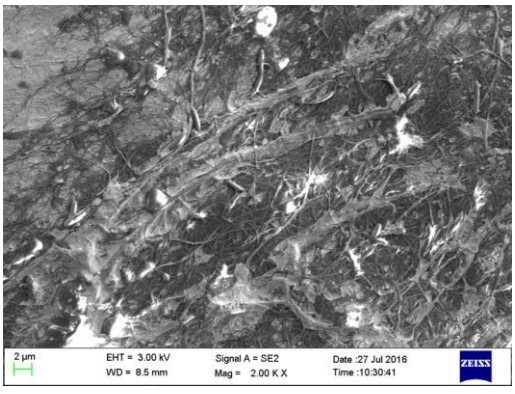


Figure 9. Sample of 80% WMJ – static mode.

Conclusions

Juices from selected fruits, for example, watermelon and mandarin juices can be successfully used as growth media for the *Gluconoacetobacter xylinus* bacteria. With no addition of nitrogen source they can provide affordable carbon sources and nutrients for production of the BC.

Using different modes of operation: under the static and dynamic cultivations of the bacteria, the BC fibrils and ribbons with diverse width and density were produced. The ribbon width produced in the shaker was approx. 25-37 microns after 2 days, however, the larger width was found in the BC produced in the incubator (~40 -50 microns) during the same production time. This morphology was responsible for the different thermal behaviour of the BC samples, when non-isothermal heating was applied in the dynamic nitrogen environment, increasing temperature from 50 to 700°C. Ten percent higher thermal stability was exhibited by the BC samples produced via the dynamic mode.

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NANOTECHNOLOGY BASED TARGETED DRUG DELIVERY SYSTEMS IN BREAST CANCER THERAPY

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Abstract: Cancer is a highly heterogeneous and complex disease condition to understand because of its cellular and physiological systems. The most common cancer treatments are chemotherapy, radiotherapy and surgical excision. Out of them chemotherapy remains as the most widely used anti-cancer therapy. But unfortunately chemotherapy has plenty of drawbacks when considering its action on cancerous cells. Many chemotherapeutic drugs are highly toxic, not specific, poorly selective and less soluble because of these incidents cancer patients have severe side effects such as alopecia, renal failures, cardiac failures and etc. In order to overcome these problems in nanotechnology based targeted drug delivery system was introduced. Nanotechnology is a rapidly growing files which promote novel methods in cancer diagnosis, treatments and prognosis. In targeted drug delivery system use differently synthesized nanoparticles to reduce the drawbacks of chemotherapy. Nanoparticles such as liposomes, carbon nanotubes, virus mediated nanoparticles and nanodiamonds are some of the nanoparticles which are being discussed in this article. By using nanoparticles many of the drawbacks in conventional chemotherapy can be altered for an example by using surface functionalized nanoparticles chemotherapeutic drugs can be loaded into the particle and easily can be delivered to the exact tumor site without harming the non-cancerous cells while increasing the half-life of the drug. Likewise there are many advantages in nanotaenology based targeted drug delivery system over the conventional chemotherapy which will give a new ray of hope towards cancer patients to have a quality life.

Keywords: Nanotecnology, Targeted Drug Delivery,Nanoparticles, Active Targeting, Passive Targeting , Cancer

Introduction

Cancer can be defined as a highly heterogeneous complicated disease condition that incorporates a large set of disorders which may result in by continues indefinite growth (Jabir *et al*., 2012). Rapid proliferations of abnormal cells are one of the defining causes in cancer which may ultimately go beyond the limits. Cancer causes morbidity and mortality rates nearly with 14 million new cases and 8.2 million relate deaths every year in worldwide. Prostate, lung, colorectum stomach and liver cancers are the most common among men and in women breast, lung, colorectum, cervix and stomach cancers are the most common types (WHO.com, 2015). Genetic and phenotypic diversity of cancer define the complexity of this disease (Hare *et al*., 2016).

Out of all cancer incidents breast cancer remains as the most common cause of women cancer incidents and the second largest cause for the cancer relating deaths in the worldwide. Moreover breast cancer is hundred times common in women than men. Due to increased risk factors breast cancer incidents have been upsurge in the time of 1975 to 2000 and now one women in every eight is suffering from a breast cancer type. Late diagnosis of the breast cancer is associated with a poor prognosis and early detection of cancer will have a better prognosis due to advances that have happened in the treatment strategies (Assi *et al*., 2011; Eissemann *et al*., 2013).

Types of Breast Cancers and Severity

Origin of the breast cancer is associated with breast tissues especially from the inner lining of the milk ducts and lobules (Eissmen *et al*., 2013). For prognosis, treatments and prediction, immunohistochemistry markers are used to assess nodal involvement, tumour grade, tumour size, surgical margins and histologic type. Further, estragon receptor, human epidermal growth factor receptor 2, progesterone receptor which are considered as immune histo chemistry markers are generally used in order to categorize breast tumour into subtypes. The common breast tumour types include, 1) either oestrogen receptor or progesterone receptor positive where HER2 is negative, 2) either progesterone receptor positive or oestrogen receptor positive where HER2 is positive, 3)

oestrogen receptor and progesterone receptor negative where HER2 is positive (HER2 positive) and 4) all oestrogen, progesterone and HER2 are negative (triple negative). Normally, cancers with either progesterone receptor or oestrogen receptor positivity have higher prognosis when compared to cancers with both progesterone receptor and oestrogen receptor negativity (Dai *et al*., 2016).

Treatments for Cancer

Treatment for cancer has become a challenge due to the complexity of the disease. Novel antineoplastic modalities such as immunotherapy, gene therapy, photothermal therapy, photodynamic therapy and hormonal therapy were introduced to treat cancer over the last decade. These remedies were able to induce cell apoptosis, alter gene mutations in angiogenesis process and cell cycle and adjust the gene transcription and translation conversions. However radiotherapy, chemotherapy and surgical excision still remains as the first line treatments for cancer (Kumari *et al*., 2016; Piktel *et al*., 2016). Out of them chemotherapy is one of the major therapeutic approaches that had been made for anticancer treatment, due to its systemic effect, usefulness as an adjuvant therapy, and can used to treat metastatic cancers. In addition chemotherapy uses in palliative care as well. The ultimate goal of chemotherapy is to deliver the drugs with the desired dose by giving sufficient circulation time for the absorption with the required half-life, to the site of action while preventing the normal cells and perform the therapeutic response (Shi, Gustafson & Mackay, 2014). However due to the side effects of chemotherapy, uses has become limited and unable to produce optimum efficacy (Zhang *et al*., 2011). Some of the major side effects facing during the chemotherapy are discussed below.

Limitations of Conventional Chemotherapy

Tumour resistance formation for drugs is one of the major disadvantage associated with chemotherapy in cancer. Tumour resistance mechanisms have several origins. ATP binding cassette known as ABC transporter pays an important role in drug resistance to several drugs. Mainly members of ABC (ABCC1, ABCB1, and ABCG2) family are expressed in different types of cancer and form the drug resistance to cancer (Liang *et al*., 2009). Multi drug resistance (MDR) is divided in to two main classes, secondary and adenosine triphosphate binding cassette base on their energy source (Leonessa, 2003). Possibly most widely considered ABC transporter is P glycoprotein (Pgp) which belongs to the ABC superfamily and use ATP to actively pump molecules in one direction across the lipid membrane. In cancer Pgp is over expressed and contributes to multi drug resistance by pumping chemotherapeutic drugs out of the cells. Because the Pgp transmembrane domain (TMD) is capable to identify chemically unrelated molecules and translocate them, mainly which are hydrophobic because ABC transporters permeates hydrophilic substances to pass through (Chang, 2003; Huang *et al*., 2016). More than 50% of cancer patients arises multi drug resistance. Furthermore drug resistance is may be due to intrinsic or extrinsic factor. At the beginning of the treatments intrinsic resistance is more common. Initially cancer cells are escaping from the exposure or repairing the damage and finally resistant cells survive and leads to acquired resistance. Both these pathways may initiate decrease drug accumulation, decrease drug activation, alter the drug targets and altered the gene expression. These may be found to be the drug barriers in MDR (Liang *et al*., 2009).

Delivering of the therapeutics agents in to the target site is one of the major challengers in the conventional chemotherapy (Kakde *et al*., 2011). Currently using drugs are distributed through the body via blood stream without preferential localization to the tumour area, while affecting the both malignant and normal cells which are rapidly dividing in the gut, bone marrow, lymphoid tissues, foetus and hail follicles (Piktel *et al*., 2016). For an example Epirubicin (EPI) and Anthracycline derivative are drugs which were used for hepatocellular carcinoma (HCC), had caused DNA destruction by disturbing the breakdown of the equilibrium and increasing of DNA topoisomerase II concentration covalent complexes. Therefore long term use of EPI was limited due to nonspecific toxicity to healthy cells. Mainly due to cardiac toxicity (Kumari *et al*., 2015).

Most of the prepared chemotherapeutic drugs have poor solubility in water and more hydrophobic (Dimendra *et al*., 2016).Therefore in order to avoid these conditions it is a necessity to produce drugs which can be administered intravenously. For an example paclitaxel in one of the most successful anticancer drugs in 1990s.

However this drug was extremely hydrophobic with no water solubility (0.5mg/L). The adjuvants which were used to solubilize the drug, including polyoxyethylate castor oil and dehydrated alcohol caused severe side effects like nephrotoxicity, cardiotoxicity, hypersensitivity reactions, hyperlipidaemia, abnormal lipoproteins patterns and erythrocyte aggregation (Kakde *et al*., 2011). Therefore in order to avoid the usage of adjuvants a new method of drug delivery was needed.

In addition chemotherapeutic drugs face lot of problems during transportation of the drugs to tumour sites. Physiochemical properties such as size, surface structure, and the charge plays an important role during drug delivery. Further due to heterogeneity of the cancer major difficulties are there when it comes to the drug delivery into whole tumour mass (Hare *et al*., 2016).

Likewise in the past few decades there were lot of problems in treating cancer patients with conventional chemotherapy. Therefore to overcome these difficulties scientists are investigating for new techniques.

Nanotechnology in Cancer Biology

In Nanotechnology materials and devices which less than 100nm in size use for engineering, characterization and synthesis of various applications in many fields. Applications of nanotechnology in medicine have accelerated diagnosis, screening and treatments of many diseases (Diaz & Vivas – Mejia, 2013). In cancer biology, nanotechnology can be used in various applications such as tumour detection, discovery of cancer biomarker and improvement of treatments.

Nanoparticles are small molecules that can be characterized as a whole unit in term of transport and properties (Tiwari *et al*., 2012; Prabhu *et al*., 2011). Nanoparticles in research filed has generated a passionate interest in scientists with the discovery of lipid based nanoparticles by Professor A.D. Bangham of the United Kingdom in 1960 (Puri *et al*., 2009).

Furthermore nanoparticles are the platform for effective and targeted drug delivery by overcoming many biological barriers with no or less side effects unlike the radiotherapy and chemotherapy. Furthermore nanotechnology based drug delivery system is a new method where target specifically for the cancer cells which have huge advantages over the conventional chemotherapy such as improvement in bioavailability and distribution of drugs ,longer shelf life , can administered with both hydrophobic and hydrophilic molecules through anywhere (oral, parental, nasal and intracellular routes)(Khanna, 2012).Therefore the new developments of nanotechnology based cancer therapies deliver a new ray of hope in the cancer research field (Calixto *et al*., 2016).

Nanoparticle Drug Delivery System

With the discovery of liposome, nanotechnology based targeted drug delivery system has made a significance impact on developing novel therapeutic approaches for many diseases as well as in cancer therapy (Puri *et al*., 2009; Shi *et al*., 2011). Different specialized nanoparticles such as liposomes, dendrimers, gold nanoparticles , silver nanoparticles , polymeric nanoparticles, carbon nano tubes, virus mediated nanoparticles, magnetic nanoparticles (table 1) and many other novel nanoparticles were introduced and will be introducing in the near future in to research field (figure 3)(Jabir *et al*., 2012).

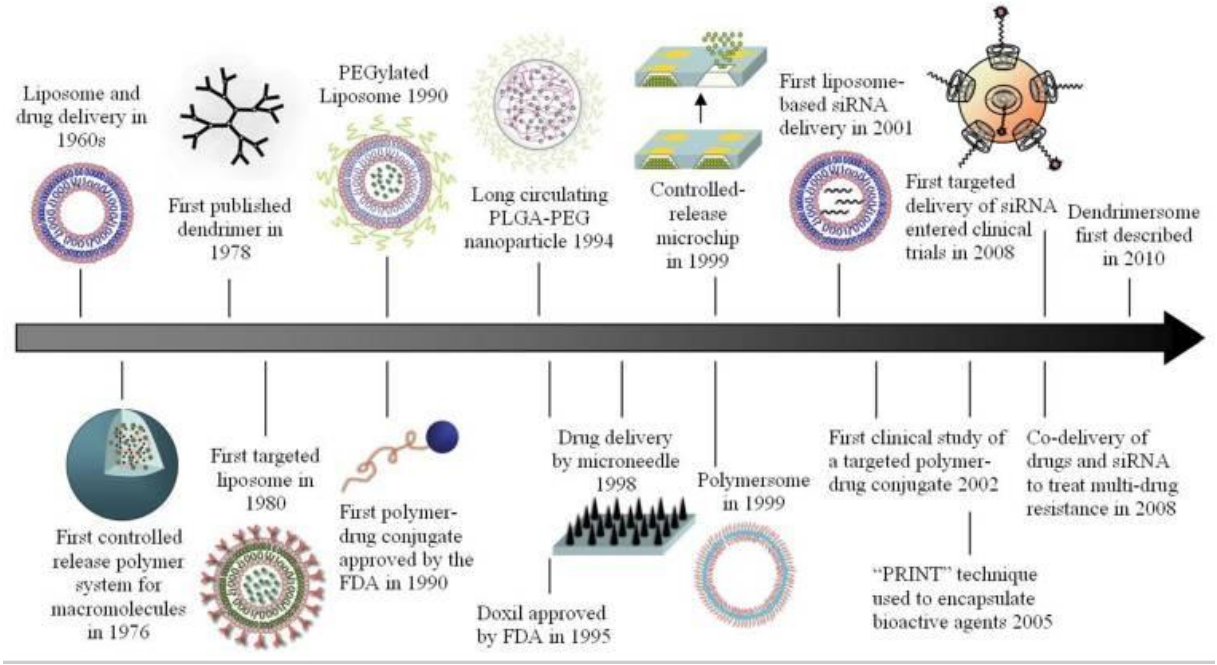

Figure 1 Development of nanotechnology based drug delivery (Shi *et al*., 2011)

Table 1 some of the nanoparticles with their characteristics associated with cancer therapies.

Name of the nanoparticle	Description	References
Lipid based nanoparticles	Liposomes are self-assembling and has a colloidal structure consist with lipid bi layer. Outer most layer of the liposome surrounds by a central aqueous area Via PEGylation, liposome can be surface functionalized and can promote receptor-mediated endocytosis by using targeting ligands such as peptides, proteins, carbohydrates and antibodies and also PEGyalation increase the circulation half-life while reducing the clearance and immune recognition. These liposome are ideal vehicles to deliver hydrophobic drugs without stimulating an immune response. Furthermore liposomes are the most clinically successful nanocarriers	(Cho *et al*., 2008; Lammers *et al*., 2008; Miller, 2013).
Nanodiamonds	Nanodiamonds can be divided in to two main type fluorescent nanodiamonds and detonation nanodiamonds. These particles are led to applications because of their favourable properties such as the facet surface possess electrical charge where the drug can be attached to the neutral surface. And has electrostatic charge allowing to disperse in the fluids. Furthermore nanodiamonds are nontoxic cheap in production. Nanodiamonds have a greater biocompatibility and stable and easy to purify .There are ongoing researches on with doxorubicin attached nanodiamonds	(Ho *et al*., 2015).

Virus mediated nanocarriers (VNPs**)**	With a great efficiency viruses are able to infect naturally to a host cell and delivery the payload of the genetic materials with response to Ph changes, chemical stimuli, temperature and redox status These phenomenon was taken to design an ideal virus mediated nanocarriers. Plant based viruses are considered as the safe delivery vehicles as they biocompatible, biodegradable, nontoxic and less likely to trigger downstream effect in humans Furthermore well characterized monodisperse structures which can be produced in large quantities are some advantages of VNPs. VNPs are one of the most advanced ad versatile nanomaterial produced by nature because of the highly symmetrical structure. The basic structure of the VNPs has an internal cavity which can be filled with drugs quantum dots and other particles and external surface consist of ligands for the cell specific delivery	(Ylidiz *et al*., 2011; Van Kan-Danvelaar *et al*., 2014; Khodabandehloo *et al*., 2016).
Magnetic nanoparticles	Under an external magnetic field magnetic drug delivery system can be work with loaded drugs to reach the tumour site in a more localized way. Also they can be synthesized and functionalized in different sizes and structures according the loading and targeting molecules). Synthesizing of these have several strategies they may have core shell structure where the core if formed either magnetite or maghemeite and the shell is made up of cationic materials such as, polyethylene oxide, poloxamers, silica, metals such as gold, dextran, polyethylene glycol; (PEG) while providing the functional groups to be attached via cross links in order to minimize opsonisation and trigger the binding to drugs and other molecules	(Dobson, 2006; Fang, 2009; Yang *et al*., 2012; Mody *et al*., 2014; Gobbo *et al*., 2015).
Carbon nanotubes	These are huge cylindrical shape large molecules consist with a hexagonal arrangement of sp2 hybridized carbon atoms. The wall is consist with multiple layers of grapheme sheets, formed by rolling simple sheet. These are used to detect protein, DNA and carriers to deliver vaccine. These are insoluble in all solvents. Carbon nanotubes can be used to deliver drugs via intravenous routes.	(Cho *et al*., 2008; Madani *et al*., 2011 Zhang *et al*., 2011; Kushwaha *et al*., 2013).

Size and the surface characteristics are one of the most important features in nanoparticles. The size of the nanoparticle should be maintain in a tuneable manner which means their size need to be large enough to prevent leakage into blood vessels and small enough to escape from macrophages and penetrate into tumour site(Singh & Lillard, 2009). In addition surface structure is important in determining the life span escape from macrophages. Therefore nanoparticles can be functionalized in order to achieve these characteristics with pegylation (Cho *et al*., 2008).

The development of targeted therapy systemically represent delivered nanoparticles reaching to the tumour tissues overcoming the obstacles with the minimal loss of volume and activity (Jin *et al*., 2014). Potential effect of nanoparticles describes by the different targeting strategies towards the tumour site (Kamal *et al*., 2012). Therefore delivery of nanoparticles into the tumour site can be presented in two major pathways, passive and active targeting.

Passive Targeting

Passive targeting can be mainly achieved through enhanced vascular permeability (EPR) effect (figure 3) (Piktel *et al*., 2016).

EPR effect mediated the delivery of nanoparticles into the tumour site because of the defective structure of the blood vessels in order to ensure adequate supply of nutrients and oxygen to the tumour cells. Which means tumour angiogenesis is defective and characterized by leaky epithelium, impaired lymphatic drainage, and reduction in up taking the intestinal fluid. Furthermore consequent accumulation of the macromolecules provides a supportive environment to the passive targeting. With the accumulation of macromolecules, nanoparticles also get into the tumour site as a result of EPR effect (Piktel *at el*., 2016).

Properties of the nanoparticles such as size, circulation half-life, pH of the medium and surface nature are also important factors which need to be considered in passive targeting (Barkat *et al*., 2011).

Active Targeting

Active targeting is specially based on ligand – mediated or antibody mediated specific interactions between cancer cell surface and NPs (figure 4) (Zamboni *et al*., 2012; Jin *et al*., 2014; Wu *et al*., 2015). Furthemore this way of targeting is more specific and can be reduce the cytotoxicity to the normal cells (figure 4) (Jabir *et al*., 2012; Barkat *et al*., 2015).

This phenomenon is attaching homing ligands on the surface of the nanosystem and enabling active binding with receptors which are overexpressed in the cancer cells. There are a wide ranges of targeting molecule examples for ligand based targeted molecules which can be used as nanocarriers surface modifiers are peptides, proteins, folic acid, glycoproteins (transferrin), nucleic acids, monoclonal antibodies or their fragments and aptamers (short single stranded RNA or DNA EGFR, transferrin receptor and CD22 or CD44 are some of the receptors present in tumour cells. . Subsequently tumour specific ligands interact with the receptors in the cancer tissues and triggers the receptor mediated endocytosis and internalization of the nanocarriers into the tumour cell (Grodzinski, 2014).

Nanoparticle Based Targeted Drug Delivery Systems for Breast Cancer in Clinical and Pre -Clinical Studies

In order to develop advanced nanoparticle based drug delivery system several clinical and pre-clinical studies have been done and some of them are already approved by the FDA. Some of the clinical and pre-clinical studies which give evidence about the advancements of this system are given below.

Doxorubicin is an anthracycline anticancer drug which has been used as a chemotherapeutic agent for the treatments of several cancer types such as breast cancer, tissue sarcoma, ovarian cancer and leukaemia. Action of doxorubicin includes DNA intercalation, topoisomerase II inhibition, and lipid peroxidation. But free doxorubicin has fatal side effects such as alopecia, nephrotoxicity, cardiotoxicity and renal failures. Therefore in order to reduce the side effect doxorubicin was encapsulated into liposomes (figure 1). In 1995, liposomal based doxorubicin (DOX) was approved by the FDA. As shown in figure 1 myocet and Doxil is the first approved drugs. Drugs' pharmacokinetics shows elimination half-life of Dox is 55 hours and 900μg h mL^{-1} area under the plasma concentration, myocet 2.5 hours and 45 μg h mL^{-1} and elimination half – life of free doxorubicin is 0.2 hours 4μg h mL^{-1} longest circulation time has Doxil when compared to free drugs (figure 3). This blood circulating time is increased in liposomes with the reduction of size, pegylated surface and with lipid bilayer. In a phase III clinical trial which was conducted to treat metastatic breast cancer had showed a comparison with DOXIL and myocet shows less cardiac toxicity, neutropenia, vomiting and alopecia (figure 2)(Chang and Yeh, 2011).

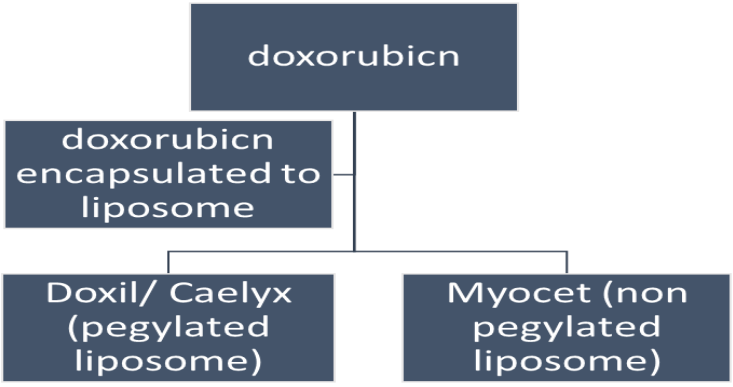

Figure 2 Encapsulation of the doxorubicin with liposomal nanoparticles.

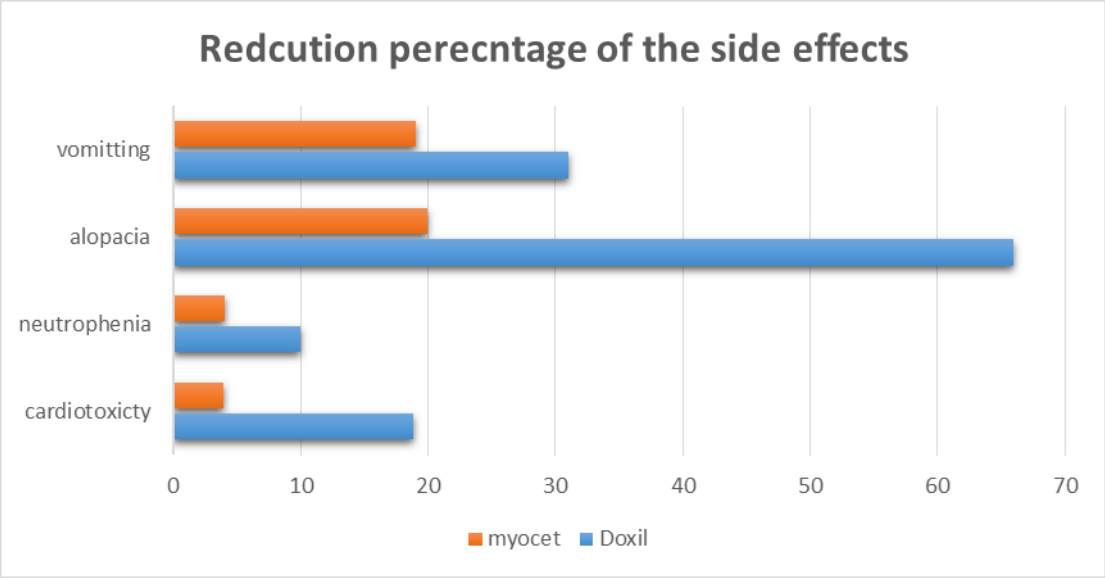

Figure 3 Reduction percentages of the side effects

Another study was conducted to target overexpressed HER-2 receptor by using liposomal doxorubicin. In this study pegylated liposomal was designed to target HER-2. The study was conducted using BT474 –M3 breast cancer cells which were inoculated to mice and had showed that HER-2 targeted liposomal doxorubicin has effectively been internalized into the tumour cells than free drug while inhibiting the tumour growth.

Another pre – clinical study has been done on integrin αvβ3 is a subset of a tumour blood vessel which is associated with malignant tumour growth and angiogenesis. Therefore a nanoparticle was designed to target αvβ3 by encapsulation doxorubicin (Dox). After synthesizing the targeted nanoparticle (RGD-NP) in order to address the specificity of the nanoparticle to integrin αvβ3 binding studies were conducted using human umbilical vein endothelial cells (HUVECs), which has higher amount of integrin αvβ3 with targeted peptides and controlled peptides and had shown a complete binding inhibition with control peptide and binding and internalization with the target peptide, which shows the advantage of active targeting strategy. Then RGD-NP targeting to the tumour vasculature were tested using M2IL-GFP mouse melanoma cells (integrin negative) and was allowed to get vascularized for 7 days. Then RGD –NP (targeted) and RAD –NP (none targeted) were injected and observed. Within 2 hours of injection RGD –NP targeted the newly formed vasculature in the margin of the tumour and reached maximum binding after 5 hours of injection, though RAD-NP was not accumulated in the tumour. After that in order to study anti-angiogenic action of the targeted nanoparticle mice which have stromal cells loaded with basic fibroblast growth factor on the flank and injected DOX (1mg/kg total

DOX) loaded nanoparticle. After 7 days angiogenesis was measured and showed 70% inhibition of angiogenesis. Therefore with the previously experimented targeting RGD-NP is an antagonist for integrin αvβ3 and targeting of DOX loaded NP produce a strong anti angiogenic effect on vasculature (figure 4) (Murphy *et al*., 2008). Which proves that nanoparticles conjugated with DOX can be easily targeted via active targeting and does it action at the desired site of action.

However liposomal based nanoparticles has showed dose limiting side effects such as palmar plantar erythrodysesthesia (PPE) or hand foot syndrome small amounts of drug can leak into palms and soles of the feet and can be resulted in tenderness, redness and peeling of the skin (Rafiyath *et al*., 2012).

Another clinical study was conducted with Trastuzumab (TZ) which is a monoclonal antibody approved by the Food and Drug Administration. Combining TZ with other chemotherapeutics, taxanes and anthracyclines, has given significant improvement of disease-free survival rates.TZ-DTX (docetaxel) combination treatment had a greater chance of survival than free DTX alone and had a lower incidence of febrile neutropenia and symptomatic heart failure.

Cytotoxicity of the DTX-loaded dendrimers was significantly higher than free DTX. Targeted dendrimers were more effective than non-targeted ones.TZ-Dend-DTX was more cytotoxic than Dend-DTX due to the interaction between TZ and HER2 receptors and internalization of receptor-mediated endocytosis is more efficient.

One of the most significant breast cancer antitumor drug, Paclitaxel (PTX) has an action in microtubule assembly stimulation however prevents the microtubule disassemble. Therefore the hindrance of microtubule functions like cell transport, mitosis and cell motility occurs. The specificity of drug is less therefore this action affects all fast dividing cells. Thus causing many side effects. To avoid these side effects SWNT-HSA (single wall carbon nanotube with human serum albumin) nanoparticle was introduced. Due to high demands of oxygen and nutrients the vascular permeability has been enhanced in fast growing tissues like tumours. A main feature of tumour vasculature is a defective endothelium due to poor alignment. The EPR effect or passive targeting strategy allows the nano-particles to accumulate in the tumour tissue. Therefore passive targeting allows PTX a more targeted specific approach than free drug. An in vitro study was carried out using MCF-7 breast cancer cell lines to study the drug efficacy with SWNT-HSA and PTX alone. In here HSA has been used as an adapter to load PTX into SWNT. The percentage of apoptosis of the cells treated with free PTX, HSA/PTX and SWNT-HSA/PTX was recorded (Figure 10). In comparison a gradual increase was identified proving the effective action of the nano-particles in the improvement of inducing apoptosis in tumour cells (Shao *et al*., 2013).

Limitations of Nanotechnology Based Targeted Drug Delivery Systems and Future Perspective

It is undebatable that nanotechnology offers various novel therapeutic approaches in drug targeting and delivering in anti-cancer therapies. But in some cases the enthusiasm need to be suppressed due to several reports on some limitations facing with nanotechnology based targeted drug delivery system. Accumulation of the nanoparticles and pharmacokinetic properties cannot be predicted easily by using their physicochemical properties (surface properties, size, and charge). Also when nanoparticles are consisting with resembling size and shape they are not strong enough to perform the desired task. Therefore there is an urge to develop a strategy to identify the exact properties of nanocarriers (Piktel *et al*., 2016).

Nanoparticles based cancer therapy may also responsible for several toxic effects too. Due to the small size these can easily penetrate into the healthy tissues and can cause damage also can be accumulated in kidneys without proper excretion. Regardless of the mobilization of cancerous agents, nanomaterials should improve biocompatibility as well. For an example administration of carbon nanotubes had been resulted in increasing the fibrotic reactions and inflammatory factors, by accumulation in airways (Piktel *et al*., 2016). Further as discussed earlier several nanoparticles such as liposomes tend to develop hand foot syndrome.

Summary

Nanotechnology is a rapidly growing filed while expanding its area in science. Also it plays an indispensable role in cancer biology therefore this drug delivery can be used specifically in breast cancer. This area of research

is expected to develop novel, erudite and multifunctional applications in cancer diagnosing, treatments and prognosis. Researches on nanotechnology is vigorously aimed towards achieving cite specific targeting while overcoming the limitations in cancer treatment strategies especially chemotherapy. Nanotechnology based targeted drug delivery systems using functionalized nanoparticles give a new ray of hope towards chemotherapy based issues such as efficacy, safety, drug accumulation drugs induce toxicity and MDR. Clinical and pre-clinical studies pointed out that nanoparticles can be used for effective treatment with no or less side effects. Furthermore some of the nanotechnology based targeted drug delivery system are approved and are already being used in treatments. Therefore applications of nanotechnology based drug delivery systems in breast cancer treatment can be used as an advanced method of treating patient with cancer with minimum suffering. Several clinical and pre-clinical studies have showed that how this nanotechnology based targeted drug delivery has been effectively used in breast cancer therapy.

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PRODUCTION AND DETECTION OF L-(+)-LACTIC ACID USING CASSAVA AS THE LOW COST FERMENTATION MEDIUM FOR THE SYNTHESIS OF BIODEGRADABLE POLYMERS AS ORTHOPEDIC DEVICES

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Abstract: Polymers based on Lactic acid are of great importance to the healthcare industry because they decompose by hydrolysis in the human body in to nontoxic metabolites. Therefore, the objective of the current study was to produce L-(+)-Lactic acid using a low cost medium in order to synthesize a biodegradable polymer for healthcare industry. Powdered cassava was acid hydrolyzed using different concentration of HCl (0.5, 1, 1.5, 2, 2.5, 3, 5, 8 and 10 %) followed by a thermal treatment at 120 ^{O}C for 10 minutes. Five different media (A- E) were prepared from the above hydrolyzed product along with yeast extract. *Lactobacillus casei* was inoculated to media A (Cassava- 5 g, Yeast extract-1.5 g, K_2HPO_4- 0.41 g, KH_2PO_4- 0.56 g), B (Cassava- 5 g, Yeast extract 1.5g, $MgSO_4.7H_2SO_4$- 1 g, $(NH_4)_2SO_4$-1 g), C (Cassava- 5 g, Yeast extract- 5g, K_2HPO_4- 2 g, NaAc- 5 g, $MgSO_4.7H_2SO_4$- 0.02 g, $MnSO_4.4H_2O$-0.05 g), D (Cassava- 5 g, Yeast extract- 1.0g, K_2HPO_4- 0.41g, $MgSO_4.7H_2SO_4$- 1g, $(NH_4)_2SO_4$-1g), and E (Cassava crumble- 5 g, Yeast extract- 1.0g, K_2HPO_4- 0.41g, $MgSO_4.7H_2SO_4$- 1g, $(NH_4)_2SO_4$-1g). Medium A, B, and C were inoculated with *L. delbrueckii*. The lactic acid produced by each species in different media were quantified using Agilent 1260 infinity HPLC. According the Benedict test the 2 % HCl was obtained to provide efficient amount of L-(+)-lactic acid via acid hydrolysis. Medium D fermented with *L. casei* was identified as the effective low cost medium for large scale fermentation. The lactic acid concentration of the medium D was detected as 259 mg/L. Medium D was identified as effective medium for large scale fermentation for the production lactic acid with *L. casei*. Purification and polymerization need to be performed in order to synthesis the biopolymer.

Keywords: L-(+)-Lactic Acid, Cassava, *Lactobacillus casei*, *Lactobacillus delbrueckii*

Background

Many scientists who work with polymers are closely working with new inventions for devices such as instruments for medical fields. Polymer scientists have gained tremendous advances over the past 30 years, regards to their inventions. These inventions have a general criteria for selecting a polymer, in which the biomaterial selected as to match the mechanical properties and the time of degradation of the application; thus fulfil the needs of the of the invention (Middleton and Tipton, 2000).

Lactic acid based polymers have deserved prodigious attention, because of the decomposing process via hydrolysis metabolism; which in the human body is convert into nontoxic metabolites. Biodegradable polymers such as Poly L-Lactic Acid (PLLA) and Polyglycolide (PGA) are produced from renewable sources which contain sugar and starch. These polymers play important role not only in the plastics industrial and in the biopolymers for medical industry (Kaihara *et al*, 2007).

In recent years, the health care sectors use Poly Lactic Acid (PLA) in many ways. Especially the PLLA is used as a representation of the orthopedic metal implants; this is because of PLLA is more strong and harmless to human. The implant using metal plate are considered expensive and could be only removed via a surgery. Despite the main drawback, it also obtains a time period for the wound to heal properly, due to process of removing the metal implants via a surgical operation. Therefore, the metal implantation is considered as a time and energy scarification. Hence, the PLA is used as a biopolymer, which would biodegradable and is nontoxic to humans. Another reason is that, PLA could be fermented easily using natural fermentation process, thus it is considerably low cost compared few metal implants (Agrawal et al, 1995).

According to Nampoothiri in his article in 2010, he has quoted that the estimated "price of PLA is ca. 2.2 $/kg"; thus this means in an industrial production, the price of confined lactic acid ought to "be less than 0.8 $/kg" (Nampoothiri *et al*, 2010). One of the key feature in reducing the price of production of lactic acid is by the cost of raw material; which is used for funding the microbial growth in the fermentation medium. This is also plays an important role when it comes to the growth of fastidious lactic acid bacteria (Zaunmuller *et al*, 2006 and Nampoothiri *et al*, 2010).

The PLA polymer production involves not only a chemically pure lactic acid, but also an optically pure lactic acid. The chemical purity is mainly contingent on the fermentation medium constituents; especially to provide cheap sources of sugar from raw material. Whereas, the optical purity of PLA is certified by the optical purity of lactic acid, for which the production of lactic acid is proceeded through several strains of microorganisms under optimized fermentation conditions. In contrast to other fermentation harvests, lactic acid production via monosaccharides is 90 % high (Abdel-Rahman *et al*, 2011).

The PLA provides both high melting point and crystallinity (Ghaffar *et al*, 2014). Lactic acid has two optical isomers, L-(+)-lactic acid and D-(−)-lactic acid (Narayanan *et al*, 2014). It is classified as GRAS (generally recognized as safe). The production of PLA requires high optical purity. Therefore, the properties of PLA are adjusted accordingly to a ratio of the L- and D-PLA to form the co-polymer, in which D-form increases the melting point of the copolymer. Both the optically pure L- and D- lactic acid are efficiently achieved by microbial fermentation, thus presently 95 % of industrial lactic acid production is supported by microbial fermentation process (Ghaffar *et al*, 2014).

The lactic acid production by LAB (Lactic Acid Bacteria) uses the biological pathway classified as homo fermentative method, since LAB's hexose metabolism is under the non-restrictive conditions, which is entirely through the pathway of Embden-Meyerhof to pyruvate pathway; this is then used to redevelop the reducing power of NADH in the lactate dehydrogenase (LDH) catalyzed reaction to lactic acid. Nevertheless, at times such as low glycolytic flux and slow growth rate help in the formation of acetic acid, formic acid and ethanol, as well to lactic acid (Zaunmuller *et al*, 2006).

The advantage in microbial fermentation is in synthesizing one of the isomers, and producing an optically pure product by selecting a particular strain the lactic acid bacteria (LAB). Meanwhile in a synthetic production the outcome is a racemic mixture of lactic acid. The optically pure lactic acid production is essential in the synthesis of polymer construction. Additionally, due to the presence of L-lactate dehydrogenase, L-(+)-lactic acid is recycled by human metabolism (Jarvi's, 2001).

Now a days, the production of L-(+)-lactic acid is used enhance the economics of the lactic acid fermentation process, it is used to increase the concentration of lactic acid in the medium via optimization of fermentation medium (Panesar *et al*, 2010). The present work was, therefore, carried out to optimize the medium for efficient lactose conversion in cassava powder to L-(+)-lactic acid.

The process fermentation originates from a sugar known as the substrates. Few carbohydrate such as, potato starch, corn starch, milk whey and molasses are used as the substrate for the production of lactic acid. Sugars

hydrolyzed from different substances of starch and molasses are plentifully used substrate when it comes to industrial production of lactic acid via microbial fermentation process (Fakhravar *et al*, 2012).

Nevertheless, choice of substrate is important depending upon factors such as (1) its availability in the area, (2) treatment required prior to fermentation, and (3) the processing costs. It is also noted widely, in the production of lactic acid via microbial fermentation using pure sugar is the best substrate but the purification process of sugar is most possibly an expensive process (Fakhravar *et al*, 2012). It is a main reason using cassava as the main sugar substrate in this study, as to the availability and high starch contend in them.

The lactic acid production for commercial manufacture via fermentation technology is mainly influenced by the raw material used; the main important factor while using raw material depends mainly on the a cost efficiency of the raw material. Therefore, it is important to select a raw material for industrial production of lactic acid with a number of characteristics such as the following (1) low cost, (2) rapid rate of fermentation, (3) lowest amount of contaminants, (4) high yields of lactic acid production, (5) least or no formation of by-products and (6) availability for whole year (Ghaffar *et al*, 2014).

In Sri Lanka cassava is not considered as a nutritionally higher crop but due to the crop's drought resistance, relative resistance from pest attack and the ability to yield under unfavorable conditions with less requirements plus attention put in it, makes it the highest grown crop in the agriculture list. Thus, it is found throughout the island apart from high raise areas concentrated with the wet and intermediate zones of Sri Lanka. Though cassava is considered as a backyard crop, it is also carried out in large scale cultivation in open-lands of the wet zone such as Gampaha, Colombo, Kegalle, Rathnapura and Matara Districts; in intermediate zone of Kurunegala district. While large scale production also occurs in the dry zone which includes Putlam, Anuradhapura, Ampara, Hambantota, Moneragala Districts. Cassva plant production is peak during falls of Maha season. (Department of Agriculture, 2006).

Furthermore, cassava produces the highest amount per unit of area of soluble carbohydrates in its conversion from solar energy. Among all the starchy sources, the cassava gives higher production carbohydrate which is about 40 % is higher than rice and 25 % more than maize. The composition of the cassava is includes of 70 % of moisture, 24 % of starch, 2 % of fiber, 1 % of protein and 3 % of other substances including minerals (Tonukari, 2004).

During the microbial production of lactic acid major impurity is the cell mass, which is separated easily from the product. The important economic factors in the industrial production of lactic acid via fermentation process includes the following (1) the optimization of the production medium, (2) high product yields, (3) productivity, and (5) the concentration of products formed, which ultimately influences the down-stream processing costs (Abdel-Rahman *et al*, 2011).

Objective

The main objectives of the current study was to select a *lactobacillus* strain which efficiently produces L-(+)-Lactic acid and to develop a cost effective medium using Cassava as the main substrate to produce high yield of L-(+)-Lactic acid, in large scale fermentation.

Methods

Processing Cassava Powder

Fresh cassava was washed with water and both outer dry and inner layers were removed. The peeled cassava was the cut into small pieces and dried at 65 ^{o}C for 48 hours (hrs). The dried cassava was ground to make a powder using a motor and pestle. The acid hydrolysis of the starch containing cassava was performed using different concentrations of HCl (0.5, 1, 1.5, 2, 2.5, 3, 5, 8 and 10 %). A weight of 1 g of cassava powder was mixed with

different HCl solutions having different concentration and kept at 120 ºC for 10 minutes (mints). The amount of reducing sugar produced by different concentration of acid was detected by Benedict's test (Pratt, 2011).

Selection of microorganisms for fermentation

Lactobacillus casei (ATCC No: 393) and *Lactobacillus delbrueckii* (ATCC No: 15808) were used as the bacterial strains to produce L-(+)-Lactic acid (Panesar *et al*, 2010 and Chang *et al*, 1999).

Preparation of a Pilot Scale Fermentation Medium

The lactic acid fermentation was conducted with a 100 ml of acid hydrolyzed cassava product namely; medium A, B, C, D, and E enriched with some nutrient sources (Table 1). Each medium was neutralized using 2% HCl and 2 N NaOH (pH 5.5). The media A, B, C, D and E were fermented with *L. casei* and *L. delbrueckii* separately. The fermentation was carried out in a shaking incubator for 48 hrs at 37 ^{O}C for 150 rpm.

Table 1. Composition of different media used

Medium	Composition
A	Cassava- 5 g, Yeast extract-1.5 g, K_2HPO_4-0.41 g, KH_2PO_4-0.56 g
B	Cassava- 5 g, Yeast extract 1.5g, $MgSO_4.7H_2SO_4$- 1 g, $(NH_4)_2SO_4$-1 g
C	Cassava- 5 g, Yeast extract- 5g, K_2HPO_4- 2 g, NaAc- 5 g, $MgSO_4.7H_2SO_4$- 0.02 g, $MnSO_4.4H_2O$-0.05 g
D	Cassava- 5 g, Yeast extract- 1.0g, K_2HPO_4- 0.41g, $MgSO_4.7H_2SO_4$- 1g, $(NH_4)_2SO_4$-1g
E	Cassava crumble- 5 g, Yeast extract- 1.0g, K_2HPO_4- 0.41g, $MgSO_4.7H_2SO_4$- 1g, $(NH_4)_2SO_4$-1g

Detection of L-(+)-Lactic Acid Using High Performance Liquid Chromatography (HPLC)

The media were centrifuged initially at 4000 rpm for 20 mints at 4 oC and supernatant was re-centrifuged at 12000 rpm for 15 mints at 4 oC in order to remove impurities. The High Performance Liquid Chromatography was conducted with Ultra Violet (UV) wave length 210 nm and Refractive Index Signal (RIS). A volume of 5 µl of the supernatant was filtered and injected into the pump of HPLC. Phenomenex Rezex ROA H+ (300 x 7.8 mm, 8 µm) was used as cation exchange column and deionized water was used as the mobile phase. A temperature of 30 oC was provided with a flow rate of 0.4 mL/min. The peak area corresponded to the L-(+)-lactic acid was used to quantify the amount of L-(+)-Lactic Acid. The medium which was given the high yield of L-(+)-lactic acid was identified for large scale fermentation.

Preparation of Medium for Large Scale Fermentation and Harvesting

The medium D was prepared for 14 Liters (L) consisting 200 g of acid hydrolyzed cassava, 40 g of yeast extract, 16.4 g of K2HPO4, 20 g of MgSO4.7H2SO4 and (NH4)2SO4. The large scale fermentation was performed using New Brunswick ™ BioFlo® 415 SIP Fermentor for 5 continuous days. A sample was drawn in the first day prior to the inoculation of microorganisms (negative control) and 3 samples each from day 1, 2, 3, 4 and 5 were obtained

after the microbial inoculation. The fermentation was repeated for two times and quantity of the L-(+)-lactic acid produces in each day was detected using the HPLC as described above.

Results

Acid hydrolysis of cassava

The Benedict's test provided different amount of reducing sugars. Green color precipitate indicated 0.5 % of sugars, while yellow color precipitate as 1 %, orange color as 1.5 % and brick red color indicated as 2 % of reducing sugar levels. The results of the Benedict's test are shown in table 2. The highest amount of reducing sugar was observed from the cassava product which hydrolyzed with 2 % HCl

Table 2. Results of the Benedict's test

Concentration of HCl added (%)	Results
0.5	+
1	+
1.5	+
2	++
2.5	++
3	++
5	-
8	-
10	-

+: Low green high yellow precipitate

++: High yellow color precipitate

-: No color change (Negative)

Table 3. Results of pilot scale fermentation of different media using 210 nm

Medium	milli Absorbance Units per second (mAU*s) by each bacterial strain	
	L. casei	*L. delbrueckii*
Standard	225.1	225.1
A	454.0	99.5
B	374.5	32.7
C	1683.7	738.3
D	1076.5	635.5
E	483.8	103.6

Quantification of Lactic Acid Detection by HPLC for small scale fermentation

The HPLC results of different media are given in table 3. The highest milli Absorbance Units per second (mAU*s) was observed from the C and D media fermented with L. casei bacterial strain. However, the medium contained some constituents in the MRS media which the ingredients are costly.

Therefore, it is not suitable to use as a low cost fermentation medium. Hence, the medium D was identified as the suitable medium. All media which fermented with L. delbrueckii were resulted low mAU*s values with compare to L. casei (Table 3). Therefore, L. casei indicated high L-(+)-lactic acid concentrations in the fermented products (Figure 1).

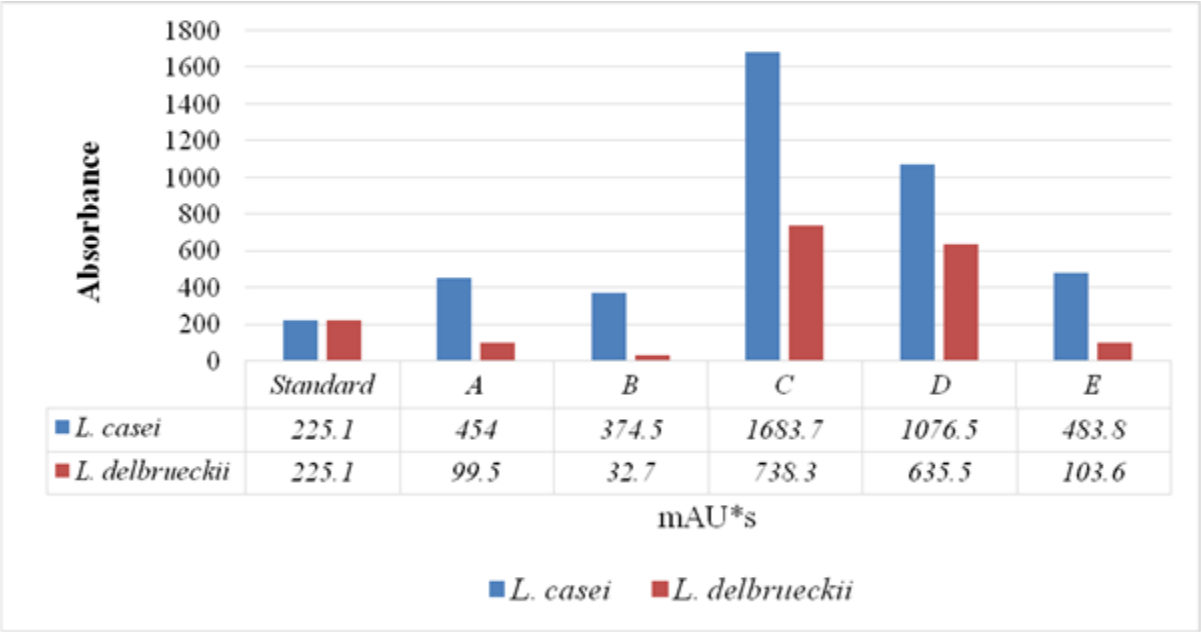

Figure 1. Productivity of L-(+)-lactic acid in five different media fermented with *L. casei* and *L. delbrueckii*

Quantification of Lactic Acid Detection by HPLC for large scale fermentation

The concentration of L-(+)-lactic acid detected from each sampling day is shown in table 4. Overall, the highest concentration of L-(+)-lactic acid was identified in the fourth day of the fermentation process.

The medium was changed in to light brown to dark brown with increasing time. It was further observed that the viscosity of the medium was also increased with respect to time. However, after the fourth day of fermentation, the quantity of L-(+)-lactic acid was gradually decreased. This may be due to conversion of lactic acid in to pyruvate by lactate dehydrogenase enzyme activity of the microorganisms.

Table 4. Results of large scale fermentation of D medium with L. casei using HPLC at 210 nm

Sampling day	milli Absorbance Units per second at 210 nm	Concentration of L- lactic acid g/L
01	604.4	0.15
02	1076.5	0.29
03	1522.4	0.43
04	8657.3	2.57
05	6526.5	1.93

Validation of Results

The concentration of the L-(+)-lactic acid was determined using a standard curve (Figure 2) using a stock solution of L-(+)-lactic acid having 1.1 mg/l concentration. The R value of the According to the standard curve, the R value was 0.99.

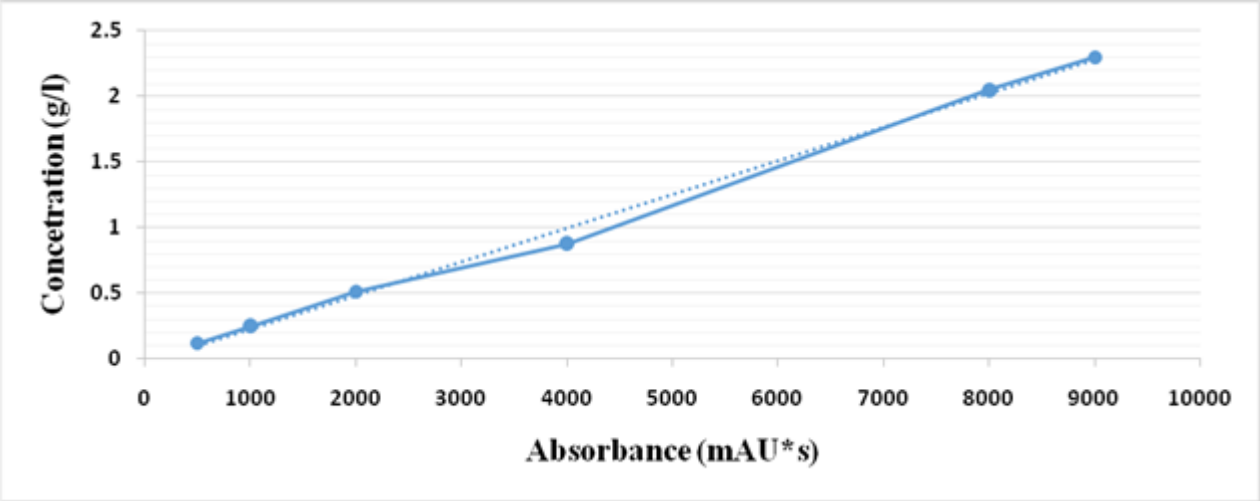

Figure 2. Standard curve

Discussion

Poly lactic acid or poly lactide (PLA) is a thermo plastic and biodegradable aliphatic polyester derived from naturally occurring organic acid (lactic acid). It is inexpensive, dimensionally stable and harder than Polytetrafluroethylene (PTFE). It melts at lower temperature (180 - 220 ^{0}C) with glass transition temperature 60 - 65 ^{0}C. Due to their excellent biocompatibility and mechanical properties, it has been used extensively in different fields such as polymer engineering, tissue engineering drug delivery systems and various medical implants of paramount significance such as sutures in surgical applications. It composites many applications in biomedical devices from fibers to subcutaneous sutures and to regenerative surgery implants (Pawar *et al*, 2014). This has also become a promising eco-friendly biopolymer for use in the human body.

The *Lacto bacillus* genus has been generally known as the major producer strain of lactic acid. These organisms have limited ability for synthesizing their own growth factors, mainly B vitamins and amino acids. Typically, they require carbon and nitrogen source sand diverse elements in the form of carbohydrates, amino acids, vitamins and

minerals. In addition, the use of assortments of amino acids, peptides and amides, stimulate growth of Lactic Acid Bacteria (LAB) yielding much higher values than those obtained with free amino acids. LAB growth is also influenced by fatty acid and phosphate which are the most important salt in the LA fermentation (Quintero *et al*, 2013).

However, the process of microbial fermentation of lactic acid is extremely expensive procedure for a developing country. This leads to the use of other sources such as soybean, potato, wood, corn liquor, and molasses are to be used for the production of lactic acid. Among these, starchy grains like sweet sorghum, wheat, corn, cassava, potato, rice and barley, and cellulosic materials, are widely used having the advantages such as low price, availability and renewable characteristics over other sources (Farooq *et al*, 2014 and Wee *et al*, 2006).

The fermentation process also requires the nitrogenous materials for the growth of the microorganisms. This need is satisfied by utilization of whey permeate, yeast extract, malt sprouts, grass extract, peptones, beef extract, casein hydrolyte with supplementation of vitamins that have been used with the carbohydrate source for fast and heavy growth. Among these Yeast extract is found to be the most effective supplement, thus it was utilized a nitrogen source in the research as well (Quintero *et al*, 2013).

It is noticed that for many lactic acid bacteria (LAB) strains cannot serve only utilizing ammonium as the primary nitrogen source; nevertheless, some display influence on the amino acid metabolism of the LAB strain. Conversely, the amount of minerals found in commercial complex media seems to be sufficient for lactic acid metabolism. With the expectation provided, it can be specified that the cassava flour is the main fundamental constituent for the optimized media in the research. Thus, cassava has become a suitable substrate for present study, aimed to biosynthesis of lactic acid via a microbial fermentation process. According to Tonukari, 2004 (Tonukari, 2004), 81.48 % cassava flour has 99% of starch hydrolyzed, when 300 g/L of cassava flour medium was prepared, accomplishing about 76% of glucose content; the residual part is conducted with malto dextrines.

The current results suggests that the medium D containing K_2HPO_4, $MgSO_4.7H_2SO_4$, and $(NH_4)_2SO_4$, with acid hydrolyzed cassava and yeast extract are more suitable as a low cost fermentation media since it does not contain costly substances. Based on this analysis, and besides reducing sugars, cassava flour has different ions including sodium, iron and magnesium, amino acids and proteins that might have favored bacterial growth and product biosynthesis (Afolabi *et al*, 2012).

Even though *Lactobacillus* is a facultative anaerobic organism, its metabolic activity is enhanced in the absence of oxygen, among other factors, by the need of the oxidized form of the cofactor NAD^+, which is reduced during the catabolic activity. The NADH donates its extra electrons to the pyruvate molecule formed during glycolysis; since the NADH has lost electrons, NAD^+ regenerates and is again available for glycolysis. However, *Lactobacillus* as a facultative anaerobic bacterium, is capable to ferment and experience cellular respiration while oxygen is present; this process is known as hetero fermentative. Biosynthesis of lactic acid in a hetero fermentative process produces carbon dioxide and ethanol, therefore implementation of lactic acid yield is reduced (Quintero *et al*, 2013).

Lactobacillus casei species belongs to different strains, which can be isolated from various environments, and some of the *L. casei* are currently used in commercial products as probiotics. These species are subjected in to studies regarding their numerous taxonomic groups, since many strains were previously classified according to their characterization, which was similar to *L. casei*. It was further notices *L. casei* sub sp. casei ATCC 393^T was having genotypic, phenotypic and phylogenetic differences when compared to the standing type of *L. casei*.

The strain *L. casei* sub sp. *casei* ATCC 393^T is especially been used in studies based on the biological process of fermentation of sugars such as glucose, lactose, citrate and pyruvate, comparative studies on and molecular characterization of the enzyme L-lactate dehydrogenase, also in the characterization of an intracellular β

glucosidase, proteolytic activity and studies on the composition of the cell wall, antibiotic resistance and adherence factors (Acedo-Fe´lixm and Pe´rez-Martı´ne, 2003).

Furthermore, genus *Lactobacillus* was reported to be primarily in the studied by *L. casei* strains, especially through the sub sp ATCC 393^T; the sup sp was used to retrieve information regarding the purpose of isolation and characterization of extrachromosomal genetic elements. Moreover, between the standing type strain and the plasmid-cured strain, the plasmid-cured strains showed a higher potential for lactose metabolism on research studies. Thus, due to this reason it was observed it carried a second lactose-specific transport and hydrolysis system, which is namely the lactose phosphoenolpyruvate phosphotransferase system (PTS) and a 6-phospho-b-galactosidase respectively. (Acedo-Fe´lixm and Pe´rez-Martı´ne, 2003)

In the present study *L. casei* and *L. delbrueckii* were used since they specifically produce L-(+)-Lactic acid (Panesar *et al*, 2010 and Chang *et al*, 1999) which we are interested in the current research. *L. casei* and *L. delbrueckii* are gram-positive lactic acid bacteria. They are non-motile, have non-spore-forming rods and cocci. These lactic acid bacteria grow under anaerobic conditions, which help them not to use oxygen for their energy manufacture, but they are also capable of growing with presence of oxygen (Coeuret *et al*, 2003). The current study observed the highest efficiency from *L. casei* than *L. delbrueckii*. According to the literature, *L. delbrueckii* requires higher nutrient level to grow (Coeuret *et al*, 2003). Hence, large scale fermentation will not be economical.

During the lactic acid production in the present study, it was noticed the microbial fermentation process was inhibited after the 4th day of inoculation of microorganisms. Further reading on this matter, it was understood this process conventionally suffer from end-product inhibition. This is due to the reason an un-dissociated lactic acid passes through the membrane of bacteria and dissociates inside the bacterial cell. Thus it concluded the mechanism of inhibition of lactic acid is related to the solubility of the un-dissociated lactic acid which is present in the cytoplasmic membrane of the bacteria and the insolubility of dissociated lactate, which produces acidification of cytoplasm, thus the proton motive forces is failed. This phenomena eventually inducements the transmembrane pH gradient and the amount of energy available for cell growth is decreased. Thus, to lighten the inhibitory effect of lactic acid during the fermentation process via microbes, it is endorsed to remove selectively in situ from the fermentation broth or media (Wee et al, 2006).

The lactate dehydrogenase enzyme in the microorganisms are not only produces lactic acid from simple sugars but also converts the lactic acid into pyruvate. Thus, the amount of lactic acid is easily converted to pyruvate. This pyruvate, and sugar content, which are not utilized by bacteria form disturbance during detection of lactic acid via HPLC; forming different peaks. The peak area of the standard lactic acid solution is much lesser than the lactic acid produced from the media, this is due to the reason as the standard lactic acid solution is diluted than the lactic acid found in the media.

The fermentation method used in the current study is the batch method. Other commonly used methods for fermentation process for lactic acid production includes fed-batch, repeated batch, and continuous fermentations. It was also been noticed from previous studies a higher concentrations of lactic acid could be obtained in batch and fed-batch cultures than in continuous cultures, whereas by the use of continuous cultures higher productivity could be achieved. Another added advantage of the continuous culture compared to the batch culture, is the possibility to continue the process for a longer period of time (Wee *et al*, 2006).

The allosteric enzyme L- lactate dehydrogenase in *L. casei* is with fructose 1, 6- bisphosphate (FDP) and the Mn^{2+} acts as the cofactor in some cases. The LDH in *L. casei* found in eukaryotes and in *L. casei* living in vertebrates express a 37 % and 76 % similarity respectively, but 70 % and 86 % similarity respectively in their active sites of LDH. This phenomena explains us that the essential parts of LDH enzyme has been conserved. In comparison to the vertebrate LDH in *L. casei* is found to lack 12-amino acid residues at the N- terminus, which is found to be a common characteristic of bacterial enzymes irrespective of the allosteric behavior. *L. casei* also carries a C end

of the protein with 7 additional amino acid residues, however it is not identified whether this characteristic of L-lactate dehydrogenase enzymes of bacteria as no complete sequence of other bacterial enzymes available (Narayanan et al, 2014).

Furthermore, after the production of lactic acid in the large scale, the medium D, it would be purified and polymerized to obtain the PLA biosynthetic polymer to be used as orthopedic devices. L-PLA can have two crystalline modifications, α and β conformation. The synthesize of high-molecular-weight PGA and PLA is constructed by opening the polymerized ring of the cyclic lactide. Catalysts used in the polymerization process include antimony, zinc, or lead. However, low-molecular-weight homo- and co-polyesters of lactic acid will also be synthesized by direct poly-condensation in the presence of water without using catalysts.

As mentioned earlier, the polymers PLA and PGA are biodegrade mainly by hydrolytic scission which occurs nonspecifically. By the simple hydrolysis, the polymer chains in the ester linkages are fundamentally cleaved. PLA undergoes hydrolytic scission to its monomeric form, thus lactic acid which is eliminated from the body is integrated into the tricarboxylic acid cycle. The eliminated lactic acid is finally excreted by its principal elimination path as respiration via the lungs as CO_2 and in urine. In a study done on rats, the PLA implants were labeled with radioactive carbon and placed internally in rats for 3 months, as a result there was no significant radio activity found in the urine or feces, which confirmed that the polymer is degraded and probably eliminated through CO_2 during respiration. Further, PGA can be broken down in two ways, by hydrolysis sand by nonspecific esterase and carboxypeptidases (Agrawal et al, 1995).

Conclusion

According to the study performed *L. casei* could be selected as the efficient and low cost LAB strain for the production of L-(+)-lactic acid in industrial scale fermentation. Further, Medium D was identified as the effective low cost medium for large scale fermentation for the production of lactic acid with *L. casei*. Purification and polymerization need to be performed in order to synthesis the biopolymer. It is suggested that *L. casei* and medium D together would be effective in producing a high yield of L-(+)-Lactic acid which could produce biodegradable polymers for orthopedic devices.

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Enzymatic Hydrolysis of Cellulose in Coconut Coir: Pre-treated via Sonication

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Abstract: The total world annual production of coconuts was approximately 61million tonnes in 2015. Using this amount of coconuts, the waste generated annually could reach 12 million tonnes of coconut coir. The top world producer of coconuts is Indonesia - 30%, followed by Philippines – 25%, India – 20%, Brazil (5%) and Sri Lanka - 4%. Coconut coir is composed of cellulose, lignin, pectin, and hemicellulose. One possible application of this lignocellulosic waste is to produce biofuels. Physical-chemical pre-treatment is required in order to remove lignin and to increase cellulose digestability. Commonly, the coconut coir was pretreated primarily with NaOH solution (5-11%) in autoclave at 121°C for 1 hour. The aims of this work were to find an enhanced pretreatment procedure for the coconut coir, and to study kinetics of the cellulose hydrolysis process at elevated temperature. For this purpose, we used NaOH solution (5%) and ultrasonic bath for 1 hour at 50°C, which can successfully replace autoclave pretreatment. Moreover, the temperature of the ultrasonic pre-treatment coincided with the temperature of hydrolysis reaction carried out subsequently. Two commercial enzymes cellulase and β-galactosidase were applied simultaneously as biocatalyst for the enzymatic hydrolysis of cellulose. One of the advantages of the ultrasound-assisted pretreatment was the lowest weight loss of the coir - 48% (w/w). When using autoclave, the weight loss was much higher - about 66-67% (w/w) of the coir samples. First order kinetic equation for the enzymatic hydrolysis of cellulose to glucose was derived, and kinetic constants were obtained for the product released at different modes of operation.

Keywords: cellulose; coconut coir; enzymatic hydrolysis; kinetics

Introduction

The total world annual production of coconuts was approximately 61 million tonnes in 2015 (FAO, 2015). Using this amount of coconuts, the waste generated annually could reach 12 million tonnes of coconut coir. The top world producer of coconuts is Indonesia - 30%, followed by Philippines – 25%, India – 20%, Brazil (5%), and Sri Lanka - 4%. Coconut coir (a coarse fibre removed from the fibrous outer shell - mesocarp part of the coconut fruit) is composed of cellulose, lignin, pectin, and hemicellulose. One possible application of this lignocellulosic waste is to produce biofuels. The cellulose content in the raw coconut coir was reported to be 41.7% (Fatmawati et. al, 2013). Physical and chemical pretreatments are required in order to increase enzyme digestability of cellulose. The main effects of the pretreatment are to dissolve hemicellulose and alter the lignin structure, providing an improved accessibility of the cellulose for hydrolytic enzymes. Alkaline pretreatment leads to increase accessible surface area of cellulose and altering structure of lignin and its solubilisation (Hendriks and Zeeman, 2009). During alkaline pretreatment the first reactions taking place are solvation and saphonication. This causes a swollen state of the biomass and makes it more accessible for enzymes. Alkali treatment can also cause solubilisation, redistribution, and condensation of lignin and modifications in the crystalline state of the cellulose (Gregg and Saddler, 1996). An increased production of biomethane was also reported after the alkaline pretreatment of wheat straw by Pavlostathis and Gossett (1985). The cellulose in a plant consists of fragments with a crystalline structure, and portions with an amorphous structure. Numerous researchers determined that

crystallinity of cellulose is one of the limited factors for the enzymatic hydrolysis of lignocellulosic materials. Other factors are degree of polymerization, moisture content, available surface area, and lignin content (Laureano-Perez et al., 2005).

Velmurugan and Muthukumar (2012) reported that ultrasound-assisted alkaline pretreatment of sugarcane bagasse showed better reducing sugar yield than commercial alkaline pretreatment. The substantial reduction in pretreatment time with improved efficiency was the most attractive feature of this treatment. Power ultrasound refers to sound waves with low frequencies (20-100 kHz) and high sound intensities (10 Wcm^{-2} - 1 $kWcm^{-2}$). Ultrasound-assisted alkaline pretreatment solubilised the lignin matrix more effectively than autoclave pretreatment and hence, the performance of saccharification was improved considerably. The same researchers found that the crystallinity index of sono-pretreated sugarcane bagasse was 67%, whereas that for alkaline pretreated at 121ºC was 65.40%. Hence, the removal of amorphous domains was more or less similar to that of the ultrasound-assisted alkaline pretreatment. The formation of microbubbles during sonication treatments (cavitation phenomenon, which involved the increase of temperature and pressure at the solid – solvent interface) improved diffusivity or mass transfer processes (García et al, 2011). Tang et al. (2005) also observed that no significant changes in crystallinity of cellulose after ultrasound treatment occurred. Stefanovic, et al. (2013) concluded that sonochemical degradation of cellulose is a very efficient non-classical method in accordance with "green chemistry" concepts. With regard to the above mentioned, the aims of this work were: 1) to find an enhanced pre-treatment procedure for the coconut coir comparing alkaline autoclave with ultrasonic pre-treatment methods, and 2) to study kinetics of the hydrolysis process of cellulose at elevated temperatures after the pretreatments.

Materials and Methods

The coconut coir used in this work was kindly provided by the Surabaya University, Indonesia. The dried coconut coir was cut (± 5 x 5 cm), and then milled using a disc-mill machine with a speed of 5800 rpm. It was sieved to obtain the particle size of 70-100 mesh.

Alkaline - autoclave pretreatment was carried out with two concentrations of NaOH solutions (5% wt and 11 wt%) and two temperatures: 100º and 121°C for 15 min at total cycle time 1 hour. Ultrasound-assisted pretreatment in water bath (USB) was chosen as an alternative to autoclave pretreatment method. It was carried out in 5% wt NaOH at T=50°C for one hour, the selected temperature also coincides with the temperature of hydrolysis reaction.

Two commercial enzymes: cellulose from *Trichoderma reesei* ATCC 26921, ≥700 units/g (Sigma-Aldrich) and β-galactosidase from *Aspergillus niger*, ≥ 750 units/g (Sigma-Aldrich), were used simultaneously as biocatalyst in Erlenmeyer flasks containing samples of coir and citrate buffer (100 mL) at pH=4.8 and temperature 50°C. Equal amounts of enzymes were added to the bioreactor in quantities of 0.3 mL. Tetracycline (40 μg/mL) antibiotic was supplementary to the buffer to prevent bacterial contamination. Experiments were carried out in the rotating shaker at 200 rpm.

A 2-L Trytoni bioreactor (Pierre Guerin Technologies, France) with working volume of liquid equal to 1.1 L was applied to study hydrolysis of cellulose at 200 rpm. The initial concentration of the coconut coir sample was 20 g/L, which was pretreated by alkaline solution (11wt% NaOH at 7.5% solids) and then autoclaved at 121ºC for 15 minutes. All other conditions were identical with those described for

the shaker. Spectrophotometric Dinitrosalicylic method was applied to analyse total concentration of reducing sugars (RSC) obtained after hydrolysis (Miller, 1959).

After the pretreatment, samples of coconut coir were filtered and washed with purified water (10 cycles) until neutral pH. Finally, the samples were dried in the oven at 100°C overnight.

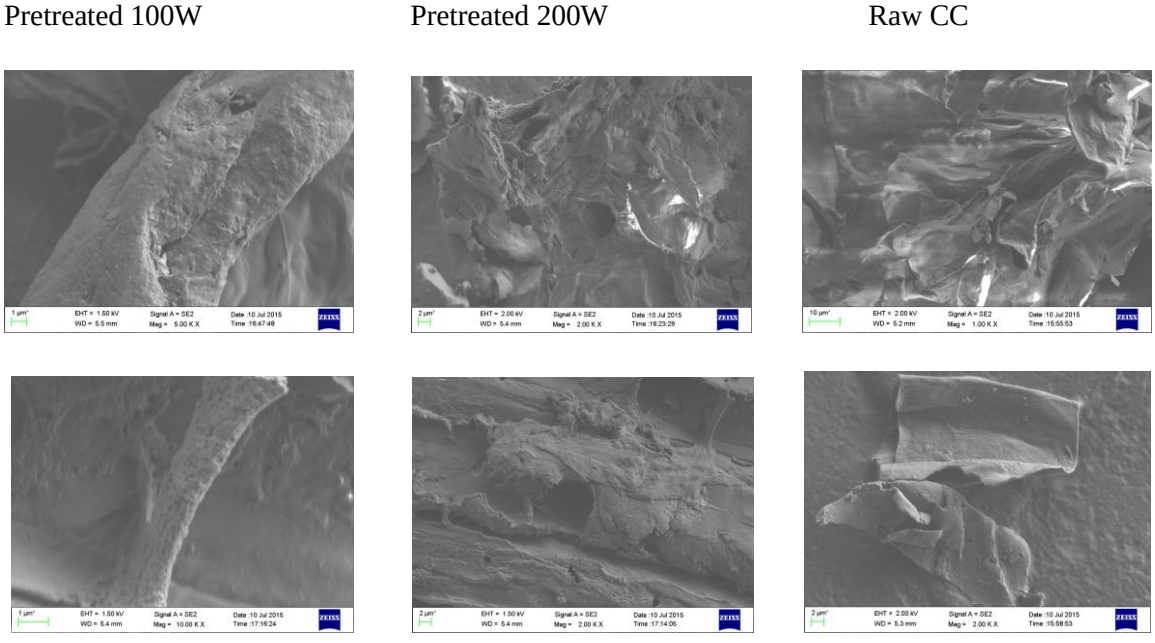
Scanning electron microscopy (SEM) was used to compare the morphological change of coconut coir before and after pretreatment with alkaline-autoclave and alkaline-USB (5% NaOH). The dried samples were mounted on specimen stubs using carbon tape and imaged by a SEM (ΣIGMA™, Carl Zeiss, type 174C CZ) at acceleration voltage of 2 kV.

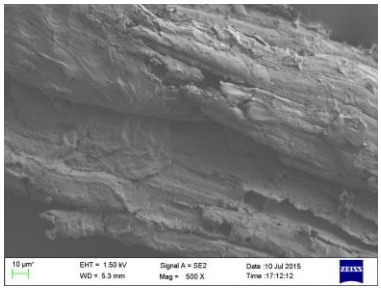
Results and Discussion

Alkaline Pretreatment

Results of the qualitative evaluation carried out by SEM show disruptive effects of the autoclave and USB at different ultrasonic power levels on the coconut coir structure after treatment with 5 wt% NaOH in the above equipment. SEM images illustrate differences in microstructure of coconut coir treated in autoclave and in the USB. The alkaline treatment was able to remove the external layer of the fibers revealing the fibrillar structures, the cavities became more apparent, also surface fibres became rougher than in the untreated sample (Figure 1). Similar observations were reported by Esmeraldo et al. (2010) who treated dwarf-green coconut with NaOH solution at different concentrations. They found that concentrations higher than 10% and temperature more than 90°C resulted in fibrillation and significant damage to the fibers of cellulose.

Figure 1. SEM micrographs (100W, 200W, autoclave, and raw CC)





Autoclave

Degree of the coconut coir degradation or % of weight loss of the dried samples was calculated gravimetrically as a ratio of the difference between initial and final weights to the initial weight of the samples in %.

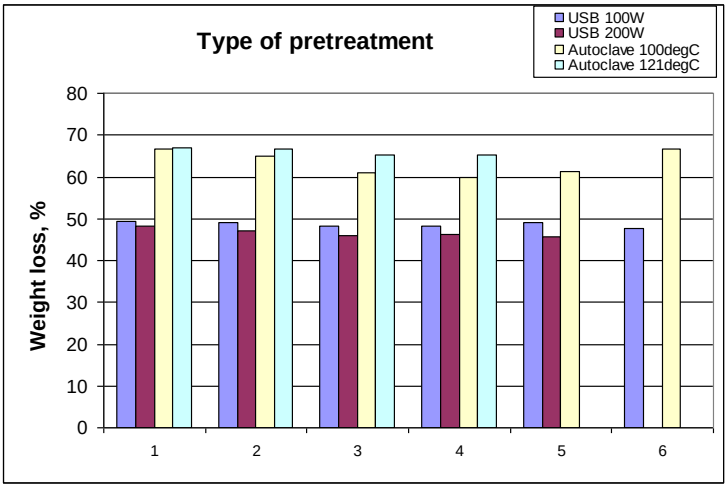


Figure 2. Weight loss of the coconut samples due to the different pretreatment methods.

One of the advantages of the ultrasound-assisted pretreatment was the lowest weight loss of the coir - 48% (w/w). When using autoclave, the weight loss was much higher - about 66-67% (w/w) of the initial samples.

Kinetics of the hydrolysis process

Considering the hydrolysis reaction (1), the main products were glucose and cellobiose in the first stage of the reaction catalyzed by cellulase and the main final product of the second stage catalysed by galactosidase was only glucose. Cellobiose was completely exhausted at the beginning of the second stage, as found by the preliminary HPLC analysis (unreported data obtained by the Surabaya University).

$$\text{Cellulose} \xrightarrow{\text{cellulase}} \text{Cellobiose} + \text{Glucose} + \text{Water} \xrightarrow{\text{ß-galactosidase}} \text{Glucose} + \text{Water} \qquad (1)$$

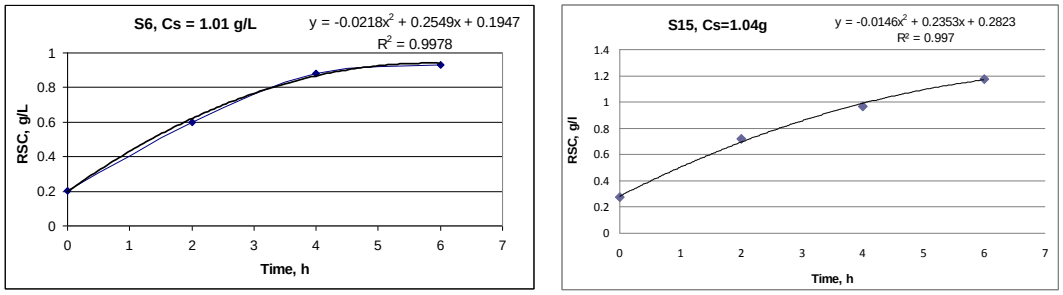
$S \longrightarrow P$
or overall, Substrate (Cellulose) to Product (RSC).

Profiles of the product concentrations or RSC produced during cellulose hydrolysis of samples containing different initial concentrations of the pretreated coconut coir versus time are shown in Figure 3 (a, b). All the kinetic curves were described by the second order polynomial regression equations with R^2 >90%. The initial velocity (V_0) of the enzymatic product appearance was obtained from the kinetic curves shown in Figure 3. Applying equation (2), the initial velocity was measured as a slope of the plot RSC versus time at the beginning of the reaction when enzyme was mixed with the substrate, before the substrate decreased significantly (Berg et al., 2006).

$$V_0 = \frac{dP}{dt} = -\frac{dS}{dt} \quad (2)$$

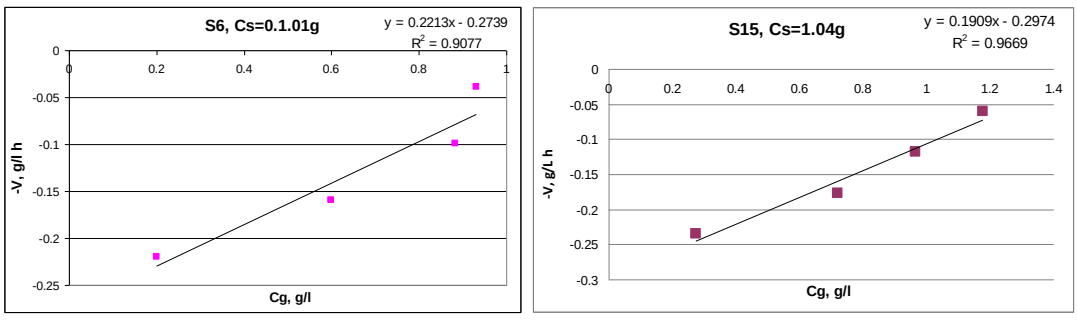
The second order regression equations obtained from RSC versus time were differentiated to obtain the slops of the plots. Some of the results are shown in Figure 4 (a, b), which correspond to the kinetic curves shown in Figure 3 (a, b) obtained after autoclave and USB pretreatments, respectively.

Figure 3. Kinetic curves of the enzymatic hydrolysis of cellulose in the coconut coir samples.



a) coconut coir pretreated in autoclave at 100ºC; b) coconut coir pretreated via sonication at 100W.

The initial velocities of the reaction versus product concentrations (or concentration of reducing sugars) were plotted. From the linear trends of the data shown in Figure 4 (a, b), kinetic constants of the overall hydrolysis reaction of cellulose to glucose were determined. The RSC measurements were generated in the experiments carried out in the shaker. During the reaction as substrate was conversed to product, the concentration of cellulose decreased, so the velocity of the reaction was also diminished.



a) b)

Figure 4. The rates of the hydrolysis process for samples: a) initial concentration of the coconut coir was 10.1 g/l, which was pretreated in autoclave, b) initial concentration of the coconut coir was 10.4 g/l, which was pretreated in USB at 100W.

For the kinetic experiments carried out in the bioreactor analogous trend was obtained, as demonstrated in Figure 5. However, the kinetic constant of the hydrolysis reaction carried out in the bioreactor had lowest value (0.046 g/l.h) compared with the constants obtained in the experiments carried out in the flasks. One possible explanation is the twice higher initial concentration of the coconut coir sample (20g/L), which was treated by alkaline –autoclave method at 121ºC (in 11 wt% NaOH).

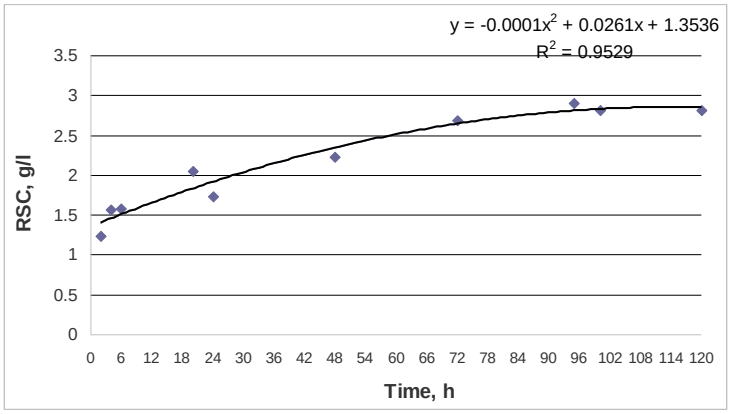


Figure 5. Kinetic curve of the total reducing sugar concentrations versus time obtained in the bioreactor, the starting coconut coir concentration was 20g/l.

The final kinetic result are illustrated in Figure 6, were kinetic constants obtained in the shaker were correlated with the initial weights of the coconut coir samples used as substrate for the enzymatic hydrolysis reaction in the citrate buffer (100 mL). The highest values of the kinetic constants were obtained for the samples pretreated via sonication at 100W. A good similarity of results was achieved for the alkaline-autoclave pretreated samples at 100ºC and those pretreated by the same concentration of 5 wt% NaOH in USB at 200W.

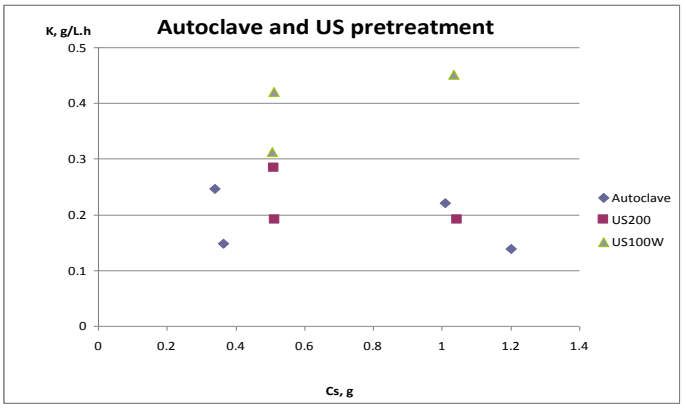


Figure 6. Plot of the kinetic constants of the cellulose hydrolysis reaction carried out in the shaker versus initial weights of the coconut coir samples pretreated in autoclave and in the USB.

Conclusions

After comparison of the pretreatment methods, the sonication was selected as an enhanced method for the coconut coir pretreatment. Temperature of this pretreatment coincided with the temperature of the hydrolysis reaction, which was carried out subsequently (50°C). The coconut coir pretreated via sonication had the lowest weight loss of the coir - 48% (w/w). When autoclave was used, the weight loss of the samples was about 66-67% (w/w). First order kinetic equations of the enzymatic hydrolysis of cellulose in the pretreated coconut coir were derived, and the kinetic constants were obtained for the products released.

Acknowledgement

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RECENT ADVANCEMENT IN FORENSIC TOXIC DRUG ANALYSIS BY DESORPTION ELECTROSPRAY IONIZATION MASS SPECTROMETRY (DESI-MS)

Ahmed S. & Kandiah M.

Abstract: Forensic toxicology is the analysis of biological samples to identify the presence of any harmful substances in the body. The three major sub disciplines in forensic toxicology are; human performance toxicology, post-mortem toxicology and forensic drug analysis. Hence, from the three major sub disciplines in forensic toxicology, forensic drug analysis is of major interest, as it involves in the identification of illicit and counterfeit pharmaceutical drugs in biological matrices. Thus, desorption electrospray ionization mass spectrometry (DESI-MS) is an emerging technique in rapid analysis of biological samples in forensic drug analysis, due to the increase in illicit and counterfeit drug users during the past decade. Although, this technique allows the direct analysis of samples from the surface at ambient pressure, development in the technique has been made to increase the sensitivity and specificity in analysing forensic drugs. Meanwhile, improvements in the extraction procedures prior to DESI-MS analysis, possibilities in analysing the drugs in raw urine sample from its native environment and reduction in the geometrical parameters have been the major developments made in DESI-MS. Also, advancement in DESI-MS enables direct analysis of drug tablets to identify the active pharmaceutical drugs present in the tablet and distinguishes between pharmaceutical drugs and counterfeit or fake drugs. This safety evaluation is important as several counterfeit drugs are prepared with illicit components and sold in the market and used to perform illegal activities. Therefore, the recent advancement in forensic toxic drug analysis by DESI-MS enables the law enforcement to pursue criminal charges against the suspect.

Introduction

Forensic toxicology is the analysis of biological samples to identify the presence of any harmful substances in the body (Peters, 2013). The three major sub disciplines in forensic toxicology are; human performance toxicology, post-mortem toxicology and forensic drug analysis. Human performance toxicology identifies the physical and mental effects of illegal drugs; post-mortem toxicology identifies the cause of death in relation to the harmful substances present in the body and forensic drug analysis monitors the presence of illegal drugs in biological matrices (Llah *et al*., 2016). Hence, from the three major sub disciplines in forensic toxicology, forensic drug analysis is of major interest, as it involves in the identification of illicit and counterfeit pharmaceutical drugs in biological matrices. Illicit drugs are substances that cause addiction, changes in consciousness and has limited or no use in medical purposes (Ifa *et al*., 2009). Thus, counterfeit drugs or falsified drugs are contaminated with limited or no active pharmaceutical ingredients (API) causing harmful effects on health. Therefore, by analysing these types of drugs, the law enforcement can pursue criminal charges and the court can determine appropriate sentencing against the suspected individual (Llah *et al*., 2016). Moreover, illicit drugs can be classified in to different types as shown in table 1, causing behavioural changes in the individual by acting on the central nervous system (CNS) due to prolonged usage of the drug (Jones, 2016).

Table 1: Summary of the illicit drug types and examples (Jones, 2016).

Classification of illicit drugs	Effect on the CNS	Examples
Opiates	Pain relieving drugs and long term use of these can cause physical dependence.	Morphine, heroine, diacetylmorphine and codeine.
Stimulants	Increase the activity of the CNS, causing the individual to be aggressive.	Amphetamine, methamphetamine, cocaine and nicotine.
Hallucinogen	Causes the individual to imagine or hear things that do not exist.	Marijuana and Methylenedioxymethamphetamine (MDMA)
Depressants	Decreases the activity of the CNS by slowing down the normal function of the brain.	Diazepam and Oxazepam

Thus, according to the World Drug Report in 2015, the use of illicit drugs has increased globally from 2006 to 2013 as indicated in figure 1 (Unites Nation Office on Drugs and Crime, 2015). The report stated 208 million illicit drug users in 2006 and 246 million users in 2013. Moreover, it has been stated that along with the illicit drug users, there has been an increase in problem drug users. Problem drug users are illicit drug users, suffering from drug use disorder or drug dependence, and 1 out of 10 illicit drug users is a problem drug user. Therefore, this places an additional cost on the treatment of such individuals by the government.

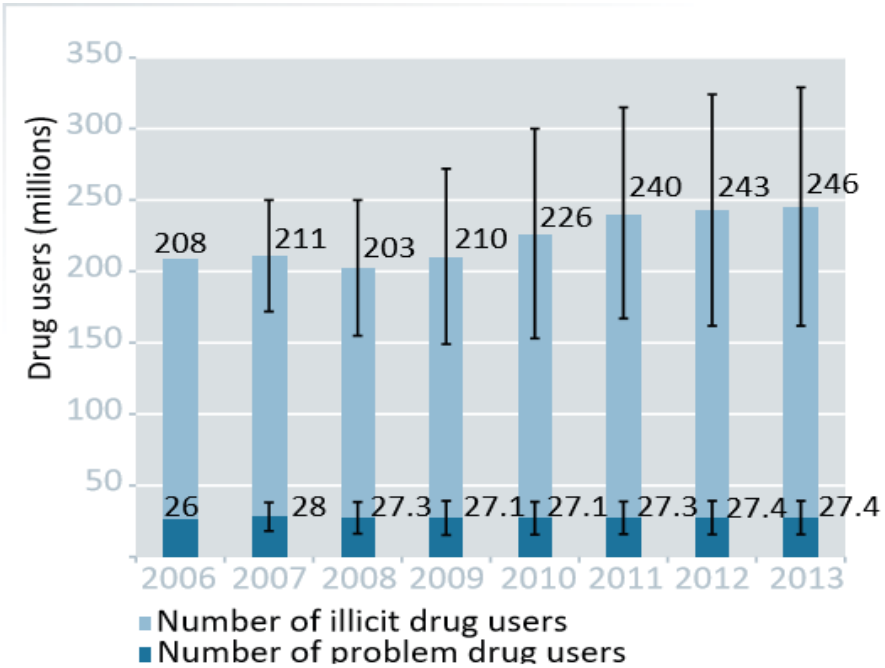


Figure 1: Global trends in the estimated number of illicit and problem drug users, from 2006 – 2013. (Unites Nation Office on Drugs and Crime, 2015).

Additionally, a report from European Union in 2015 stated a drastic increase in the number of counterfeit drug incidents from 2002 to 2013 (Europol, 2015). It was believed that in 2002 there were about 7553 cases on counterfeit drug usage in United Kingdom (UK) and it increased to 87,000 cases in 2013 as shown in figure 2.

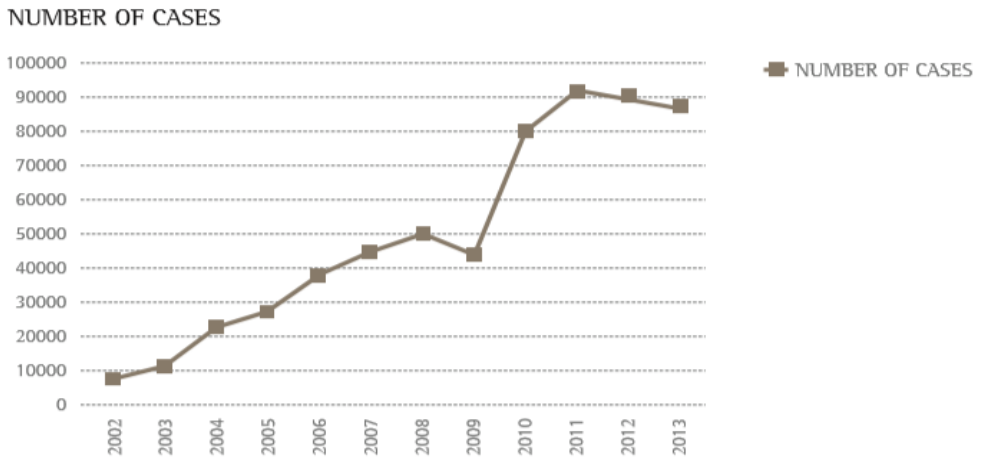


Figure 2: Statistics of the number of counterfeit cases in UK from 2002 to 2013 (Europol, 2015).

Henceforth, prolonged usage of these drugs will cause consequences to the health of the individual, to the society and legal problems of crime scenes and rape cases also arise due to the misuse of the drugs (Lachenmeier and Rehm, 2015). Therefore, rapid testing of the illicit and counterfeit drugs is required for criminal justice. There are two types of test done in forensic drug analysis (Lum and Mushlin, 2004). The screening tests identifies if the suspected drug is present in the sample, by means of a colour change. Therefore, it is less reliable as it does not indicate the quantity of the drug present in the sample. Hence, the confirmatory tests is more reliable as it indicates the quantity of the drug present in the sample, by using analytical technique of liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS) (Cooks, 2005). However, these conventional mass spectrometry techniques are time consuming as it requires numerous steps prior to analysis. Therefore, a recently developed analytical technique of ambient ionization mass spectrometry (MS) allows the direct analysis of samples from the surface at ambient pressure providing high sensitivity in forensic drug analysis by overcoming the limitations caused by the conventional mass spectrometry technique (Green *et al*., 2009). Moreover, there is numerous ambient ionization mass spectrometry techniques developed during the last decade. However, this review will be focused on the advancement in forensic drug analysis by desorption electrospray ionization mass spectrometry (DESI-MS).

Desorption electrospray ionization mass spectrometry (DESI-MS) and its advancement in illicit drug analysis

DESI-MS was developed by Takáts and co-workers with the help of Professor Graham Cook in 2004 (Takáts *et al*., 2004). It is the most frequently used ambient mass spectrometry technique in forensic analysis and the process is summarized in figure 3. Hence, this technique has been used to identify illicit drugs from varieties of surfaces such as paper, plastic, clothes and biological matrices. However, this review will mainly be focused on the analysis of illicit drugs from biological matrices using DESI-MS.

Figure 3: Schematic representation of DESI-MS setup. The DESI-MS process, involves by directing a solvent spray of charged micro-droplet (1:1 methanol : water) from the spray capillary at a specific voltage onto the surface of sample at ambient conditions. These droplets then desorb from the surface (Teflon) and are transported through an atmospheric pressure interface into the mass spectrometry inlet where the solvent is removed under heat and vacuum leaving the ionized form of analyte to be analysed. (Chen and Lin, 2015).

Moreover, in DESI-MS there are specific geometrical parameters α, β, d_1 and d_2 as shown in figure 4 and table 2, which has to be taken into consideration, as different analytes require different parameter settings prior to analysis (Takáts *et al*., 2005).

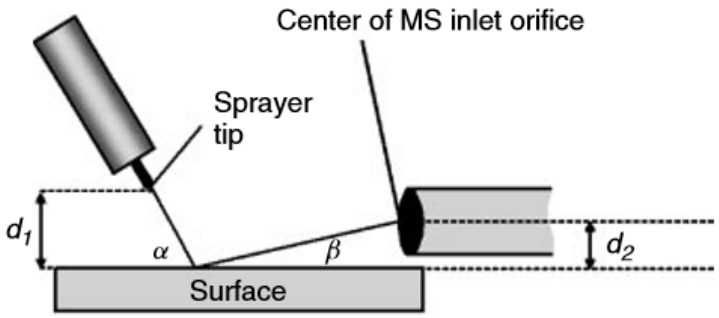
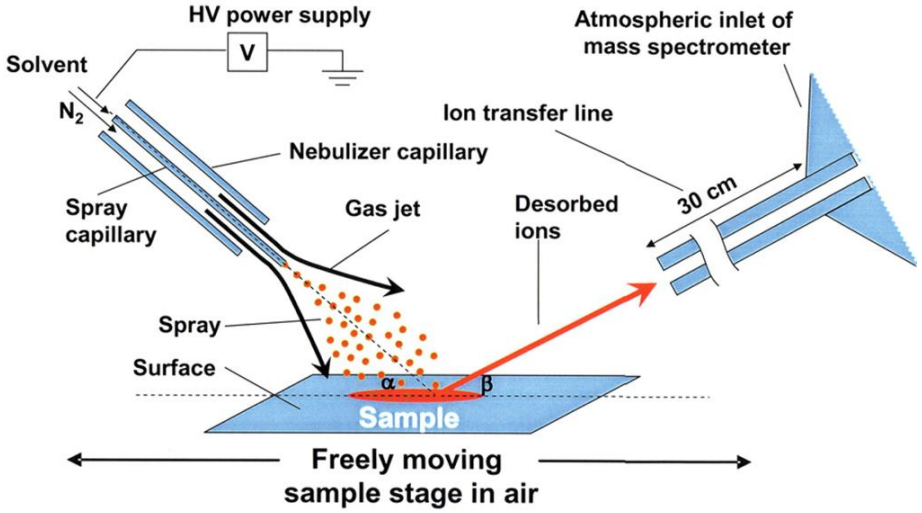


Figure 4: DESI-MS parameters. α: incident angle or solvent spray to surface angle, β: ion desorption angle to mass spectrometry inlet d_1: solvent sprayer to specimen at surface distance, d_2: mass spectrometry inlet to surface distance (Takáts *et al*., 2005).

Table 2: Parameters in desorption electrospray ionization mass spectrometry (Takáts *et al*., 2005).

Parameters	Optimum settings for high molecular weight molecules (eg: peptides, proteins and carbohydrates)	Optimum settings for low molecular weight molecules (eg: drug molecules, explosives, dyes and lipids)
Electrospray voltage	1-4 kV	3-8 kV
Electrospray solvent flow rate	0.1-3 ml/min	1.5-5 ml/min
Heated capillary temperature	200-300 °C	200 °C
Nebulizing gas (N_2) linear velocity	>350 m/ sec	-
Incident angle (α)	60-90 °	20-50 °



Ion desorption angle (β)	<10 °	10-15 °
solvent sprayer to specimen at surface distance (d_1)	1-2 mm	2-8 mm
mass spectrometry inlet to surface distance (d_2)	1-2 mm	5-8 mm

Thus, using DESI-MS, in 2007 Jackson and co-workers performed the first study to identify illicit drugs from a drug mixture as shown in table 3 (Jackson *et al*., 2007). In this study, 50 nanogram (ng) of drug mixture was dissolved in 1.0 milliliter (ml) of methanol : water (1:1) and then, 1.0 microliter (µl) of the dissolved sample was applied on the Teflon surface and subjected to DESI-MS for analysis. Hence, cocaine, diacetylmorphine and methamphetamine were successfully detected at ng range as shown in table 3 and figure 5.

Table 3: Summary of drugs analysed using DESI-MS (Jackson *et al*., 2007).

Drug	Molecular weight	Amount analyzed using DESI (ng/mL)	Limit of detection (ng/mL)
Cocaine	303	50	10.8
Diacetylmorphine	369	50	10.8
Methamphetamine	150	50	10.8

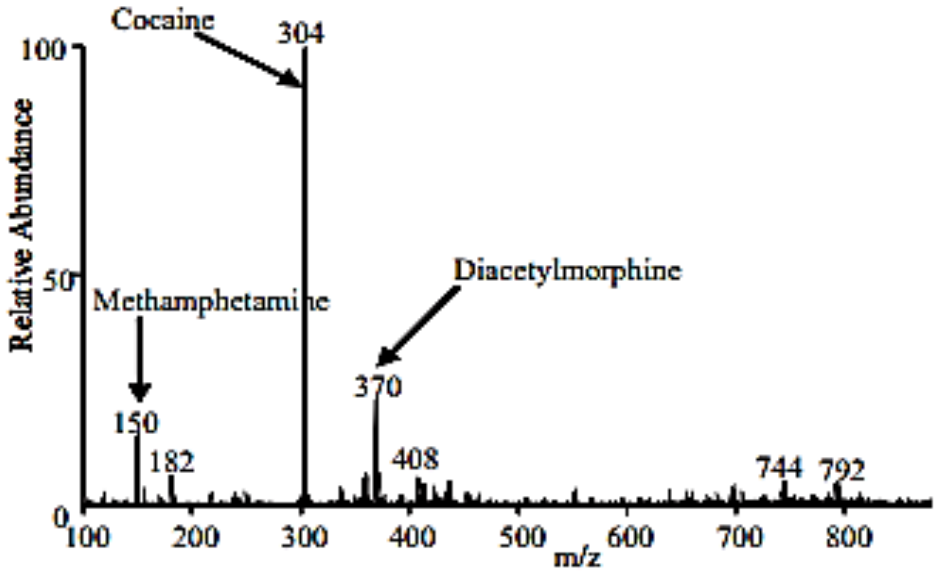


Figure 5: DESI-MS mass spectra obtained for the three different illicit drugs analysed. The solvent spray used was methanol : water (1:1) (Jackson *et al*., 2007).

Afterwards, in the same year another study was conducted by Kauppila and co-workers to identify the presence of illicit drugs in patient urine samples (Kauppila *et al*., 2007). In this study urine sample was extracted by liquid-liquid extraction (LLE) and 1.0 ml of extracted sample was dissolved in 1 ml of methanol : water (1:1). Then, 1.0 µl of the dissolved sample was applied on the Teflon surface, allowed to dry and subjected to DESI-MS for analysis. Hence, the outcome of this study indicated a group of illicit drugs determined at ng level as shown in table 4

Table 4: Summary of drugs identified in patient urine samples using DESI-MS (Kauppila *et al*., 2007).

Drug	Molecular weight	Limit of detection (ng)	Solvent spray	Sample
Temazepam	300	10.4	Water: formic acid (100:0.1 %)	1
Oxazepam	286	17.8		
Desmethyldiazepam	270	0.68		
Codeine	299	1.9	Water: acetonitrile (10: 90%)	2
Morphine	285	2.4		
Amphetamine	135	2.2	Water: acetonitrile (10: 90%)	3

Typically, DESI-MS is used to analyse solid samples, and to analyse liquid samples such as urine and blood; the sample has to be dried prior to analysis as discussed earlier. The dried sample by DESI-MS results in incomplete analysis of analytes from its native environment, due to the consumption of analytes on the surface (Zheng and Chen, 2016). Additionally, the high velocity of nitrogen gas projected by DESI-MS can result in splashing of the liquid sample on the surface. Therefore, due to these limitations, in 2009 Miao and Chen improved the DESI-MS developed by Takáts and co-workers in 2004, to directly analyze liquid samples such as urine and blood by a technique known as liquid DESI-MS (Miao and Chen, 2009). In this technique as shown in figure 6, liquid DESI-MS allows direct analysis of liquid sample from its native environment, allowing a continuous flow of sample to the Teflon surface through a fused silica capillary, overcoming the limitations caused by the traditional DESI-MS.

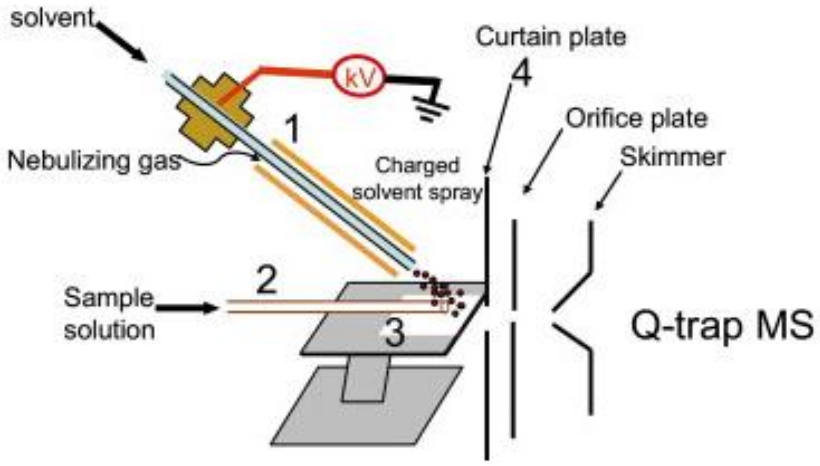


Figure 6: Schematic representation of liquid DESI-MS setup. (1) The solvent spray source (2) Fused silica capillary (3) Teflon surface to desorb and analytes into the mass spectrometry inlet (4) The analytes are analysed by Quadrapole- trap mass spectrometry (Miao and Chen, 2009).

Hence, to confirm the study, the technique was used to analyse raw urine sample spiked with illicit drug methamphetamine. Hence, a mass spectrum of methamphetamine analyte at mass to charge ratio (m/z) 150 was detected as shown in figure 7.

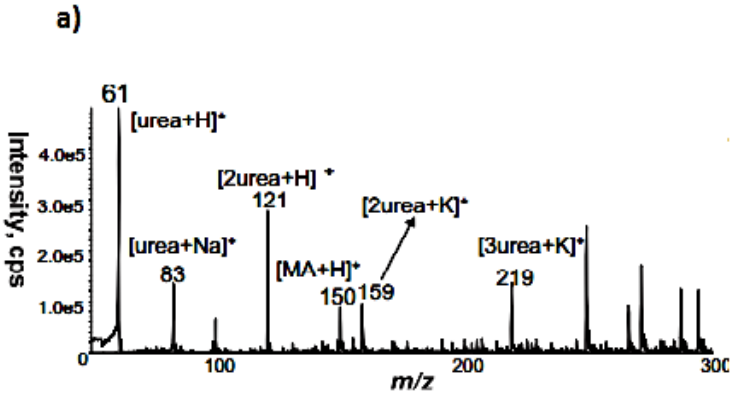


Figure 7: a) liquid DESI-MS mass spectra of methamphetamine (1 µg/mL) in raw urine sample and DESI solvent spray was methanol : acetic acid (1:0.03 by volume). The formation of fragment ions along with the analyte ion at m/z ratio 150, confirms the presence of methamphetamine (Miao and Chen, 2009).

Additionally, the liquid-liquid extraction used in traditional DESI-MS prior to analysis is relatively time consuming as it requires the separation of organic layer with the sample from the aqueous layer (Laskin and Lanekoff, 2016). Moreover, large amount of solvent and sample is required for extraction. This can be a major drawback, as samples obtained in forensic cases are of limited quantity. Therefore, considering the above stated drawbacks of liquid-liquid extraction procedure, DESI-MS was improved with an extraction procedure known as solid phase microextraction (SPME). SPME consists of a polymer fibre coated material, with extracting components involved in extracting analytes from different media (Mirabelli, Wolf and Zenobi, 2016). Importantly, SPME has several advantages when compared with the traditional extraction procedure of liquid-liquid extraction. It is easy to automate and allows the usage of solid, liquid and gaseous samples. Also, it can be used as a portable device due to its small size which makes the extraction procedure more convenient in forensic drug analysis (Yazdi and Amri, 2010). Furthermore, the analytes are separated by desorption and not by separation proving to be less time

consuming in providing rapid result in forensic drug analysis. Hence, in 2010 the SPME DESI-MS was used to analyse opiates (illicit drugs) from raw urine sample (Kennedy *et al*, 2010). In this study 500.0 ng of respective drug was dissolved in 1.0 ml of raw urine sample and to this prepared sample, the SPME fibre coated with C_{18} silica was dipped for a short period of time as shown in figure 8. During this time the analytes in the urine adsorbs on to the fibre surface, setting up equilibrium between the analytes and the coating on fibre material. Afterwards, the SPME fibre with the analyte was subjected to DESI-MS as shown in figure 9 for analysis. The results of the study are indicated in table 5 with the respective lower limit of quantification of each drug.

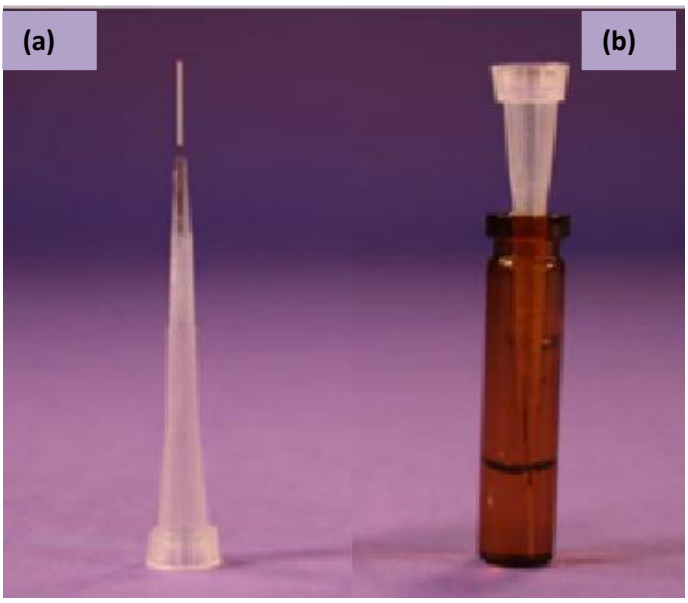


Figure 8: The SPME fibre used in the analysis of opiates in raw urine sample. (a) The overview of SPME fibre (b) SPME fibre dipped in the respective sample solution set up equilibrium between the analytes and fibre coating (Kennedy *et al*., 2010).

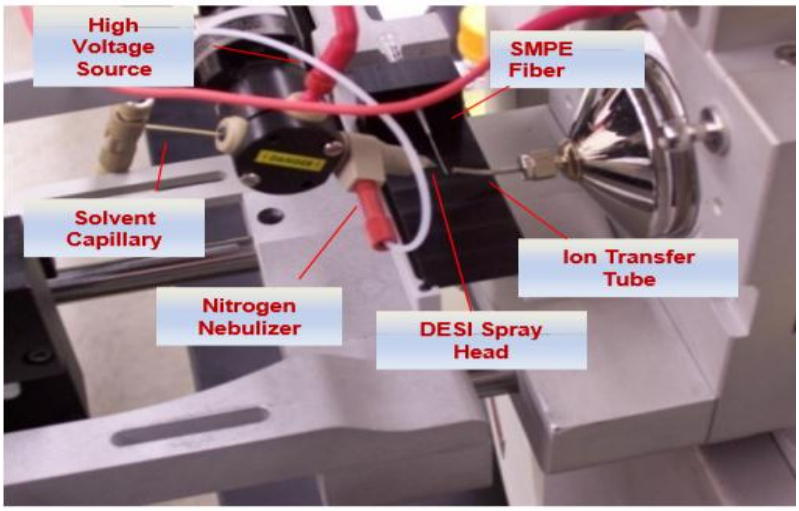


Figure 9: SPME DESI-MS setup for the analysis of opiates in raw urine sample (Kennedy *et al*., 2010). \

Table 5: Summary of the illicit drugs (opiates) analysed by SPME DESI-MS. The solvent spray used was 1:1 methanol : water (Kennedy *et al*., 2010).

Drug	Amount analyzed using SPME DESI-MS (ng/mL)	Lower limit of quantification (LLOQ)/ (ng/mL
Morphine	500	36.1
Hydrocodone	500	2.83

Oxymorphone	500	46.8
Oxycodone	500	42.8
Methadone	500	2.77
2-Ethylidene-1,5-Dimethyl-3,3-Diphenylpyrrolidine (EDDP)	500	0.26

However, SPME fibres can be relatively costly and the extracting material coated on the fibre surface has the chance of wearing off (Rutkowska *et al*., 2014). Therefore, in 2011 another extraction technique known as single droplet microextraction (SDME) was coupled with DESI-MS for the analysis of an illicit drug methamphetamine in raw urine sample (Sun *et al*., 2011). This study was performed to obtain a single droplet of the analyte from the sample by three steps. Initially, as shown in figure 10 (a), 50.0 µl of hexamine was added to the sample tube with methamphetamine, from the syringe and was stirred using the magnetic stirrer for 15 minutes at 11,000 rates per minute (rpm). Secondly, 4.0 µl of 5% acetic acid was added to the organic hexane layer and stirred for another 40 minutes at 11,000 rpm to obtain a single droplet of methamphetamine drug. Finally, the extracted single droplet of methamphetamine was withdrawn back into the syringe and coupled with DESI-MS for the analysis of the drug a shown in figure 10 (b).

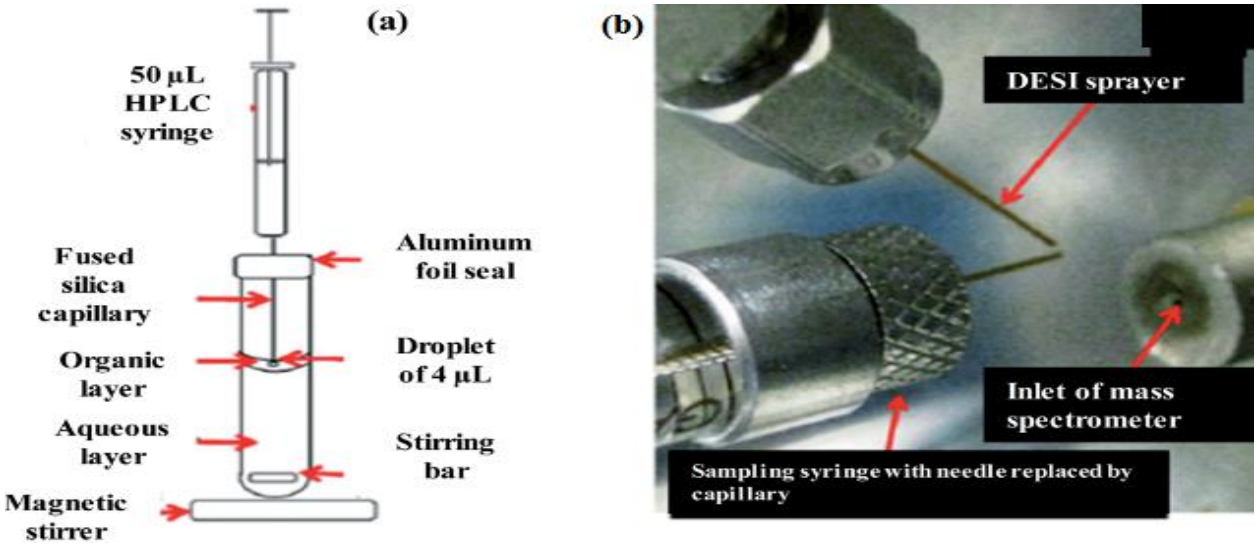


Figure 10: a) Three-phase SDME setup for methamphetamine analysis b) image showing the direct desorption and ionization of the single droplet methamphetamine by DESI-MS (Sun *et al*., 2011).

And, the results obtained from this study was compared with the liquid DESI-MS of methamphetamine and as shown in table 6, SDME DESI-MS had a lower detection limit of methamphetamine in comparison to liquid DESI-MS. Therefore, the study reveals that SDME DESI-MS is more sensitive in the detection of methamphetamine when compared to liquid DESI-MS.

Table 6: comparison of liquid DESI-MS and SDME DESI-MS to detect methamphetamine in raw urine sample

Drug	Molecular weight	Technique used	LOD/ (ng/ml)	LOQ (ng/ml)

Methamphetamine	150	Liquid	DESI-MS	200	-
Methamphetamine	150	SDME	DESI-MS	51	127

Interestingly, SDME DESI-MS technique had two improvements when compared to the earlier discussed DESI techniques (Sun *et al*., 2011). Firstly, as seen in figure 10 (b) there was no platform used and secondly the metallic surface of syringe with the micro droplet was replaced by a fused silica capillary avoiding it acting as a platform which might cause partial neutralization of analytes, leading to weak mass spectrometry signals.

However, the SDME procedure is relatively time consuming as it takes one hour for the extraction to complete and the technique can sometimes be less precise in obtaining the droplet of analyte due to prolonged agitation of sample (Rutkowska *et al*., 2014). Therefore, another extraction method known as thin liquid membrane extraction (TLME) was coupled with DESI-MS in 2013 to analyse illicit drugs of depressants and opiates from urine, blood and saliva (Rosting *et al*., 2013). In this technique the analysis of each sample was performed within 15 minutes. Firstly, as seen in figure 11, 1.5 µl of organic solvent of hexadcane was deposited on a porous Teflon membrane forming a liquid and to the thin liquid layer, sample was added for extraction. Afterwards, the lid was placed and vortexed for 10 minutes to allow extraction and the extracted sample on the thin layer membrane was subjected to DESI-MS for analysis. Interestingly, this technique had a similar principle as the liquid DESI-MS introduced by Miao and Chen. However, the major difference is the analysis being performed on an organic layer rather than a liquid phase.

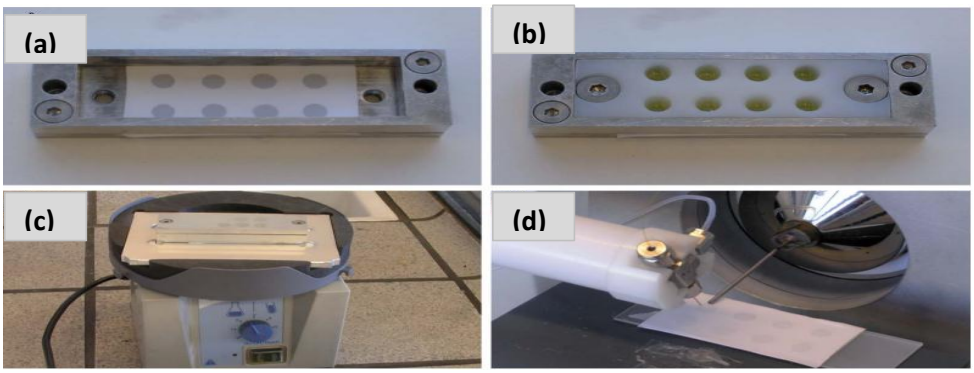


Figure 11: Steps involved in TLME-DESI (a) deposition of hexadecane on a porous Teflon membrane forming a liquid b) addition of sample to the layer c) the lid was placed and vortexed for 10 minutes to allow extraction d) DESI-MS analysis (Rosting *et al*., 2013).

Thus, according to table 7, the respective drugs were analysed from saliva, urine and blood samples.

Table 7: Summary of the non-polar drugs analyzed in three biological fluids using TLME-DESI (Rosting *et al*., 2013).

Sample type	Drug	Molecular weight	Physiological concentration (ng/ml)	Limit of detection(LOD) (ng/ml)
Urine	Amphytiphyline	278	25	17
	Notriptyline	264	25	17
	Pethidine	248	25	17
	Methadone			4
Saliva	Amphytiphyline	278	25	-
	Notriptyline	264	25	-
	Pethidine	248	25	-

Blood	Methadone	310	100	-

Although, TLME DESI-MS technique was successful in analysing various biological matrices (blood, urine and saliva), it is only applicable in the determination of non-polar drugs. Therefore, improvements need to be done on the technique for it to analyse polar drugs.

Desorption electrospray ionization mass spectrometry (DESI-MS) and its advancement in counterfeit drug analysis

Direct analysis of drug tablets involves in the identification of APIs present in the tablet and distinguishes between active pharmaceutical drugs and counterfeit drugs (Xie *et al*., 2015). This is important as several counterfeit drugs are prepared with illicit components and sold in the market and used to perform illegal activities. Therefore, in forensic drug analysis the analysis of counterfeit drugs are of great concern as mentioned prior for criminal justice and to trace the possible source of the counterfeit (Fernandez *et al*., 2006). Hence, using DESI-MS developed by Takáts and co-workers in 2004 can be used to analyse counterfeit drugs. And, using this technique aspirin tablet was analysed directly to identify the APIs in the tablet and as indicated in figure 12, aspirin tablet was identified at an abundance of 90 with a mass to charge ration of 179 (Green *et al*., 2009).

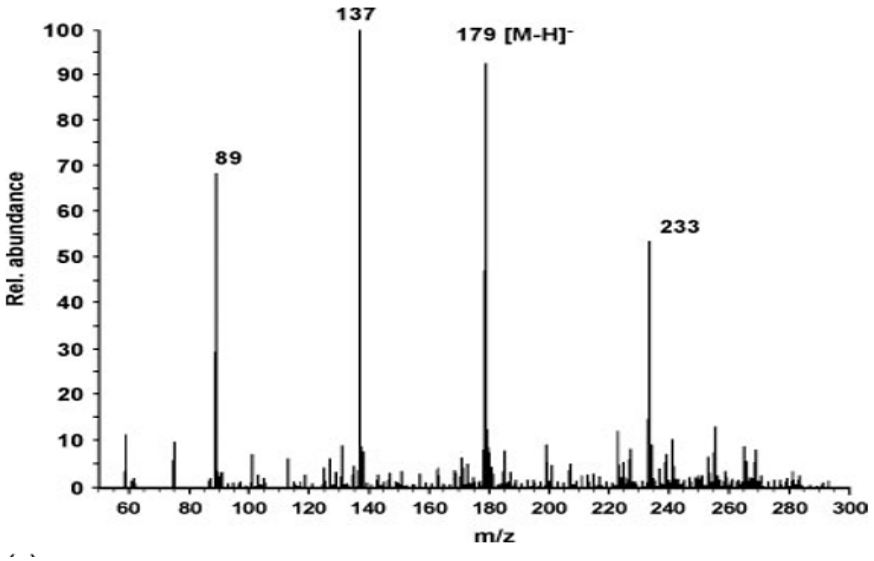


Figure 12: Direct analysis of aspirin tablet using DESI-MS (Green *et al*., 2009).

However, the geometrical parameters in DESI-MS as mentioned in figure 4, are of great concern, as slight changes within the parameters will result in weak mass spectrometry signal if the analyte fails to desorb from the surface to the mass spectrometry inlet (Morelato *et al*., 2013). Therefore, in 2010 Roach and co-workers developed the DESI-MS and named it as nano-spray DESI-MS (Roach *et al.,* 2010). Nano-spray DESI-MS uses a self-aspirating nanospray to ionize the analyte and transport the analyte to the mass spectrometry inlet as shown in figure 13 (Roach *et al*., 2010). This technique thereby reduces the geometrical parameters of ion desorption angle to mass spectrometry inlet (β) and mass spectrometry inlet to surface distance (d_1), required by the traditional DESI-MS (Green *et al*., 2009). As a result, the number of analyte reaching the mass spectrometry inlet becomes precise resulting in sensitive detection of analytes.

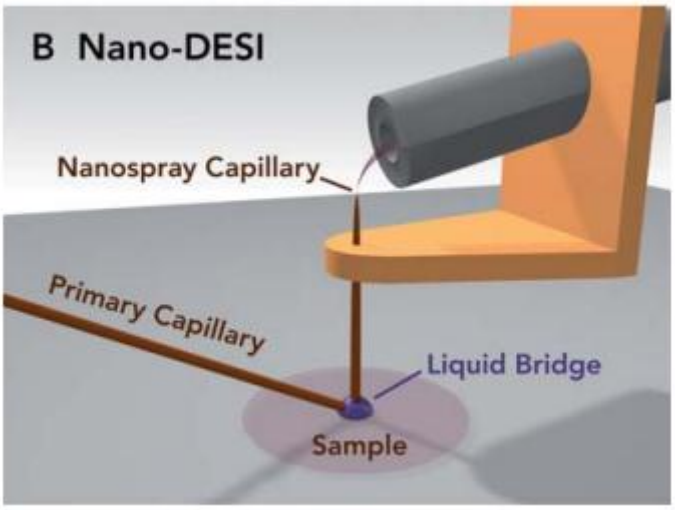


Figure 13: Nano-DESI setup. A liquid bridge formed between the primary capillary and nano-spray capillary. The analyte containing solvent is ionized and removed by self-aspiration nanospray into the mass spectrometry inlet (Roach *et al*., 2010).

Therefore, in 2016 Palcios and Lanekoff applied the technique to detect the biologically active pharmaceutical ingredients in different tablets as shown in table 8 (Palcios and Lanekoff, 2016). The surface of the tablet was cut using a blade and directly exposed to nano DESI-MS for analysis. Hence, it was concluded that this technique successfully analysed the active pharmaceutical ingredients of the tablets, indicating that nano DESI-MS is a powerful and useful technique in forensic drug analysis of counterfeits.

Table 8: Summary of the APIs analyzed, molecular weight and detected m/z ions from the surveyed tablets (Palcios and Lanekoff, 2016).

Active pharmaceutical ingredient (API)	Detected peaks (m/z)
Ibuprofen ($C_{13}H_{18}O_2$) MW= 206.3 g/mol	119 161 207 $[M+H]^+$
Paracetamol ($C_8H_9NO_2$) MW= 151.2 g/mol	110 152 $[M+H]^+$ 174 $[M+Na]^+$
Sildenafil ($C_{22}H_{30}N_6O_4S$) MW= 474.6 g/mol	475 $[M+H]^+$ 497 $[M+Na]^+$ 513 $[M+K]^+$ 949 $[2M+H]^+$ 971 $[2M+Na]^+$
Tadalafil ($C_{22}H_{19}N_3O_4$) MW= 389.4 g/mol	268 412 $[M+Na]^+$

Summary

DESI-MS is one of the rapidly developing ambient ionization MS techniques due to its speed of analysis and minimum sample preparation. It is a well-used technique in forensic drug analysis of illicit drugs and counterfeit drugs. Hence, this review has mainly highlighted on the advancement in forensic drug analysis by DESI-MS.

In the application of DESI-MS in forensic drug analysis of illicit drugs, several improvements have been made in the technique. Firstly, the built up of an instrumental setup of liquid DESI-MS to analyse illicit drugs directly from urine sample have been achieved without the need of sample being dried in traditional DESI-MS which might be

time consuming. Secondly, the liquid-liquid extraction procedure in traditional DESI-MS was improved to obtain results with minimum wastage of sample and cost, by coupling the solid phase micro extraction procedure with DESI-MS. Afterwards, another extraction procedure of single droplet micro extraction was coupled with DESI-MS to improve the sensitivity of the technique. Henceforth, SDME DESI-MS technique proved to be more sensitive in analysing the illicit drug methamphetamine in comparison to liquid DESI-MS. Finally, another less time consuming extraction technique known as thin liquid membrane extraction was coupled with DESI-MS to analyse illicit drugs in different biological matrices. Importantly, compared with the other stated DESI-MS techniques which only analysed urine sample, this technique was successful in analysing drug in other biological matrices of blood and saliva.

Furthermore, in the application of DESI-MS in forensic drug analysis of counterfeit drugs, only one improvement was achieved. The development of nano-DESI-MS was developed to overcome the difficulties obtained in adjusting the geometrical parameters in traditional DESI-MS. Henceforth; this technique was successful in analysing several APIs in several types of drugs regularly sold in the market. Therefore, in summary there have been numerous developments of DESI-MS in forensic drug analysis

Future scope

Although, successful advancement of DESI-MS has been achieved in forensic drug analysis, it requires additional studies to identify the actual sensitivity of the techniques to different types of drugs, as each drug behaves differently to each developed technique. Therefore, in future, extensive research needs to be performed to identify the sensitivity and specificity of each discussed DESI-MS technique in this review on all the type of illicit drugs and counterfeit drugs. Henceforth, the TLME DESI-MS was successful in analysing non-polar drugs and developments need to be done on the technique to analyse polar drugs. Importantly, the developed techniques were mainly subjected to analyse drugs in urine sample and additional studies need to be conducted on other biological matrices such as blood and saliva. Finally, the studies stated in this review are performed on homogenous or relatively clean samples and in forensic drug analysis the samples obtained are relatively unclean. Therefore, the discussed techniques in this review needs to be performed on realistic forensic cases, before they are routinely used by forensic laboratories.

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DETECTION OF COTININE IN PASSIVE SMOKERS EXPOSED TO ENVIRONMENTAL TOBACCO SMOKE

Shadiya Fawzul Ameer & Mathi Kandiah

Abstract: Environmental tobacco smoke (ETS) is the main cause of second hand smoking (SHS) induced disease conditions such as chronic obstructive pulmonary disease (COPD), lung cancer, vascular cancer and other types of cancers. It was estimated by the Health and Social Care Information Center (HSCIC) in UK that in the year 2016, 16 million people worldwide between the ages of 16 - 35 years died of smoking related diseases. Hence, different analytical techniques based on mass spectrometry (MS) and chromatography were developed, to detect the presence of tobacco metabolites in body fluids. These tobacco biomarkers in different body fluids could be used to correlate the level of ETS exposure. Among all the tobacco metabolites, cotinine was studied to be the most reliable bio-marker, since it does not get affected by other environmental factors and it has along half-life f 17 hours. Therefore, by studying the levels of SHS, smoking cessation programs in public places like schools, parks and public transports could be reinforced in order to protect the innocent lives who are being exposed to tobacco smoke. This review mainly focuses on the detection of cotinine in saliva, serum, urine and hair. In conclusion, it was evident that cotinine detection in hair was the most reliable biological matrix to detect ETS exposure in passive smokers.

Key Words: Detection, Cotinine, Analytical Techniques, Body Fluids, Passive Smoking

Introduction

Environmental tobacco smoke is a global threat. Since the early 1970s environmental tobacco smoke (ETS) is considered to be a major health problem in young and the elderly , mainly due to varied concentration and exposure profiles to the carcinogens present in tobacco smoke (Ramdzan *et al.*, 2016; Woodward *et al.*, 2005; Al-Demaiy, Crane & Woodward, 2002). This results in approximately 1.1 billion individuals being exposed to tobacco smoke, which makes every sixth human being a second hand smoker (SHS). In addition, the carcinogens present in tobacco are estimated to cause ~ 5 million deaths per annum around the globe (Royal college physicians, 2016). Figure 1 illustrates the prevalence of tobacco smoke and the disease condition caused by it around the globe.

Therefore, research scientists are developing different analytical techniques based on mass spectrometry (MS) and chromatography to detect the presence of tobacco biomarkers in body fluids. This could be used to determine exposure of tobacco smoke and to validate abstinence in smoking cessation programs (O'Malley *et al.*, 2014).

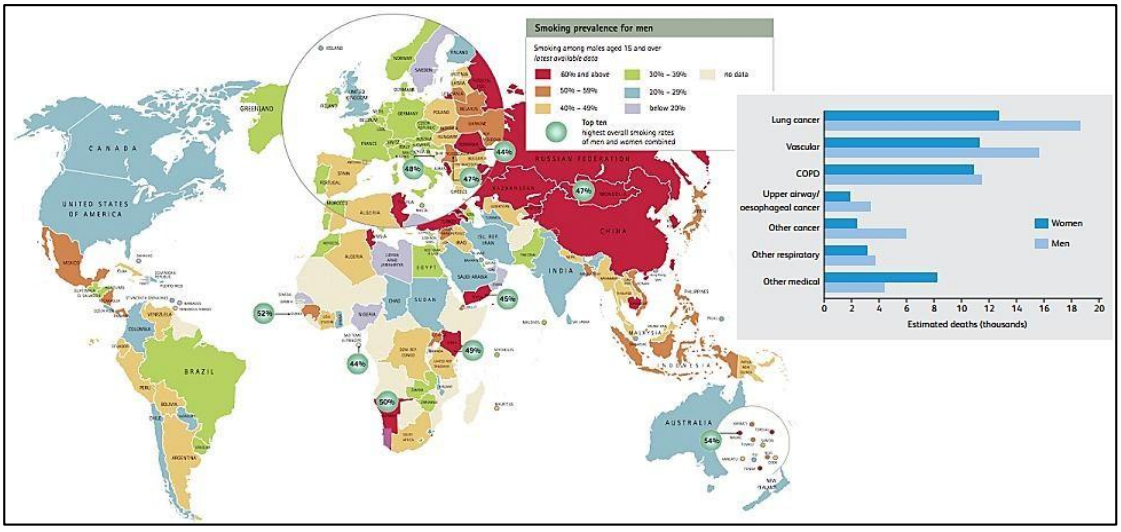

Figure1: Describes the prevalence of ETS around the globe. Different countries are exposed to varied concentration of tobacco. The red regions are those that are highly prevalent to tobacco and green regions are least prevalent. Disease conditions such as; chronic obstructive pulmonary disease (COPD), lung cancer, vascular cancer and other types of cancer are caused due to SHS (Royal College Physicians, 2016).

According to the 2016, statistical compendium report presented by the Health and Social Care Information Center (HSCIC), in the UK. It stated that there were 16 million hospital admissions for adults aged 35 and above due to SHS related diseases. This is approximately 4500 admissions per day. In 2013, 16 percent of all deaths of adults aged 35 and above was due to passive smoking related problems. Figure 2 illustrates the disease conditions presented due to passive smoking (Niblet, 2015).

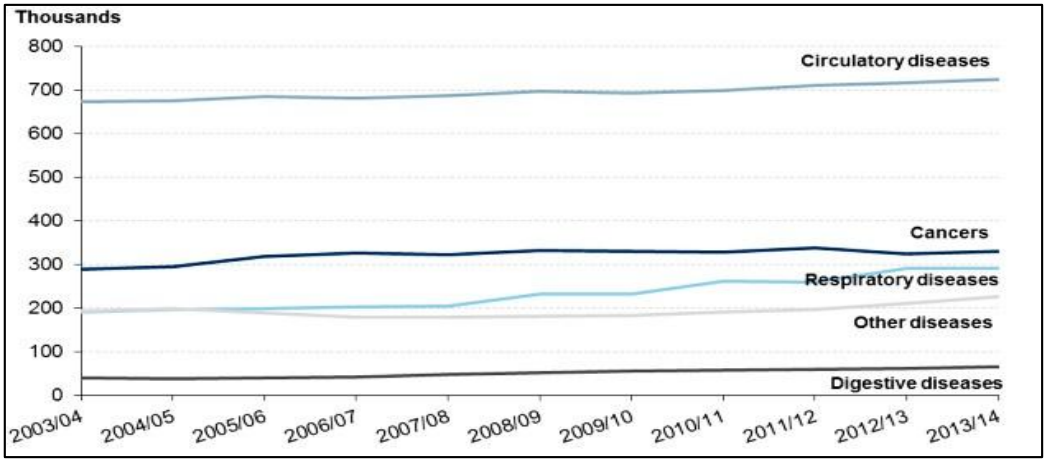

Figure 2: The NHS hospital admissions among adults aged 35 and over in England with primary diagnosis of disease which can be caused by SHS 2003/04 and 2013/13 (Niblet, 2015).

Metabolites of Tobacco Smoke

Tobacco smoke contains approximately 7000 toxic chemicals including oxidative gases, heavy metals, carcinogenic chemicals, ciliary injuring substances and various other harmful substances (Avila-Tang *et al.*, 2013). Figure 3 illustrates components of tobacco smoke and their respective metabolites. Tobacco is primarily metabolized in the liver, and to a smaller extent in the kidney and lungs. Enzymes such as cytochrome P450 2A6 (CYP 2A6), flavin monooxygenase 3 (FMO 3) and UDP-glucoronyltransferase (UGT) enhances the metabolism of tobacco. Furthermore, nicotine the main metabolite of tobacco is a psychoactive compound and it is the mediator of tobacco addiction. This is because it can cross the blood brain barrier as it has a pyridine and pyrrolidine ring which enhances its solubility (Thomas *et al.*, 2011). Moreover, tobacco specific nitrosamines (TSNA), polycyclic aromatic hydrocarbons (PAH) and volatile organic compounds (VOC) are metabolized to their respective by-products as illustrated in figure 3. Thus, in the early 1970, the international agency for research on cancer (IARC) understood that tobacco metabolites can be used as biomarkers to determine the level of SHS in different individuals (Yuan *et al.*, 2014).

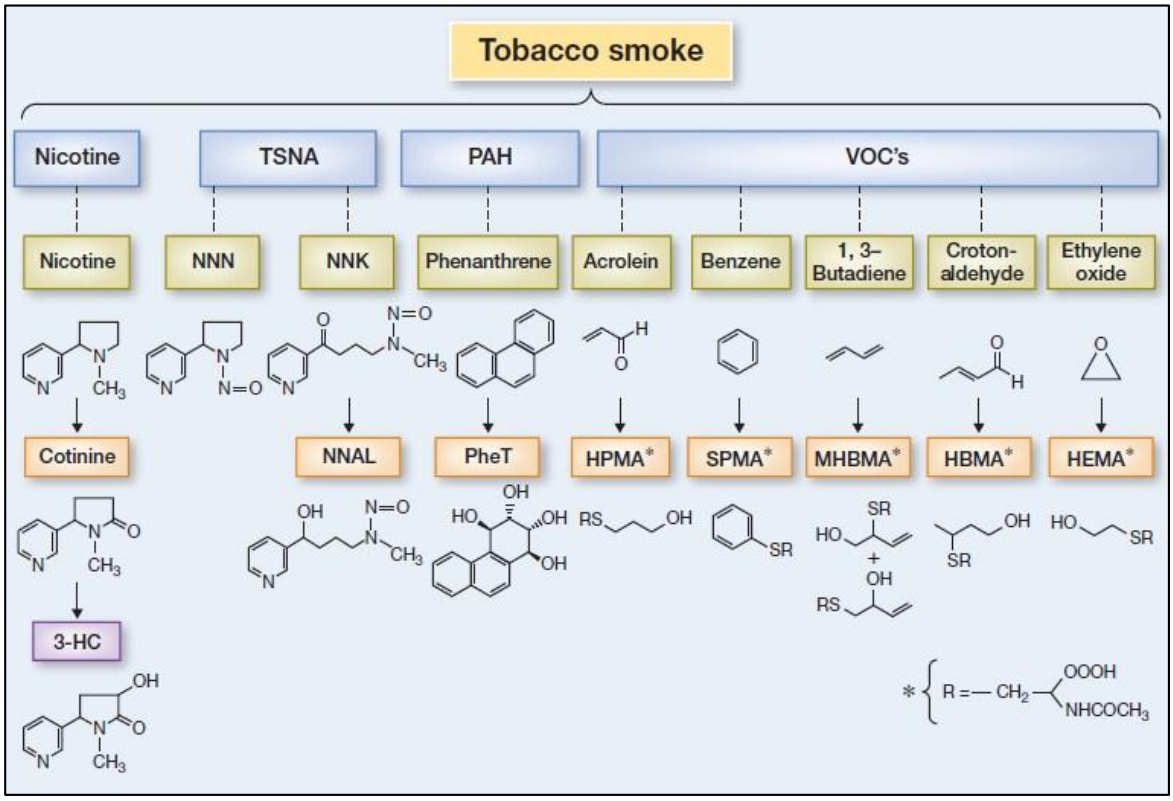

Figure 3: Metabolites of tobacco. Tobacco alkaloids are the active principal components in all tobacco products. Among the components of tobacco, nicotine is the most abundant (98% of the total alkaloids) tobacco metabolite (Yuan *et al.*, 2014).

Tobacco Metabolites Used as Tobacco-Specific Biomarkers

A biomarker is desirable in quantitating systemic exposure to SHS in different individuals. Therefore, the national research council has proposed four criteria's which should be fulfilled so that a specific tobacco metabolite can be used as a valid biomarker to detect ETS (Florescu *et al.*, 2009). Table 1 explains the four criteria's for tobacco biomarkers. Whereas, in table 2 presents the most ideal markers used to assess ETS exposure.

Table 1: The four criteria's proposed by national research council (Benowitz, 1999).

	Criteria's
01.	Should be unique or nearly unique so that other sources are minor in comparison
02.	Should be easily detectable and have a reasonable half life
03.	Should be emitted at similar rates for a variety of tobacco products
04	Should have a fairly constant ratio to other ETS components of interest under a range of environmental conditions encountered

Table 2: Biomarker used to detect environmental tobacco in passive smokers.

Biomarker	Reference	Specificity	Sensitivity	Duration after exposure reflected	Comments
Cotinine	Dhar, 2004	High	High	3-4 days	Can be measured in urine, plasma, saliva and hair
Nicotine	Yuan *et al.*, 2014	High	High	Hours	Short half-life (2-3 hours) indicating that results are very dependent on time of sampling; saliva nicotine can be elevated by local deposition of ETS. Plasma levels are very low. Urine levels are highly influenced by urine volume and pH. Hair measurement is promising as a long term marker of exposure. But analytical techniques are relatively expensive.
Carbon monoxide (CO)	Hatsukami *et al.*, 2010; SRNT subcommittee on biochemical verification, 2002	Low	Low	Hours	Many environmental sources, CO also produced by endogenous metabolism. Only small changes in CO levels are seen after ETS exposure. Short half-life (4-5 hours) limits its usefulness to determine ETS exposure. Sensitivity limited due to rapid elimination.
Thiocyanate (SCN)	Jarvis *et al.*, 1987	Low	Low	Weeks	Many dietary sources. Specifically reliable for heavy smokers and not for light smokers due to dietary sources also contain SCN.
Aminobiphenyl-haemoglobin	Hammod et al., 1993; Bartsch et al., 1990	Moderate	Moderate	Months	Levels in non-smoker may be 10 to 20% those of smokers. Analytical techniques technically difficult.

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Benzo[a]pyrene-DNA adduct	Binkova *et al.*, 1995; Van Maanen *et al.*, 1994	Low	Low	Probably months	Analysis is technically difficult. Difference between smokers and non-smokers are found in all studies
PAH-albumin	Benowitz,1999	Moderate	Moderate	21 days	Analysis is technically difficult

Cotinine, the major metabolite of nicotine has a longer half-life (15 - 19 hours) in body fluids, and it remains stable throughout the day when compared to nicotine which oscillates (Florescu *et al.*, 2009; Benowitz, 1996). In addition, it is very specific when compared to other markers, because it has a long elimination time and its levels are not affected by the other factors such as, environment factors and dietary intake (Yuan *et al.*, 2014). Therefore, cotinine is known to be the best surrogate biomarker to assess the level of tobacco exposure in passive smokers (Flores, Liu & Taroli, 2016; Haley, Axelard & Tilton, 1983).

Cotinine Detection in Biological Matrices

The levels of cotinine present in biological fluids due to SHS is in low concentration, therefore assessing the level of risk due to tobacco smoke is a challenging task (Yuan *et al.*, 2014).Thus, a range of analytical techniques such as; gas chromatography –MS (GC-MS), radioimmunoassay (RIA) and high performance liquid chromatography (HPLC) with UV/MS are being designed to detect cotinine. RIA is a sensitive method as it uses antibodies which bind to cotinine. However, it is not specific because the antibodies may bind to structures similar to cotinine in the sample of study which will result in a false positive analysis (Tricker, 2006). GC and HPLC are highly specific and sensitive methods because it separates the chemical using a column, and then measures that separated molecule using a detector. In this review, the development of different analytical techniques in four body fluids will be described.

Salivary Cotinine Detection

Salivary cotinine is commonly used for large population studies as it is a non – invasive technique which can be carried out quickly. Moreover, it can be used in studies where multiple sample collection is required. However, as it is pH dependent, standardized sample collection and storage is required for saliva cotinine measurements (Tricker, 2006).

Initially cotinine determination in saliva was performed by RIA, developed by Langone and his team in 1973. In this technique they used a radioactive tracer known as cotinine 4'carboxyllic acid molecule that covalently bound to ^{125}I-probe, this complex then binds to the cotinine present in saliva. This is detected using an immuno-electrophoretic method (Haley, Axelrad & Tilton, 1983; Matsukura *et al.*, 1979). Later, in 1986 Bjercke and his coworkers proposed that by using monoclonal antibodies the specificity and sensitivity of cotinine detection can be increased 10 times to that of conventional RIA. Therefore, they used monoclonal antibodies to develop enzyme linked immunosorbent assays (ELISA) and a fluorescence immuno assay (FIA) (Benkirane *et al.*, 1991). But, in 1987 he used polyclonal antibodies, which have shown a 100 – 1000 fold higher sensitivity compared to monoclonal antibodies (Bjercke, 1987). This technique was later opposed by Feyerabend and Russelll in 1990, because they observed that chromatography techniques were more sensitive and specific compared to immunoassays. Thus, his team proposed GC with nitrogen/phosphorus thermionic detection (GC-NPD) technique for chemical analysis and quantification of cotinine.

This declaration was then confirmed by Watts and his fellow scientists in 1990, when they observed that there were no cross reactions that occurred, because the elements (nitrogen and phosphorus) in the cotinine molecule was itself used for detection (Matsumoto *et al*., 2010; Zuccaro *et al*., 1997; Watts *et al*., 1990). However, all the above mentioned methods are generally time consuming because sample extraction is done manually and they are expensive since they require trained expertise and costly reagents for equipment maintenance. Therefore, in 2009 Kataoka and his associates developed an automated in tube solid phase micro extraction (SPME) technique coupled with LC-MS. This extraction method is automated thereby reducing analysis time and carry over contamination because the extracted sample is directly injected to the chromatography column. Hence, it increases the precision and sensitivity of the analytical method (Kataoka *et al.*, 2009; Floresceu *et al.*, 2009; Elsert & Pawliszyn, 1997). Figure 4 illustrates the experimental study carried out to prove SPME method.

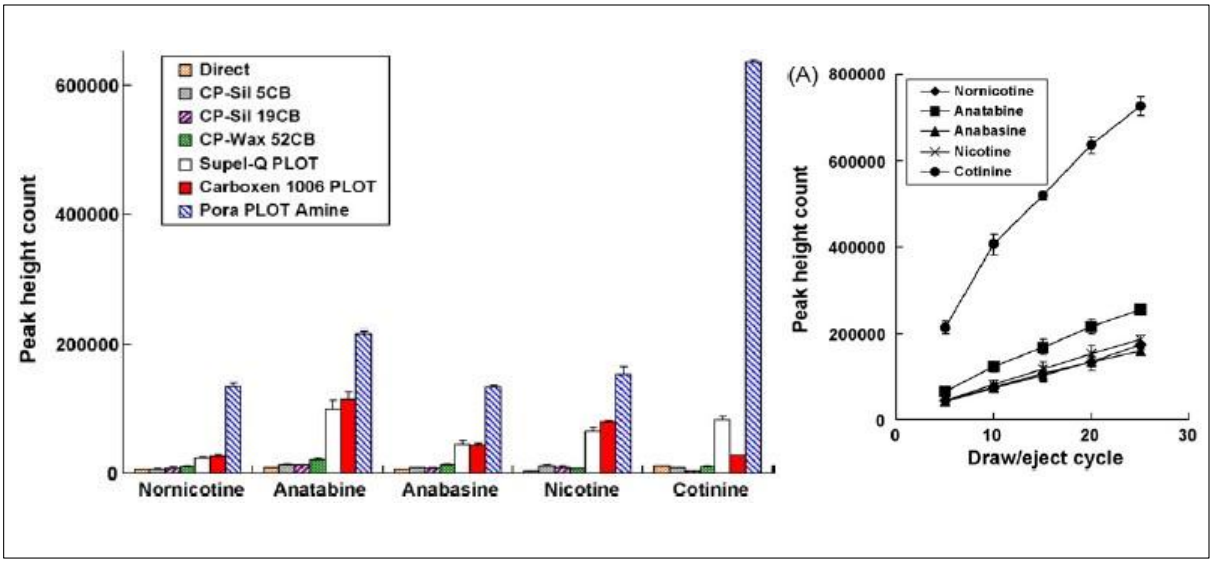

Figure 4: The Experimental study conducted by Kataoka and his team. The extraction was optimized by a number of factors like the stationary phase of the in tube SPME capillary column and number and volume of draw/eject cycles. In this study six different capillary columns were tested; CP-Sil 5CB, CP-Sil 19CB, CP-Wax 52CB, Supel-QPLOT Carboxen 1006 PLOTand Pora PLOT Amine. Among these types Pora PLOT amine gave superior extraction efficiency because of its affinity to polar compounds and it had a greater adsorption surface. In addition the extraction was observed to be most efficient at 30µL/min (Kataoka *et al*., 2009).

Although the method proposed by Kataoka and his team, reduced sample pretreatment analysis time, it could only be used to quantify large cotinine concentration. For this reason, in 2011 Ill and his co-workers decided to modify the LC-tandem mass spectrometry method (LC-MS-MS) which was initially proposed by Bernet and his team at the center of disease control (CDC). They utilized electroscopy ionization (ESI) and atmospheric pressure chemical ionization (APCI) methods to increase the sensitivity and specificity for the determination of cotinine in low level exposure to ETS (Stragierowicz *et al.*, 2013; Pitt, 2009). However, this technique was found to be highly prone to matrix suppression of ionization (Ill *et al.*, 2011; Mallet, Lu
& Mazzeo, 2004; Liang *et al.*, 2003; Matuszewski, Constanzer & Charez-Engem, 1998). Therefore, in 2016 Ramdzan and his fellow workers established lab – on – valve technique also known as third generation of flow injection analysis to automate sample pre-treatment (Ramos, 2012; Kataoka *et al.*, 2009). As a result, they developed the bead injection-lab on valve (BI-LOV) for hyphenation of solid phase extraction (SPE) coupled to HPLC in the determination of cotinine. In addition, this technique is less time consuming and cost effective as sample pretreatment is automated. Table 3 illustrates a summary of certain salivary detection techniques.

Table 3: Summary of analytical techniques to detect salivary cotinine.

Technique/ Assay	Limit of Detection (LOD/ µg/ml)	Limit of Quantification (LOQ µg/ml)	Reference
RIA (^{125}I, ^{3}H)	2.00 x 10^{-3}	0.37	Langone &Van Vunakis, 1987; Langone *et al.*, 1973
RIA (^{3}H)	0.37 x 10^{-3}	0.2	Haley, Axelard & Tilton,1983
RIA (^{125}I, ^{3}H), ELISA, FIA	0.50 – 1.50 x 10^{-3}	0.1	Bjercke *et al.*,1986
GC-NPD	0.10 x 10^{-3}	10 x 10^{-3}	Feyerabend *et al.*, 1986
LC-MS/MS	0.1 x 10^{-3}	50 x 10^{-3}	Matuszewski, Constanzer & Charez-Engem, 1998.
SPME-HPLC/MS	15 – 40 x 10^{-2}	-	Kataoka *et al.*, 2009
LC-API-MS/MS	1.07 x 10^{-3}	-	Smith *et al.*, 2014; Bernet *et al.*, 2009
GC-MS	14.7 x 10^{-3}	10 x 10^{-3}	Parzynski *et al.*, 2008; Torano &Van, 2003
LC-ESI-APCI-MS	0.12 x 10^{-3}	>0.4 x 10^{-3}	Stragierwicz *et al.*, 2013
BI-LOV-µSPE-HILIC	15 x 10^{-3}	3.0 x 10^{-3}	Ramdzan *et al.*, 2016

Urinary Cotinine Detection

Urinary cotinine detection is a non – invasive method that depends on urinary pH and rate of urine flow. Therefore, appropriate storage conditions should be given to get reliable results, since high temperature can cause thermal degradation of N-glucuronide and thus increasing urinary cotinine levels, resulting in false positive analysis. For instance, polypropylene-polyethylene sponge salisoft tubes are used for urine collection and it should be stored at -20^{0}C (Tricker, 2006).

In the past urinary cotinine levels were detected using conventional RIA methods, however, as it lacked specificity, different analytical techniques were developed (Langone & Van Vunakis, 1987; Knight *et al.*, 1985; Langone, Gjika & Van Vunakis, 1973). For instance, in 1991 Hariharan and Van Noord developed gas chromatography on capillary column coupled to nitrogen phosphorus detector (GC-NPD) method to overcome cross reactivity that could occur in RLA methods. However, GC-NPD was found to have a lower detection limit as they are affected by certain drugs such as Theophylline if present in sample (Matsumoto *et al.*, 2010; Kuo, Yang & Chiu, 2001; Feyerabend & Russel, 1990). Therefore, in 1998 Oddoze and his team developed reversed phase HPLC method paired with UV detector

(RH-HPLC-UV), which is applicable for epidemiological studies. Since, these preliminary HPLC techniques can be non-specific as it can interfere with caffeine if present in sample (Kim *et al.*, 2004; Knight *et al.*, 1989; Langone, Gijka & Van Vunakis, 1973). As a result, compound specific methods such as mass spectrometry (HPLC – atmospheric pressure chemical ionization tandem mass spectrometry (HPLC-API-MS) which is more specific was developed in 1997 by Bernet and his friends (Tuomi *et al.*, 1999; Rustemeier *et al.*1993; Mc Manus *et al.*, 1990). Figure 5 illustrates the comparison between compound specific detection and compound non-specific detection.

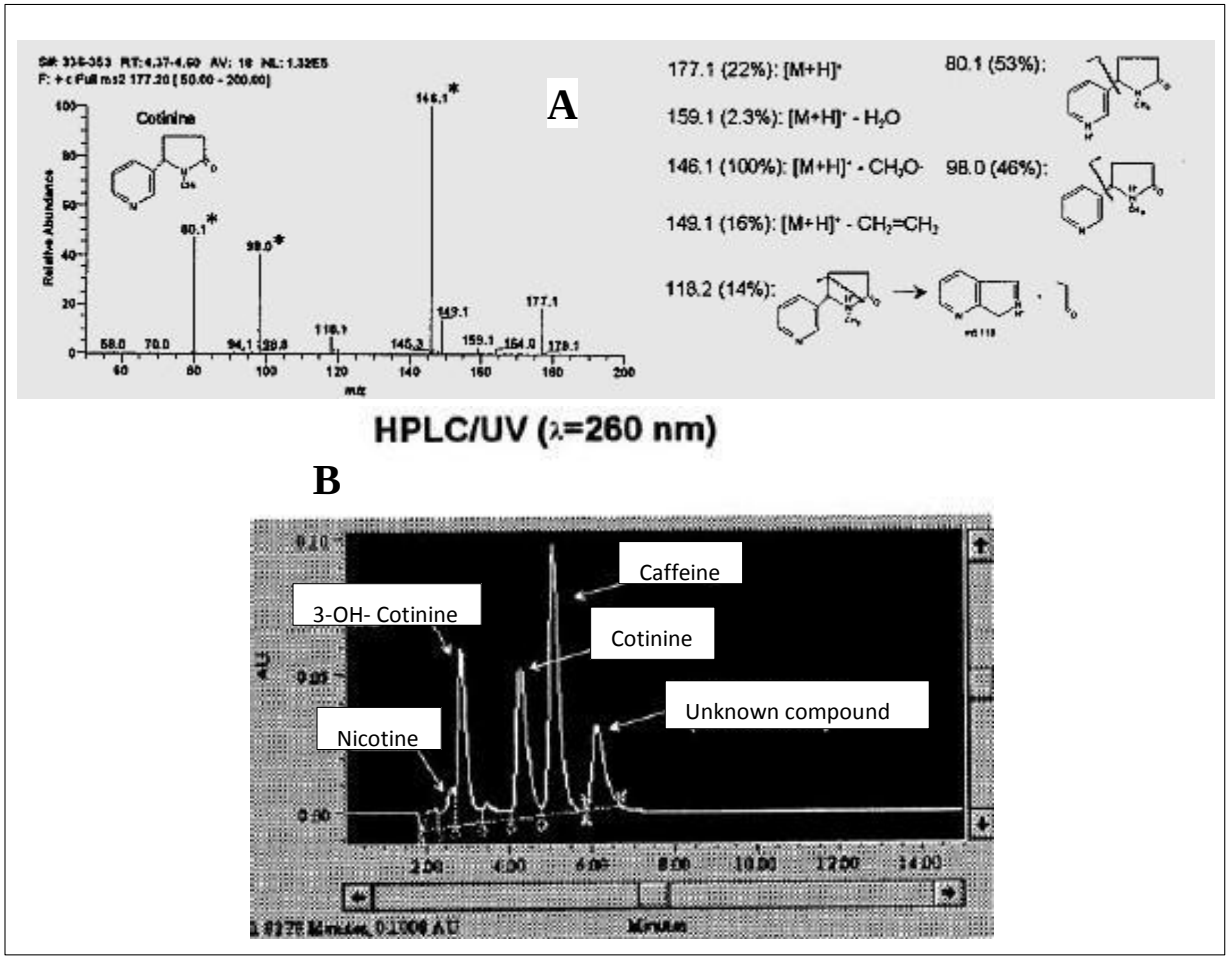

Figure 5: (A) Compound specific detection gives rise to peaks only specific to elements present in cotinine. Whereas, compound non-specific detection (B) can have peaks for other components present in ETS such as caffeine (Tuomi *et al.*, 1999).

However, since all of the above techniques are usually time-consuming in sample pretreatment Kataoka and his co-workers developed an automated in tube solid phase extraction coupled to HPLC and LC-MSMS in 2009. This technique is selective, sensitive and allows easy monitoring of ETS in passive and active smokers. Whereas, in 2010 Apinan and her team designed a simpler, sensitive and selective technique to detect cotinine in a single step analysis. This method was based on HPLC electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS). Although these techniques did not require sample pretreatment, it was highly likely that contamination could occur, giving rise to inaccurate results. Hence, in 2013 Iwai and his team used micro-extraction by packed sorbent coupled to GC-MS (MEPS-GC-MS) technique to study passive smokers in ETS. This technique increased sensitivity as it was directly subjected to GC-MS without sample evaporation. Moreover, in 2016 Li and his team developed liquid-liquid micro extraction (LLME) combined with GC-MS to avoid any cross contamination. Later that year, Carrizo and his team redesigned the mass spectrometry technique, desorption electrospray ionization (DESI) and the direct analysis in real

time (DART) techniques proposed by Mc Ewen in 2005. They coupled this atmospheric pressure solid analysis probe (ASAP) technique to a high resolution quadruple time of flight mass spectrometry (Q-TOF-MS) technique in order to detect and study the specific structure and shape of nicotine and their major metabolites - cotinine in biological samples. This technique is was proved to be a highly efficient and sensitive method because it does not involve and sample pre-treatment and the sample can be directly injected in to the ionization chamber (Carrizo *et al.*,2016). Table 4 summarizes the analytical techniques to detect urinary cotinine.

Table 4: Analytical methods to detect urinary cotinine.

Technique/ Assay	**Limit of Detection (LOD/µg/ml)**	**Limit of Quantification (LOQ /µg/ml)**	**Reference**
RIA (^{125}I)	0.4 x 10^{-3}	0.2 x 10^{-3}	Knight *et al.*, 1989
ELISA	7-8 x 10^{-3}	4-8 x 10^{-3}	Yoshioka, Dohi & Yonemasu, 1998
LE-HPLC-UV	0.5 x 10^{-3}	0.5 x 10^{-3}	Oddoze, pauli & Pastor,1998
RH-HPLC-EI-MS/MS	1.0 x 10^{-3}	1.0 x 10^{-3}	Tuomi, Johnsson & Reijula, 1999
SPE-HPLC	6.0 x 10^{-3}	1.5-6.0 x 10^{-3}	Bazylak, Brozik & Sabanty, 2000
HPLC	0.078 x 10^{-3}	14.3 x 10^{-3}	Kuo, Yang & Chiu, 2001
GC-NPD	0.2 x 10^{-3}	37.6 x 10^{-3}	Kuo, Yang & Chiu, 2001
GC-MS	0.2 x 10^{-3}	1-20 x 10^{-3}	Man *et al.*, 2006
SPME-LC-MS	15-40 x 10^{-2}	0.05-20 x 10^{-3}	Apinan, Choemug & Na-Bangchang, 2010; Katoaka *et al.*, 2009
HPLC-ESI-MS/MS	10.0 x 10^{-3}	0.02 x 10^{-3}	Apinan, Choemug & Na-Bangchang, 2010
UPLC-EI-MS/MS	0.4 x 10^{-3}	1.1 x 10^{-3}	Kuhn *et al.*, 2012
SPE-GC-MS/MS	0.06 x 10^{-3}	10.0 x 10^{-3}	Chiadmi & Schlatter, 2014
MEPS-GC-MS	20 x 10^{-3}	50.0 x 10^{-3}	Iwai *et al.*, 2013

SPE-UPLC-MS/MS	1.0×10^{-3}	10.0×10^{-3}	Cremer, Overmeire & Loco, 2013
ASAP-MS-Q-TOF	0.51×10^{-3}	3.0×10^{-3}	Carrizo, 2016

Serum Cotinine Detection

In passive smokers, cotinine concentrations in plasma increases during the first 4-6 hours after exposure and then it gradually plateaus down (Teeuwen, Alder & Van Rossum, 1989; Feyerabend, Ing & Russell, 1985). Therefore, the best time to collect blood samples is in the mid afternoon as the cotinine levels remain almost in a steady state. This can then be stored at 37^0C for 6 weeks, as serum cotinine is stable at normal body temperature (Tricker, 2006; Foulds *et al.,* 1994).

Methods for assessing cotinine in passive smokers have developed over the past two decades. Initially, in the early 1980s RIA techniques were used to detect serum cotinine, but this technique has a 0.3% chance of cross reactivity with other tobacco metabolites producing inaccurate results (Watts *et al.,* 1990; Langone & Van Vunakis, 1987; Langone Gjika & Van Vunakis, 1973). Hence, in 1989 Jacob and his team designed an improved gas chromatographic method for quantification of cotinine. In this method they used 1-butanol instead of methylene chloride to extract cotinine, since this results in less emulsion formation and cleaner separation layers. However, in 1997, the Third National Health and Nutrition Examination Survey
(NHANES 111) stated that LC-MS-MS techniques are more suited for analyzing low cotinine concentrations when compared to GC-MS techniques. Since, serum cotinine levels below ≥10µg/L will not be detected using capillary GC-MS (Bernet *et al.,* 1997; Watts *et al.,* 1990; Davis, 1986). Therefore, that year Bernet and his team designed a HPLC technique coupled to atmospheric pressure chemical ionization tandem mass spectrometry (API MS-MS). However, in 2002 Shin and his colleagues observed that APIMS-MS technique did not produce reliable results when the serum samples were in an alkaline state. Therefore, they developed an idea of concentrating the sample using nitrogen stream and directly injecting it to the GC-MS-selected ion monitoring (GC-MS-SIM) column. This proved to be a simple and convenient method, that and can be learned easily by unexperienced individuals. All the above mentioned techniques required large sample volumes and sample pretreatment prior to analysis (Nakajima *et al.,* 2000). Thus, in 2005 Chang and his team developed a rapid, sensitive and cost effective technique, utilizing microdialysis sample extraction coupled with HPLC- UV detector. Sample preparation using microdialysis helps to continuously monitor the samples and reduce the chances of fluid loss. But further research provides more sensitive chromatography techniques. For instance, in 2012, Xu and Fan decided to study the sensitivity of cotinine using micellar electrokinetic chromatography (MEKC), combined with on line concentration technique, cation selective exhaustive injection (CSEI) and sweeping. The LOD was quantified using ultraviolet absorbance detection and it was 0.2ng/ml. Therefore, this method was proven to be successful. In addition, in 2013 Liu and his associates designed laminated analytical device (LPAD) with origami enabled chemiluminescence immunoassay to detect cotinine in SHS. This device was then fabricated by a craft-cutter to define flow channels and then laminated. This was done to improve the reliability of results and simplicity of the technique. This method proved to be cost effective and sensitive. Moreover, in 2014 Dunlop and his colleagues designed a LC-MS-MS technique using cotinine d_3 as an internal standard couple to SPE to study large populations within a short period of time. The specificity and sensitivity of the results increased as cotinine quantification was done using electrospray ionization with multiple reaction monitoring. This technique was better than the other LC-M-MS techniques as the sample preparation and quantification was all one in a single analysis. Table 5 summarizes the analytical techniques used to detect serum cotinine over the years.

Table 5: Summary of analytical methods to detect serum cotinine.

Technique/ Assay	Limit of Detection (LOD µg/ml)	Limit of Quantification (LOQ µg/ml)	Reference
RIA (^{125}I, ^{3}H)	2.00 x 10^{-3}	2.00 x 10^{-3}	Langone & Van Vunakis, 1987
RIA (^{3}H)	0.37 x 10^{-3}	1.0 x 10^{-3}	Haley, Axelard & Tilton, 1983
RIA (^{125}I)	0.3 x 10^{-3}	1.0 x 10^{-3}	Knight *et al.,* 1985
RIA (^{125}I, ^{3}H), ELISA, FIA	0.50– 1.50 x 10^{-3}	0.50-1.5 x 10^{-3}	Bjercke *et al.*,1986
GC-NPD	0.10 x 10^{-3}	0.1 x 10^{-3}	Feyerabend, 1987
HRGC-NPD	0.2 x 10^{-3}	0.5-5.0 x 10^{-3}	Jacob, Wilson & Benowitz, 1989
Capillary GLC/NPD	0.1 x 10^{-3}	0.1 x 10^{-3}	Bernet *et al.*, 2009
HPLC-API-MS MS	<0.1 x 10^{-3}	<0.1 x 10^{-3}	Bernet *et al.*, 2009; Bernet *et al.*, 1997
HPLC-ESI-MS-MS	0.5 x 10^{-3}	0.5 x 10^{-3}	Bernet *et al.*, 2009
LLE-GC-MS	0.16 x 10^{-3}	1.25 x 10^{-3}	James, Tizabi & Taylor, 1998
LLE-GC-MS-SIM	1.0 x 10^{-3}	1.0 x 10^{-3}	Shin *et al.*, 2002
HPLC-UV	0.01	0.05	Chang *et al.*, 2005
SPE-LC-ESI-MS-MS	0.13 x 10^{-3}	0.2 x 10^{-3}	Dunlop *et al.*, 2014

Hair Cotinine Detection

Analysis of cotinine in human hair provides reliable information on ETS over a long time period. However, it can be affected by chemicals used in hair dyes, and the melanin content (Umetsu, 1993; Kintz & Mangin, 1993). The cut off value to distinguish between a smoker and non-smoker is >2ng cotinine/mg of hair, which can be analysed by a number of different analytical methods (Kintz, Ludes & Mangin, 1992). These techniques differ in either the washing or extraction steps. As a range of solvents such as, ethanol and dichloromethane are used to the wash hair to remove passive absorbed cotinine prior to analysis (AlDelamiy, 2002). Upon washing the samples are subjected to neutralization using concentrated hydrochloric acid and then they are analyzed using a respective analytical technique (Tricker, 2006).

In the past, GC and radioimmunoassay (RIA) techniques were used to detect cotinine, however, these techniques are expensive and cannot be used in routine laboratories. In addition, RIA measurement of cotinine can cause cross reactivity, especially with other cotinine and nicotine metabolites (Torano & Van Kan, 2003; Dimish-Ward *et al.*, 1997). Therefore, in 2001 Mahoney and Al-Delaimy used solid phase extraction, reversed-phase high performance liquid chromatography (SPE-RH-HPLC/ECD) coupled with electrochemical detection, because it gave a better LOD (0.05ng/mg) than HPLC coupled to UV detection. But RH-HPLC-ECD or HPLC/ECD are not used in common

laboratories, and the sorbents used in SPE to retain the analytes result in low sensitivity and specificity as the binding is not specific. Thus, SPE coupled with molecular imprinted polymer (MISPE) enhanced the sensitivity of the HPLC technique (Yang *et al.,* 2007; Mahoney & Al-Delaimy, 2001). Although the sensitivity of HPLC increased in terms of extraction it was not capable of analyzing minute cotinine concentrations and large number of sample in a single analysis. Hence, in 2013 Tsuji and his co-workers developed HPLC/UV to column switching method which was 12.9 to 16.9 more sensitive than preliminary HPLC techniques. Figure 6 illustrates the increased sensitivity of sample analysis using HPLC/UV couple with column switching method and other HPLC techniques. Thereafter, in 2016 Pradel and his partners, introduced spectroscopic methods using non-linear multi-photon lasers for ultrasensitive detection, separation and identification of cotinine. In this technique extraction method was very sensitive as it used compact, portable capillary-or microchip based separation.

Table 6 illustrates a summary of the techniques proposed above for hair cotinine.

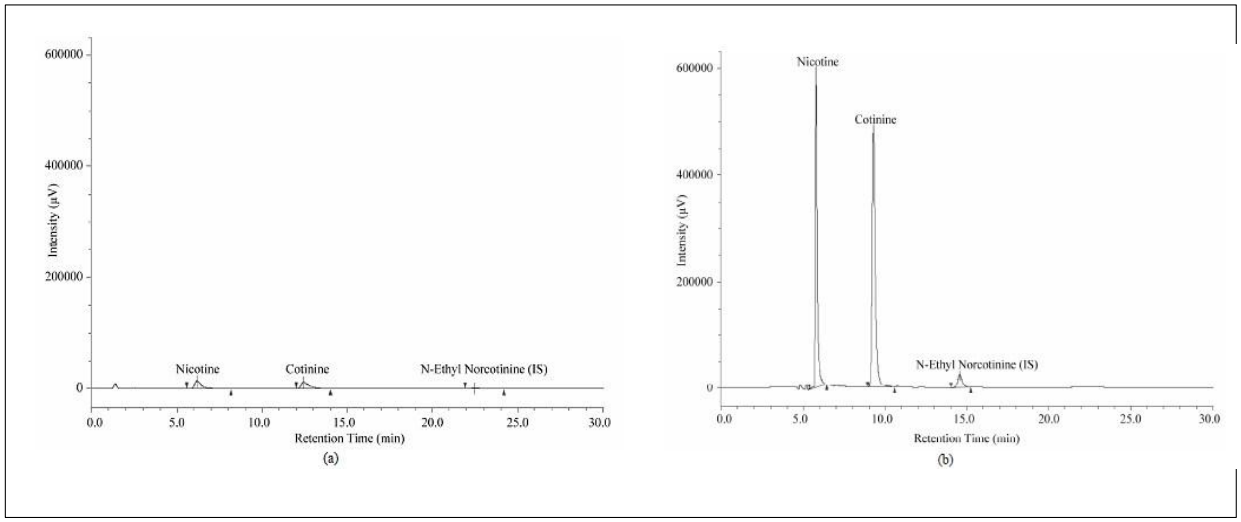

Figure 6: Chromatograms obtained for hair cotinine and nicotine sample using different HPLC techniques. (a)Results obtained from preliminary HPLC techniques, which lack sensitivity when analyzed for minute cotinine levels. (b) Results obtained from HPLC/ UV coupled with column switching, which had a greater sensitivity than the other HPLC techniques (Tsuji *et al.,* 2013).

Table 6: Summary of hair cotinine detection techniques.

Technique/ Assay	**Limit of Detection (LOD ng/mg)**	**Limit of Quantification (LOQ ng/mg)**	**Reference**
RIA	0.1	0.3	Ellopoulos *et al.,* 1994
GC-MS	0.01	0.60	Torano & Van Kan, 2003
SPE-RH-HPLC/ECD	0.05	0.1	Mahoney & Al-Delaimy,2001
MISPE-HPLC	0.2	0.5	Yang *et al.,* 2007

LLE-GC/MS	0.02	-	Kim *et al.,* 2008
GC-NPD	0.2	-	Kim *et al.,* 2009
HPLC/UV	0.08	0.1	Tsuji *et al.,* 2013

Summary and future perspectives

Cotinine, the first metabolite of nicotine is the best biomarker to indicate tobacco smoke exposure. This is because it has a long half - life and not affected by other environmental factors. Various methods are such as, HPLC, GC, colorimetric assays and RIA's are designed and used in developed countries, in order to setup smoke free regulations in public, indoor areas and workplaces. Cotinine present in each body fluid varies in concentration, therefore the most reliable body fluid should be chosen for population studies.

Urine cotinine was known to be a gold standard for ETS exposure measurement, therefore it was used widely for all epidemiological studies. But it was affected by inter-individual variability in cotinine excretion levels for similar exposures. In addition it had a short half-life of 20 hours. Salivary cotinine is subjected to similar drawbacks, plus saliva samples were prone to be concentrated by salivary glands giving rise to false high estimates of cotinine in saliva. Despite it being a non-invasive technique. Therefore, assessing hair cotinine levels is a better choice as it is less affected by daily changes in the ETS exposure (due to the slow growth rate of hair), body metabolisms and it provides a long term history because 1cm of hair represent a month's tobacco exposure. However, hair cotinine detection cannot be done between different races and ethnic groups, because melanin content play an important role. In that case the next best biological matrix would be saliva as it is a non-invasive method and not affected by and external factors like hair dyes. Thus, different analytical technique are being developed for detection of cotinine in each body fluid, table 7 illustrates the summary of the best available techniques for each body fluid.

In the future, genomic based methods and modernized immunological methods using mutant single chain Fv fragments are to be designed to detect cotinine in dried blood samples. This is mainly because it reduces the time required for cotinine detection and produces more accurate results, since it does not depend on sample collection and storage. In addition, micro extraction methods like, dispersive liquid - liquid micro – extraction (DLLEM) techniques are to be developed enhance the sensitivity of the test results. For instance DLLEM technique is coupled to injection port silylation (IPS) to detect cotinine using GC – MS.

Table 7: Summary of the most efficient, sensitive techniques for each body matrix.

	Saliva	**Serum**	**Urine**	**Hair**
Technique	BI-LOV-µSPE-HILIC	HPLC-UV	SPME-LC-MS	MISPE-HPLC
Internal Standard (IS)	Acetonitrile (ACN)	Acetonitrile (ACN)	Methanol	Acetonitrile (ACN)
Extraction Method	Automatic micro solid phase extraction	Micro-dialysis	Automated solid phase micro extraction	Solid phase extraction

LOD	15.0 x 10^{-3} µg/ml	0.01µg/ml	15.0-40.0 x 10^{-2} µg/ml	0.2 ng/mg
LOQ	3.0 x 10^{-3} µg/ml	0.05µg/ml	0.05-20.0 x 10^{-3} µg/ml	0.5 ng/mg
Advantage	Automated sample pretreatment to reduce pretreatment time and volume of reagents used. Higher sensitivity because hydrophilic interaction liquid chromatography column is used. ACN enhances the sensitivity and retention efficiency.	The samples of microdialyste are protein free making it possible for direct coupling of the microdialysis to the liquid chromatographic graphic analysis with no sample cleanup required. This is a simple, rapid and cost saving technique.	SPME-automated extraction method, reduces sample analysis time, better precision and sensitivity.	Selective extraction, as the molecular imprinted polymer has a recognition site to which the cotinine molecule can bind.

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INSECTICIDAL PROPERTIES OF SECONDARY METABOLITES PRODUCED BY AN ENDOLICHENIC FUNGUS, *PENICILLIUM PINOPHILUM* AGAINST STORED GRAIN INSECT PESTS, *CALLOSOBRUCHUS MACULATUS*.

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Abstract

Fungi produce secondary metabolites with insecticidal properties. To investigation the insecticidal activity from the isolation of secondary metabolites in fungus, *Penicillium pinophilum* and their toxicity against cowpea weevil, *Callosobruchus maculatus*. Pure fungi cultures were grown in small scale on PDA for two weeks. The fungi grown on PDA were used for further investigations and grown in large scale. After extracted to EtOAc and isolated bioactive compounds. The bioactive EtOAc extracts were partitioned with hexane, $CHCl_3$ and 50 % MeOH and bioactivity of the three fractions (hexane fraction, chloroform fraction and 50 % MeOH fraction) were evaluated for bioactivity separately using the insecticidal bioassay (Residual Film Bioassay) and anti-inflammatory bioassay (Heat Induced Human Red Blood Cell Bioassay). After bioactive $CHCl_3$ fraction and it sub-fractions were further purified using column chromatography and preparative TLC respectively. Pure compound 3 isolated from the chloroform extract of *P. pinophilum* was shown highly % mortality values (lowly LD_{50} values) and pure compounds 1, 2 and 3 were shown highly % inhibition values for above bio assays respectively. So results suggest pure compound 3 can be used as bio-insecticide against *C. maculatus* and pure compounds 1, 2 and 3 better than that aspirin as anti-inflammatory drugs.

Keywords: insecticidal bioassay, anti-inflammatory bioassay, *P. pinophilum*, *C. maculates*

Introduction

Cowpea, (*Vigna unguiculata*) is one of the most nutritious grain legumes for human consumption worldwide. It is a valuable source of dietary protein, vitamins and minerals. Loss of seed yield in legume crops during storage due to different types of stored-grain insects, notably bruchid beetles, is a very serious problem for farmers and traders.[1] Cowpea weevil, *Callosobruchus maculatus* (Coleoptera: Bruchidae) is one of the most destructive pest species of cowpea.

***Callosobruchus maculatus*:** Kingdom: Animalia, Phylum: Arthropoda, Class: Insecta, Order: Coleoptera, Family: Chrysomelidae, Genus: Callosobruchus, Cowpea weevils, Growth temperature: 32 ºC, Life time: 10 days, Humidity: 60% - 90%. Eggs: Adult females oviposit their egg on the surface of the cowpea and eggs are cemented to the surface of cowpea. Larva and Pupa: The larvae and pupae are normally only found in cells bored within the cowpea. Adult: *C. maculatus* adults are 2.1-3.5 mm long. The females are maculated with four elytral spots and males are plain with no distinct spots.[11, 12]

Recent estimations have revealed that about 2.5 million tons of pesticides are used on crops and the worldwide damage caused by pesticides reaches $100 billion annually. The indiscriminate use and the excessive reliance on chemical pesticides (phosphine, organophosphates) in crop protection have resulted in serious problems linked to

water and environmental contamination, phytotoxicity, and toxic hazards to human and non-target organisms. Selection of resistant insect strains, including *C. maculatus* against chemical pesticides is also a thorny problem in crop protection.[12] These concerns have resulted in a renewed interest being given to the search for new approaches to control insect pests without these negative draw-backs. In recent years, many research studies focused on the use of natural materials as low-risk pest control agents.[15, 16]

Bio-insecticides are certain types of insecticides derived from such natural materials as animals, plants, bacteria, fungi, viruses and certain minerals (Examples: Bacillus thuringiensis, Azadirachtin, Bacillus popilliae, Essential oils (citrus peel oil), Plants and plant extracts (Aframomum melegueta, Aglaia elliptical, Annona spp.)). Advantages of bio-insecticides: Do not persist long in the environment and have shorter shelf lives, they are effective in small quantities, safer to humans and animals compared to synthetic insecticides, they are very specific, often affecting only a single species of insect and have a very specific mode of action, slow in action and the timing of their application is relatively critical.[20]

Some fungi produce many secondary metabolites with insecticidal properties. Therefore, the present study was focused to deduce bio-insecticide from extract of *P. pinophilum.*

***Penicillium pinophilum*:** reproduces asexually and non-motile spores (conidia) produced exogenously by constriction at the tips of special hyphen branches (conidiophores).[3] Growth temperature: 32 ºC, Growth time: 7 days, Colony nature: diameter 35-36 mm, deep, sub center somewhat raised, sulcate, dense and floccose, margin entre; low, narrow, Mycelium: white to orange white, Conidiogenesis moderate and grayish green, exudates soluble pigment absents and reverse pale orange to light orange, Enzyme production: dextranase.[4]

Objective

To investigation of the insecticidal activity from the isolation of secondary metabolites in Endolichenic fungus, *Penicillium pinophilum* and their toxicity against cowpea weevil, *Callosobruchus maculatus*.

Material and Methods

3.1 Test insect and rearing conditions

Adult cowpea weevils were collected from stores at piliyandala, Sri Lanka in July 2015 infested black eye peas cowpea variety. The stock culture of *C. maculatus* raised by placing 100 unsexed adults in two-liter jars half full of disinfected black eye peas cowpea seeds. Muslin cloth was used to cover the top of the jars so that cowpea weevils could not escape. These parent cowpea weevils were allowed to mate for seven days under laboratory conditions (30 – 33 °C and 60 % – 90 % relative humidity) and lay eggs, after which they were removed. A day after emerging, the insects were sexed by the examination of the elytral pattern; females are maculated with four elytral spots whereas males are plain with no distinct spots. The experiment was carried out in the laboratory at institute of chemistry Ceylon using reared population of *C. maculatus*.[12]

3.2 Isolation of the fungal strain

The lichen host, *Pseudocyphellaria sp*., was collected from the Hakgala Botanical Garden situated at an elevation of about 1745 m above mean sea level within the Hakgala Strict Natural Reserve in the Nuwara Eliya District, Sri Lanka. The collected lichen samples were kept in sterilized polythene packs and transported to the laboratory at the Department of Chemistry, University of Kelaniya and processed within 24 h.

Fungal isolations were carried out according to the surface sterilization method.9 Healthy lichen thalli were cleaned in tap water and surface sterilized by consecutive immersion for 10 s in 95 % ethanol, 3 min in 0.5 % sodium hypochloride and 30 s in 75 % ethanol. The thalli were surface dried with sterile filter papers and aseptically cut into small segments. Lichen segments of size 1 × 1 cm were then placed (20 pieces from each of the 5 replicate samples of the lichen species) on 2 % malt extract agar (MEA) supplemented with 0.01 % streptomycin. The plates were sealed with parafilm and incubated up to 14 days at room temperature under ambient light. Fungi grown from each lichen particle were isolated into pure cultures. Slides containing pure cultures were prepared using the sticky tape method and identified using identification keys.[9] The emergent fungi were sub-cultured on 2 % MEA, photographed and deposited as a living voucher in the Department of Chemistry, University of Kelaniya under the accession number US/PA/06.

3.3 Molecular identification of the isolated endolichenic fungus

The fungal identification was achieved by analysis of the ITS region of its rDNA.[9] The sequence data obtained from the fungus has been submitted to GenBank with accession number KC 427134.

3.4 Extraction of the secondary metabolites

Penicillium pinophilum was cultured on PDA for 2 weeks. After 2 weeks the solid PDA were cut into small squares using a steriale spatula and transferred to 1 L Erlenmeyer flask. The PDA pieces were submerged in EtOAc and shaken for overnight at 170 rpm using a vertical shaker. The extract was filtered under suction using a Whatman no 1 filter paper and dried with anhydrous Na_2SO_4 to remove water. The solvent was evaporated under reduced pressure using a rotator evaporator at 40 ˚C. The EtOAc extract was transferred to a vial and N_2 was passed through the samples to remove remaining solvent in the extract.

3.5 Evaluation of bioactivity of crude extract using selected bioassay

3.5.1 Determination of Insecticidal Bioassay using Residual Film method

Mortality Test

The amounts of 50 µl, 100 µl, 200 µl, 350 µl and 500 µl of each extract dissolved in CH_2Cl_2 (1 mg/ml) were applied separately to clean glass vials. The vials were slowly rotated on a horizontal plane to apply the extracts on the inner surface. Vials were air dried to remove the remaining solvent and then three pairs of one-day old *C. maculatus* were introduced into them and screw caps of the vials were tightened (place at 32 ºC). After that percentage mortality of *C. maculatus* was recorded daily for 3 days.[12] The calculation of mortality rate was corrected for control mortality according to Abbott's formula: $Mc = \left(\frac{Mo-Me}{100-Me}\right) \times 100\%$

Where, Mo = Observed mortality rate of treated insects (%), Me = mortality rate of control (%), and Mc = corrected mortality rate (%).

3.5.2 Determination of Anti-inflammatory activity using Heat Induced Human Red Blood membrane stability bioassay.

Preparation of Red Blood cells (RBCs) suspension

The blood was collected from healthy human volunteer who has not taken any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10 % v/v suspension with normal saline.[18, 19]

Heat induced haemolysis

The reaction mixture (2 ml) consisted of 1 ml test sample of different doses (0.4 - 0.025 mg/ml) and 1 ml of 10 % RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 ºC for 30 min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 540 nm. The experiment was performed in triplicates for all the test samples.[18, 19] The Percentage inhibition of Haemolysis was calculated as follows:

$$\% \text{ Inhibition of hemolysis} = \left(\frac{\text{Absorbance for control} - \text{Absorbance for test sample}}{\text{Absorbance for control}}\right) \times 100\%$$

3.6 Solvent solvent partitioning of crude extract

A portion (3.60 g) of crude was dissolved in 100 mL of 80% methanol in water and partitioned with 150 mL of hexane three times. The hexane fractions were combined and dried with anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure using rotary evaporator at 40 ˚C. The aqueous methanol fraction was diluted to 60 % aqueous methanol by addition of appropriate volume of water and partitioned with 150 mL of $CHCl_3$ three times. The $CHCl_3$ fractions were combined and dried with anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure using rotary evaporator at 40 ˚C. Each fractions were transferred to glass vials and dried the fractions by passing N_2 gas. They were labeled as PC/M (methanol fraction), PC/C (chloroform fraction) and PC/H (hexane fraction). The anti-insecticidal and anti-inflammatory activities of each fractions were evaluated using the method describe 3.5.1 and 3.5.2.

Bioassay guided fraction of Chloroform fraction of *P. pinophilum*

A portion (2.7 g) of the bioactive $CHCl_3$ fraction was subjected to column chromatography on silica gel (81.0 g) by elution with CH_2Cl_2 followed by increasing amounts of MeOH in CH_2Cl_2, which afforded 38 fractions. These fractions were combined on the basis of their TLC profiles to give seven fractions; F/1 (116.3 mg), F/2 (160.0 mg), F/3 (76.7 mg), F/4 (476.8 mg), F/5 (71.7 mg), F/6 (33.7 mg) and F/7 (175.8 mg). The anti-insectisidal and anti-inflammatory activities of each fractions were evaluated using the method describe 3.5.1 and 3.5.2.

Purification of F/1 using Sephadex LH-20 Column

A portion of (110.0 mg) fraction F/1 was further fractionated by gel permeation chromatography over a column of Sepadex LH-20 (4.0 g) made up in hexane, and eluted with hexane contacting increasing amounts of CH_2Cl_2, CH_2Cl_2 only, followed by CH_2Cl_2 containing increasing amounts of MeOH, and finally 100 % MeOH. Twenty-two fractions (10 ml each) were collected and combined on the basis of their TLC profiles. The anti-insecticidal and anti-inflammatory activities of each fractions were evaluated using the method describe 3.5.1 and 3.5.2.

Fractionation of F/1/A using Silica gel column Chromatography and Purification using Preparative TLC

A protion (70 mg) of the resulting active fraction F/1/A was next chromatographed on silica gel to give three active fraction F/1/A/1 (12.3 mg), F/1/A/2 (20 mg) and F/1/A/3 (13.1 mg). Final purification of each of these three fractions by normal phase preparative TLC (2 % MeOH in CH_2Cl_2) led to the isolation of three pure compounds, 1 (8 mg), 2 (12 mg) and 3 (10 mg).

4.0 RESULTS AND DISCUSSION

4.1 Evaluation of bioactivity of crude, fractions and pure compounds using selected bioassay

4.1.1 Determination of Insecticidal Assays using residual film method

The toxic effects of the crude extract, chloroform fraction and pure compound 3 were evaluated against, *C. maculatus* by using the residual film method. Crude extract, chloroform fraction and pure compound 3 at different doses revealed toxicity. But any of hexane and methanol fractions, pure compounds 1 and 2 did not showed any toxic effect to *C. maculatus*.

The mortality percentages of *C. maculatus* were counted after 1st, 2nd and 3rd days at all doses 50, 100, 200, 350 and 500 µg/µl respectively. Then the percentages of corrected mortality were calculated by using Abbott's formula. Mortality percentage of *C. maculatus* treated with the crude extract, chloroform fraction and pure compound 3 isolated from the chloroform extract of *P. pinophilum* respectively by Film Residue method are shown in figure 1, 2 and 3.

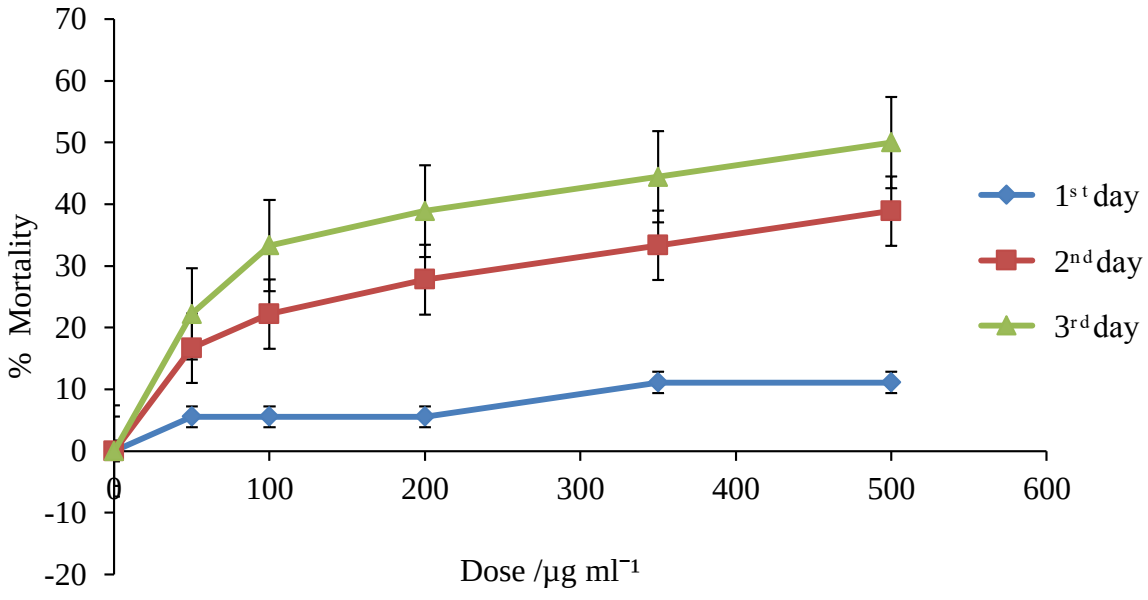

Figure 1: Mortality percentage of *C. maculatus* treated with the crude extract of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates.

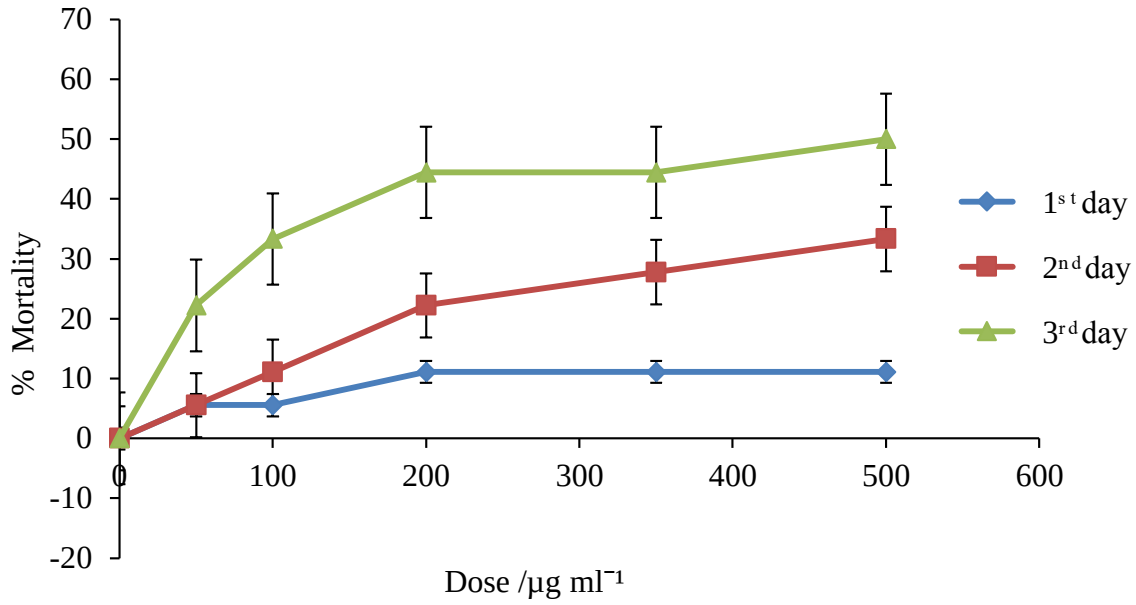

Figure 2: Mortality percentage of *C. maculatus* treated with the chloroform fraction of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates.

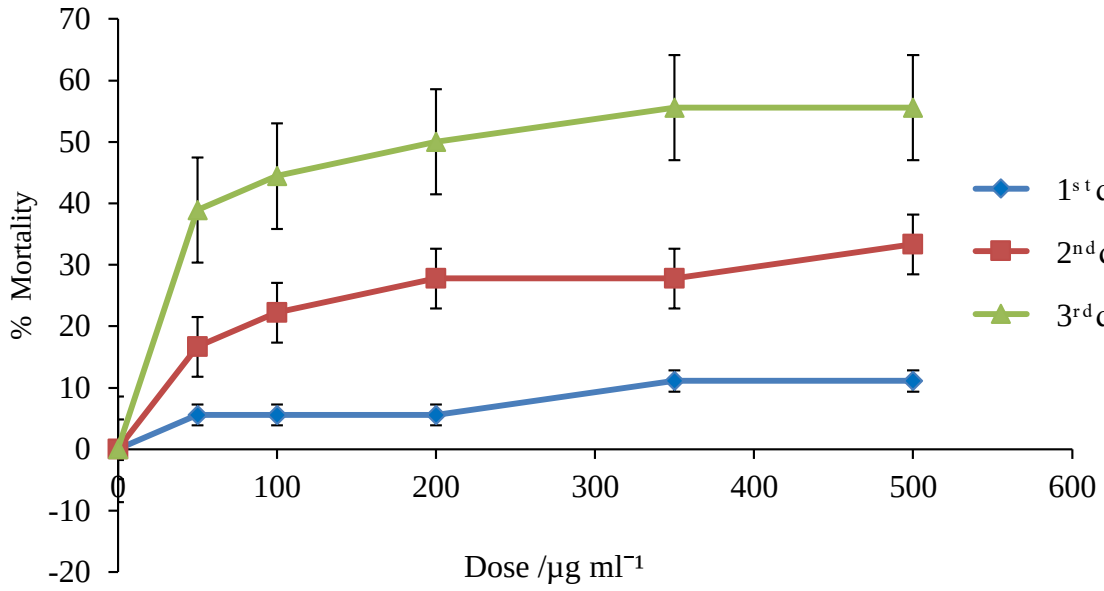

Figure 3: Mortality percentage of *C. maculatus* treated with the pure compound 3 of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates.

The results revealed that, the crude extract, chloroform fraction and pure compound 3 possessed the highest toxicity at doses of 500 and 350 µg/µl, but showed the moderate toxicity at doses of 200 and 100 µg/µl, where as the lowest toxic effect at dose of 50 µg/µl against *C. maculat* LD_{50} values of the crude extract, chloroform fraction and pure compound 3 isolated from the chloroform extract of *P. pinophilum* respectively against *C. maculatus* stored grain insect pests after 1st, 2nd and 3rd days of treatment are shown in figure No: 4.

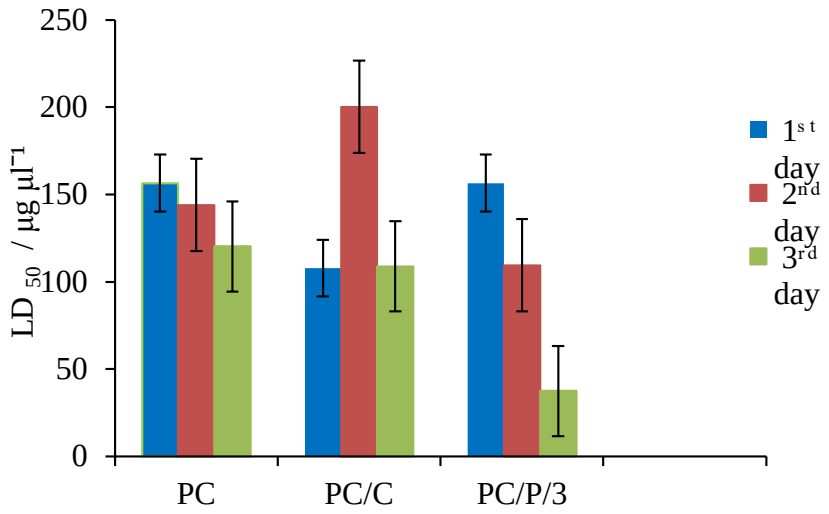

Figure 4: LD_{50} values of the crude extract, chloroform fraction and pure compound 3 of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates. PC- crude extract, PC/C- chloroform fraction and PC/P/3- pure compound 3.

The results of the probity analysis for the estimation of LC_{50} values at 1st, 2nd and 3rd days for the mortality of *C. maculatus* are presented in figure No: 4. The LC_{50} values of crude extract of *P. pinophilum* at 1st day after treatment is 156.52 µg/µl, at 2nd day after treatment is 143.98 µg/µl and at 3rd day after treatment is 120.24 µg/µl respectively. The results indicated that the crude of *P. pinophilum* at 3rd day at treatment was the most toxic against *C. maculatus*.

The LC_{50} values of chloroform fraction of *P. pinophilum* at 1st day after treatment is 107.84 µg/µl, at 2nd day after treatment is 200.16 µg/µl and at 3rd day after treatment is 108.88 µg/µl respectively. The results indicated that the chloroform fraction of *P. pinophilum* at 1st day at treatment was the most toxic against *C. maculatus*. The LC_{50} values of the pure compound 3 isolated from the chloroform extract of *P. pinophilum* at 1st day after treatment is 156.52 µg/µl, at 2nd day after treatment is 109.50 µg/µl and at 3rd day after treatment is 37.46 µg/µl respectively. The results indicated that the pure compound 3 isolated from the chloroform extract of *P. pinophilum* at 3rd day at treatment was the most toxic against *C. maculatus*.

From the insecticidal activity results, pure compound 3 isolated from the chloroform extract of *P. pinophilum* was shown insecticidal activity against cowpea weevil, *C. maculatus* by residual film bioassay (at day of LD_{50} of 3rd and 37.46 µg/µl) and so it can be used as bio-insecticide.

4.1.2 Determination of Anti-inflammatory Assay using Human Red blood cell membrane stability method

The HRBC membrane stabilization has been used as a method to study the *in-vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane[18, 19] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorder. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.[19] In recent years, the search for phytochemicals possessing anti-inflammatory property has been on the therapy of various chronic and infectious diseases. Epidemiology and experimental studies have implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of cardiovascular, diseases, cancer, and aging etc.[20]

Denaturation of proteins is a well-documented cause of inflammation. The inflammatory drugs (aspirin, ibuprofen, naproxen etc) have shown dose dependent ability to thermally induced protein denaturation. Similar results were observed from many reports from plant extract. The extracts may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further tissue inflammation and damage.[19]

Heat Induced Human Red Blood Cell Bioassay activity of the crude extract of *P. pinophilum* and the resulting hexane, chloroform and methanol fractions respectively compared with the aspirin are shown in figure 5.

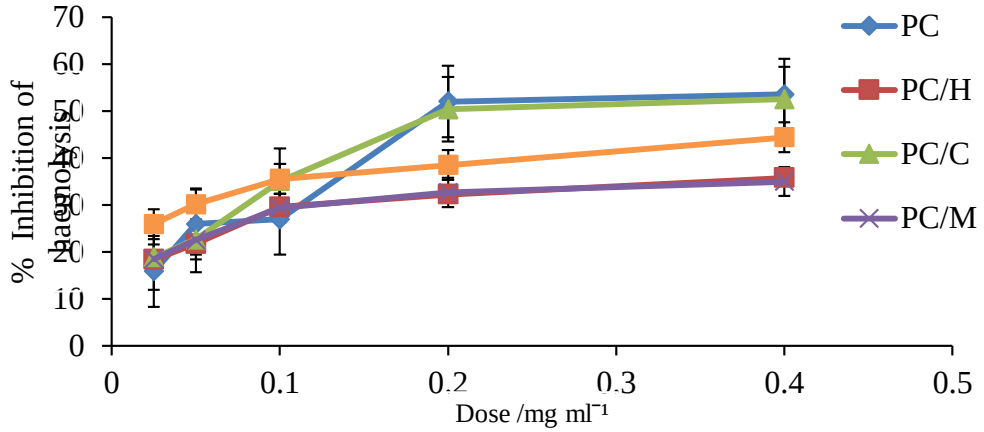

Figure 5: Anti-inflammatory assay activity of the crude extract, hexane, chloroform and 50 % methanol fractions of *P. pinophilum*. Note: Each data point represents the mean of 3 replicates. PC- crude extract, PC/H- hexane fraction, PC/C- chloroform fraction and PC/M- 50 % methanol fraction.

The percentage of membrane stabilization form isolated of secondary metabolites in fungus and aspirin were done at 0.4, 0.2, 0.1, 0.050, 0.025 mg/ml. In the anti-inflammatory assay, aspirin showed the best results (44.44 % at 0.4 mg/ml).

The activity increased in a dose dependant manner compared to aspirin. Crude extract of P. pinophilum and hexane, $CHCl_3$ and 50 % MeOH fractions were showed the maximum inhibition of haemolysis at 53%, 35%, 52% and 35 % respectively at dose of 0.4 mg/ml. The results revealed that the crude extract of *P. pinophilum* and $CHCl_3$ fraction have the highest activity.

Heat Induced Human Red Blood Cell Bioassay activity of the pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum* respectively compared with aspirin are shown in figure 6.

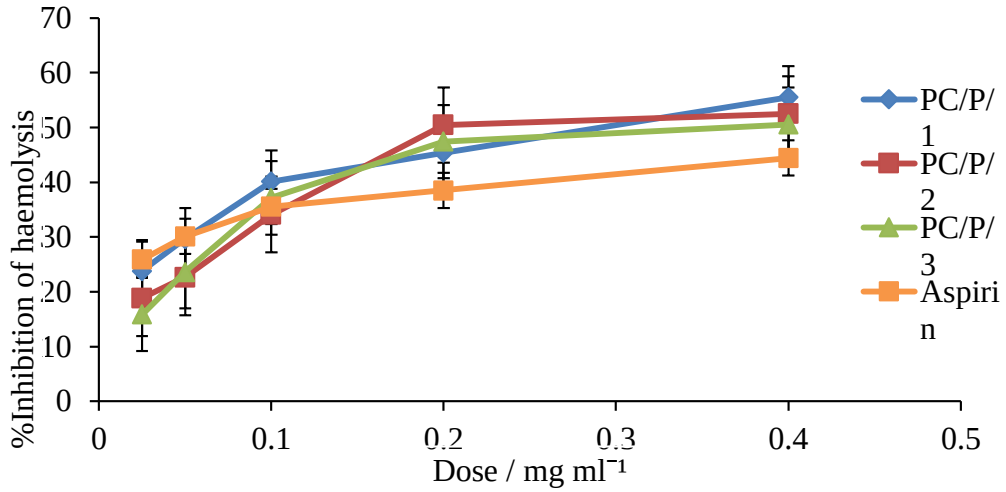

Figure 6: Anti-inflammatory assay activity of the pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum.* Note: Each data point represents the mean of 3 replicates. PC/P/1- pure compound 1, PC/P/2- pure compound 2 and PC/P/3- pure compound 3.

The 1, 2 and 3 pure compounds are effective in inhibiting the heat induced hemolytic of HRBC at different doses. They showed the maximum inhibition of hemolysis 55, 52 and 50 % respectively at dose of 0.4 mg/ml. The results

revealed that the 1, 2 and 3 pure compounds have the highest activity than that aspirin (at doses of 0.4 and 0.2 mg/ml) for Heat Induced Human Red Blood Cell Bioassay.

Heat Induced Human Red Blood Cell Bioassay activity of the pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum* respectively compared with aspirin. From the anti-inflammatory activity results, pure compounds 1, 2 and 3 were showed higher % inhibition values than that aspirin value (at doses of 0.4 and 0.2 mg/ml). So pure compounds 1, 2 and 3 better than that aspirin as anti-inflammatory drugs.

5.0 Conclusion

The EtOAc fractions of the endolichen fungus, isolated from the lichen *Pseudocyphellaria* showed highest anti-insectisidal and anti-inflammatory activity. Therefore, bioassay guidline fraction of the EtOAc extract was carried out. The chloroform fraction indicates that it contains more bioactive compounds than other two fractions. Therefore, chloroform fraction was further separated and three pure compounds were isolated. Based on results, it can be concluded that pure compound 3 isolated from the chloroform extract of *P. pinophilum* was showed higher % mortality value (at day of % mortality of 3rd and 55.56) and lower LD_{50} value (at day of LD_{50} of 3rd and 37.46 µg/µl). Chemical insecticides are costly and not sustainable in the long run due to environmental contamination, the use of this extract of fungus would be cost effective and sustainable, especially considering that this fungus is easy to grow. The pure compound 3 isolated from the chloroform extract of fungus, many of which is selective and has little or no harmful effect on non-target organisms and the environment. So this compound would play a role in the future of management of stored-grain insects, including *C. maculatus*.

Pure compounds (1, 2 and 3) isolated from the chloroform extract of *P. pinophilum* were effectively inhibiting the heat induced hemolysis (at doses of 0.4 and 0.2 mg/ml). These results provide evidence for membrane stabilization as an additional mechanism of their ant-inflammatory effect. The anti-inflammatory activity was comparable with standard aspirin.

These finding provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an anti-inflammatory agent from pure compounds of *P. pinophilum* fungus. These pure compounds (1, 2 and 3) of fungus by anti-inflammatory results appear as interesting and promising and may be effective as potential sources of novel anti-inflammatory drugs.

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THE USE OF ANALYTICAL TECHNIQUES TO DETECT TOXIC SYNTHETIC DRUG, SIBUTRAMINE, ADULTERATED IN TRADITIONAL HERBAL MEDICINES

Zeenath Ismail Gadit[1], Dr. Mathi Kandiah[2]

Abstract: Traditional herbal medicines (THM's) are still being used worldwide in at least 80% of the population, mainly in developing countries, due to the assumption that they are safer and cheaper. However, these medicines are now being adulterated with synthetic drugs causing toxic effects. One such drug is Sibutramine, a drug used for weight loss. Although Sibutramine has several promising weight loss effects, it causes several toxic side effects; this resulted in its banning and withdrawal from the market. Despite this, it is still being adulterated in herbal medicines and needs to be detected. It is detected using several analytical techniques such as TLC, GC, LC-MS, LC-MS/MS, QTOF-LC/MS and more recently, spectroscopic techniques. Out of all the techniques, QTOF-LC/MS detected concentrations of Sibutramine adulterated in 9.4 mg (not clear what this means) and had LOD and LOQ values of 0.4- 2.0 µg kg-1 and 1.3- 6.0 µg kg-1 respectively; therefore, QTOF-LC/MS is the best technique developed up to date when compared to all the other techniques. However, research is currently ongoing to combine spectroscopic techniques other than MS, to overcome disadvantages such as lack of global information, false-positive results, time and labor consumption to detect Sibutramine adulteration in the future.

Keywords: Toxic, Sibutramine, Adulteration, Analytical Techniques

Introduction

Traditional herbal medicines (THMs) are medicines made solely of a natural origin; they are rapidly gaining popularity all over the world as they are considered safer and cheaper alternatives to synthetic pharmaceuticals, and have no side effects (Haneef *et al.*, 2013). The World Health Organization estimates at least 80% of the population that live in developing countries depend only on THM's (Skalicka-Wozniak, Georgiev, and Orhan, 2016). However, recently, it has been detected that these herbal medicines are adulterated with synthetics drugs that are not declared in the labels; the term adulteration refers to addition of substances that are not part of the actual mixture, or removal of an important component, which may present a risk of illness to the consumer (Miller *et al.*, 2001). Adulteration is intentionally performed if the natural substances are expensive, short in supply or to increase pharmacologic efficacy (Haneef *et al.*, 2013).

It was estimated in 2012 that about 69% of the adults in the United States were thought to be obese, which is an increase by 15% from 1970 (Creatore *et al.*, 2016). This article focuses more on women, due to several cases of women being reported to take slimming drugs. Therefore, figure 1 illustrates the prevalence of obesity in women worldwide.

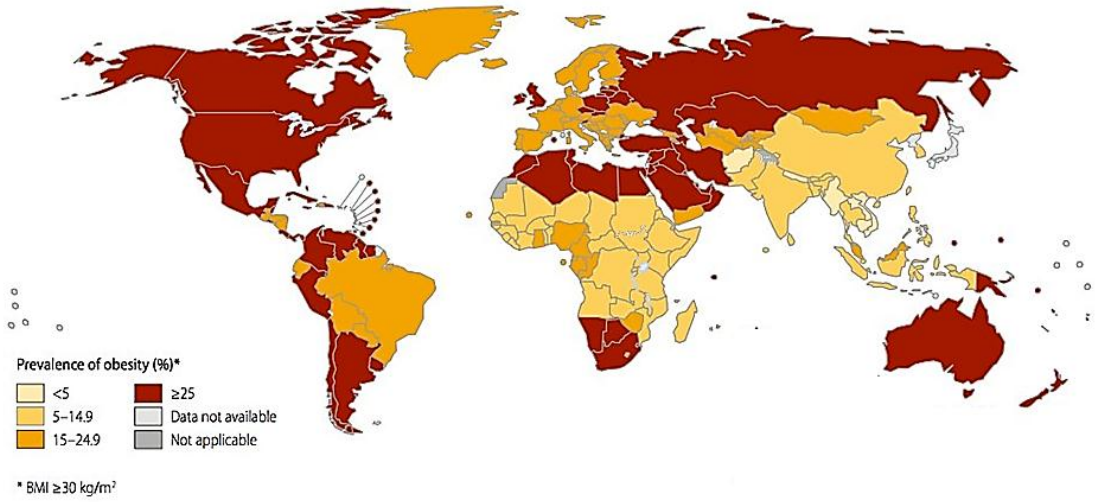

Figure 1: Prevalence of obesity in women older than 18 years in 2014 (Mendis, 2014)

A Food and Drug Administration (FDA) study in 2008 reported the adulteration of 72 herbal products, of which 94.4% of them were adulterated with Sibutramine (Jordan, 2013). Sibutramine (N-{1-[1-(4- chlorophenyl) cyclobutyl]-3-methylbutyl}-N.N-dimethylamine) was first thought to be used as an antidepressant in the 1980's, but later it was found that it could be used as a drug to induce weight loss; it was therefore FDA approved and available for use in 40 countries since 1998 (Oberholzer *et al.*, 2014; Florentin, Liberopoulos, and Elisaf, 2008). Sibutramine can be metabolized into its two analogues: N-mono-desmethylsibutramine and N-di-desmethylsibutramine (Huang *et al.*, 2008). The structures of sibutramine and its analogues are shown in Figure 2.

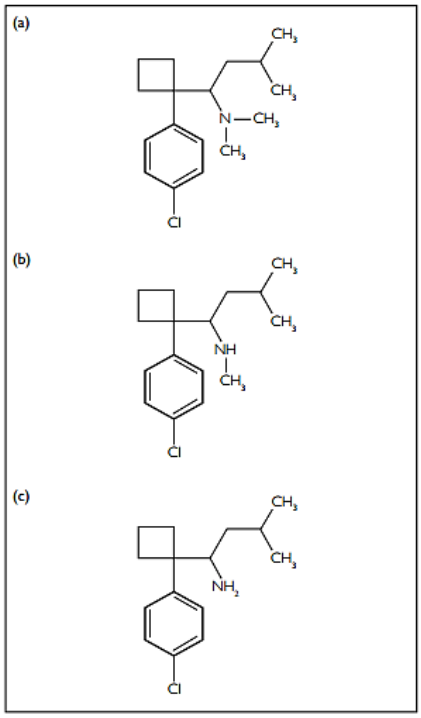

Figure 2: Chemical structures of (a) sibutramine, (b) N-desmethyl-sibutramine, and (c) N-bisdesmethyl-sibutramine (Yuen *et al.*, 2007)

Although sibutramine has promising results in weight loss, many side effects of the use of sibutramine have been observed such as headaches, insomnia, anorexia, dry mouth, confusion, dizziness, shivering, panic attacks, mood swings, hypertension, tachycardia and arrhythmias; therefore, in 2010, sibutramine was withdrawn and banned from markets due to its serious cardiovascular risks (Deconinck *et al.*, 2014; Yu *et al.*, 2010; Bogusz *et al.*, 2006).

Despite this, sibutramine is still detected as an adulterant in THMs and therefore, it is essential to detect its presence. It is performed by using several analytical techniques that have developed throughout the years.

2.0 Analytical techniques for the detection of Sibutramine

Several analytical techniques have been developed for the detection of this adulteration. The techniques mentioned in detail in this review are thin layer chromatography (TLC), gas chromatography, liquid chromatography and spectroscopic techniques. These techniques have been improved over the years to overcome limitations such as sample handling, sample preparation, cost effectiveness, time taken, accuracy, sensitivity and precision.

2.1 Thin layer chromatography (TLC)

TLC is an older technique compared to other analytical techniques, but is still useful for the separation of compounds; in TLC a solid adsorbent phase is used such as a silica gel or alumina plate which first causes separation of the compounds (AlOthman and Rahman, 2013). The separated compounds are then carried up by a mobile phase and separated according to their respective retention times (Sjursnes, Kvittingen and Schmid, 2014).

In 2012, Phattanawasin and coworkers developed a simple TLC-image analysis method to detect sibutramine; chromatographic separation was performed using a silica gel 60 F_{254} TLC plate, toluene-*n*-hexane-diethylamine as the mobile phase and Dragendorff reagent for detection of spots (Figure 3). This was the first validated report for the method and detected amounts of sibutramine adulterated in herbal medicines ranging from 6- 24 mg. The limits of detection (LOD) value was obtained as 190 ng/spot and the limits of quantification (LOQ) was obtained at 634 ng/spot (Phattanawasin *et al.*, 2012).

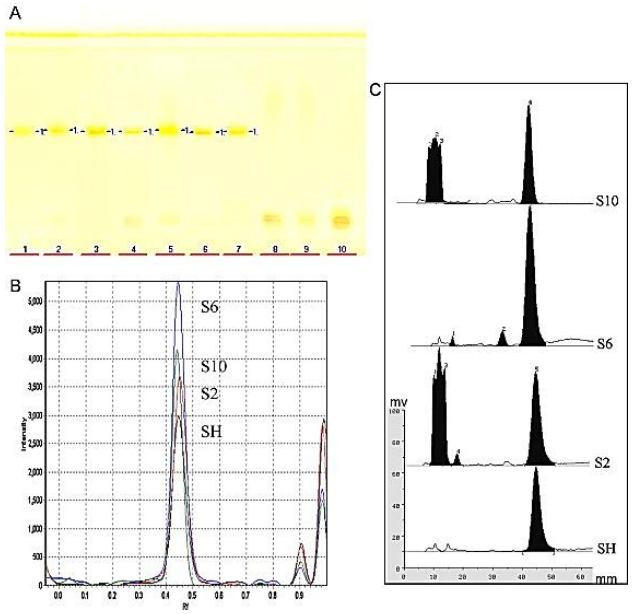

Figure 3: (A) TLC image of standard Sibutramine (track 1), adulterated slimming formulations (tracks 2–7: S2, S6, S7, S9, S10 and S19) and Sibutramine-free samples (tracks 8–10: S14, S15 and S16), (B) TLC chromatogram of standard Sibutramine, slimming coffee (S2), slimming gel (S6) and slimming capsule (S10) obtained from TLC-image analysis and (C) from TLC-densitometry (Phattanawasin *et al.*, 2012).

High Performance TLC (HPTLC) is an improved form of TLC due to the addition of a number of features that end up automating steps, increasing resolution and also result in more accurate results (Raja, 2015). Therefore, in 2012, Ariburnu and coworkers performed an HPTLC-densitometry method using glass HPTLC plates coated with silica gel 60 F254 and a mobile phase of *n*-hexane–acetone–ammonia; the LOD and LOQ calculated were 77.34 and 257.79ng respectively (Ariburnu *et al.*, 2012). Following this, in 2014, Mathon and coworkers performed an HPTLC-UV method using HPTLC silica gel plates and a mobile phase of toluene-methanol mixture; quantification and confirmation of sibutramine were then performed using TLC-MS; the results found half of 52 herbal samples adulterated with sibutramine, with concentrations up to 35mg (Mathon *et al.*, 2014). However, HPTLC has limitations such as poor precision, which therefore render it less suitable for quantitative analysis (Haneef *et al.*, 2013).

TLC is used because it is a simple, rapid method with a low cost of operation; however, its major disadvantages are that it has a low sensitivity and low precision (Rocha, Amaral, and Oliveira, 2015). In addition, tests involving only chromatography, such as TLC cannot be used to identify the compound; rather, they confirm the necessity to perform further tests such as spectrometry, for qualitative or quantitative analysis (Csupor *et al.*, 2013). Therefore, hyphenated GC and LC techniques were developed and used more frequently than TLC.

2.2 Gas chromatography-Mass spectrometry (GC-MS)

GC-MS is a hyphenated technique that combines both GC and MS; GC separates components in the sample, and the fragments are then analyzed according to their mass using MS (Chauhan, Goyal and Chauhan, 2014).

In February 2006, a metabolite of sibutramine was detected and identified in the urine of a 16 year old girl (Figure 4) who was admitted to the hospital due to side effects observed by the consumption of the Chinese herbal drug "LiDa Dai Dai Hua Jiao Nang"; identification was performed using a GC-MS system composed of an Agilent 6890 gas chromatograph with an Agilent 5973 mass selective detector and an Ultra 1 column (Vidal and Quandte, 2006). However, no quantification was performed in this method.

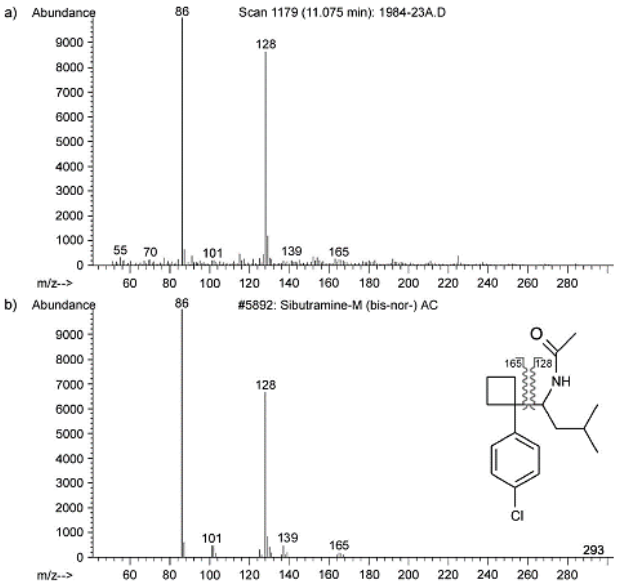

Figure 4: Acetylated derivative (a) of the bis-demethylated Sibutramine-metabolite (Mr = 293,84; $C_{17}H_{24}ClNO$) and reference spectrum (b) selected from spectra library (Vidal and Quandte, 2006)

Furthermore, an additional case was reported in 2012 and an Agilent model 6890 GC and 5973 mass selective detector and a DB-17MS GC-MS method was used (Figure 5); the test showed that each capsule contained 22 mg of sibutramine (Da lio lu and Akcan, 2012).

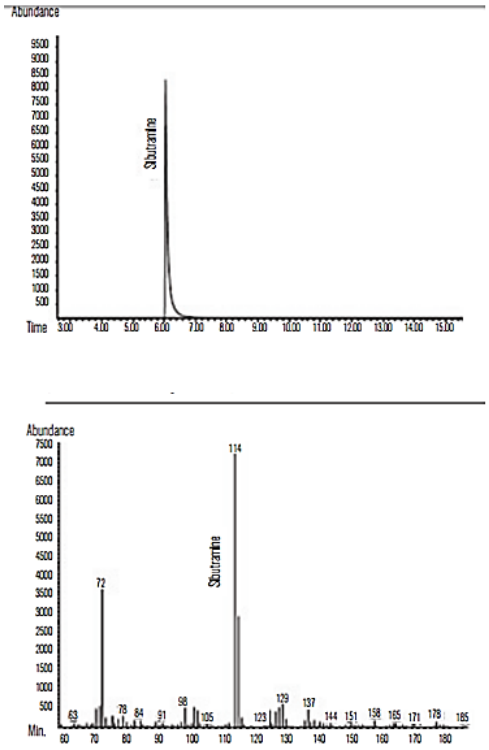

Figure 5: GC-MS chromatogram of sibutramine in herbal drug (LiDa) capsule - the total ion chromatogram (Da lio lu and Akcan, 2012)

A study conducted in 2014 used GC/MS (Agilent 7000, Triple Quaed, GC7890A) with a capillary column for the stationary phase; linear calibration range was between 1 - 500 µg/mL and the LOQ was 5 ng/mL. Sibutramine was detected in amounts ranging from 6-78 mg/capsule (Khazan *et al.*, 2014).

The main advantage of GC-MS is the fact that it can analyze volatile compounds and has a high sensitivity; conversely, the biggest disadvantage of GC-MS is that it cannot detect polar and non-volatile compounds; therefore, LC methods are preferred over GC (Kamboj, 2012).

2.3 Liquid Chromatography (LC)

Prior to the ionization and mass analysis of molecules, LC plays an important role in separation of a bulk sample matrix (which could later cause interference or suppression of the signals of the compound actually being detected); furthermore, if a standard is available, LC can identify the compound based on the retention time (Vaclavik, Kryntisky and Rader, 2014). However, LC is not usually used alone to detect the presence of Sibutramine in THM's. It can be developed and used as High Performance Liquid Chromatography (HPLC), or more commonly hyphenated with MS to give rise to techniques such as LC-MS, LC-MS/MS (tandem mass spectrometry) and Quadrupole time of flight (QTOF) - LC/MS.

2.3.1 HPLC

A case was reported in September 2006, of a 20 year old woman who had seen her family doctor after the use of the same drug "LiDa" mentioned earlier, and experiencing side effects; detection was performed using GC-MS with an Optima-1-MS capillary column and was followed up by an HPLC-DAD method for quantification of the drug; the quantification yielded 27.4 mg of sibutramine adulterated in the drug, which is approximately twice the accepted prescribed amount (Jung, Hermanns-Clausen and Weinmann, 2006).

A further study was developed in 2011; Sibutramine was detected in the quantity of 30mg per capsule and one of its metabolites, N, N-didesmethylsibutramine was also detected. LOQ's varied from 0.1-5.0 µg/ml. However, the procedure was validated by an unconventional method, as it did not investigate the precision or accuracy of the method (Ancuceanu, Dinu and Arama, 2013; Rebiere *et al.*, 2011).

Therefore, a validated HPLC method was developed in 2013 (Figure 6b). 24.71mg (97.28 mg g^{-1}) of Sibutramine was detected; the LOQ values ranged from 4.47-7.81 µg/mL and the LOD ranged from 1.34-2.34 µg/mL (Ancuceanu, Dinu and Arama, 2013).

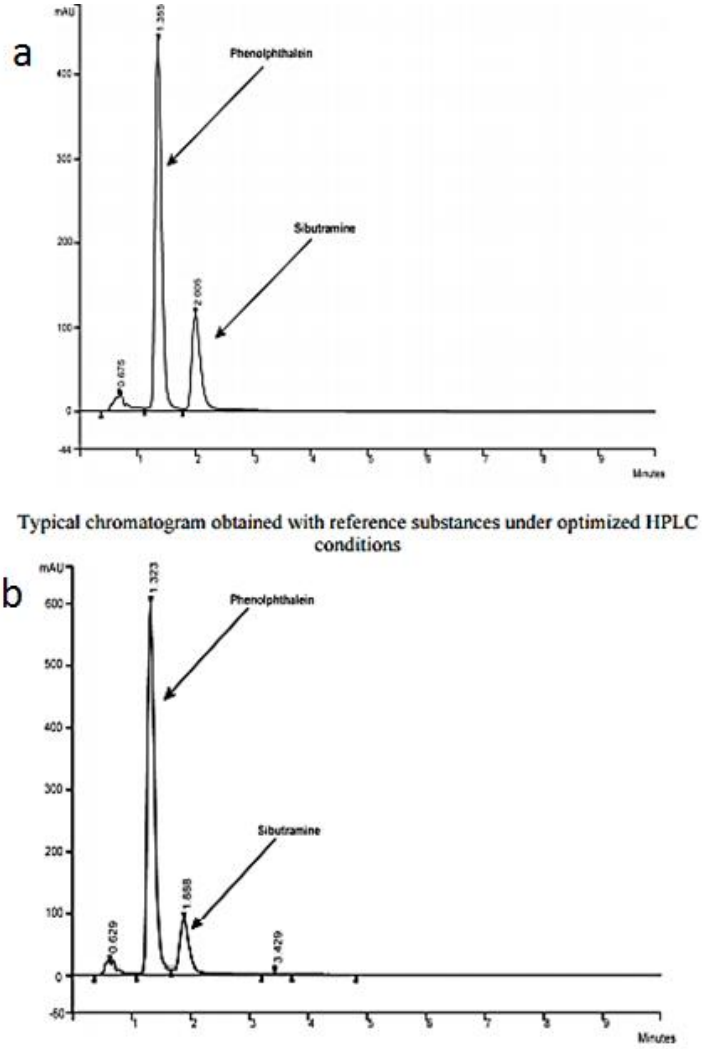

Figure 6: (a) Typical chromatogram obtained with reference substances under optimized HPLC conditions and (b) the chromatogram obtained with capsule content of an adulterated for the herbal supplement (CS) (Ancuceanu, Dinu and Arama, 2013)

The different equipment used and results obtained are summarized below in table 1.

Table 1: The equipment used for the HPLC techniques and their respective LOD and LOQ values

Equipment	Solvent	LOD	LOQ	Sibutramine concentration detected (mg)	Reference
LiChrospher 100 Å RP-8 column	-	-	-	27.4	Jung, Hermanns-Clausen and Weinmann, 2006
A trifunctional C18 end capped column and a photodiode array detector	Phosphate buffer & acetonitrile	-	0.1-5.0 µg/ml	30	Rebiere *et al.*, 2011
Varian HPLC system + Prostar 240SDM quaternary high-pressure pump, diode-array detector + CN Nucleosil column	Phosphate buffer and acetonitrile	1.34-2.34 µg/mL	4.47-7.81 µg/mL	24.71	Ancuceanu, Dinu and Arama, 2013

Due to the ability of LC systems to withstand high pressures has become more available, UPLC/MS methods are being developed; advantages of UPLC over HPLC include decreased run time, reduced solvent consumption, improved peak capacity and sensitivity, which allows multi-analyte screening (Nielsen *et al.*, 2010).

2.3.2 LC-MS

By the use of only HPLC, sibutramine was able to be detected as an adulterant; however, by the use of only HPLC, less precise results tend to be obtained. To overcome this, mass spectrometry is hyphenated with techniques such as LC to give higher precision and accuracy. LC-MS is used as a tool for detection of undeclared synthetic adulterants in THM's due to its application in detecting a wide range of compounds; in addition, it provides both quantitative and structural data of the analytes. It can also be paired with different ionization techniques such as electrospray ionization (ESI), fast atom bombardment (FAB) and direct analysis in real time (DART), depending on the analyte being detected (Haneef *et al.*, 2013). It is also paired with different mass analyzers such as triple quadrupole (QqQ), single quadrupole (Q), ion trap (IT) and time-of-flight (TOF); furthermore, hybrid systems such as quadrupole-time-of-flight (QqTOF) have also been developed (Vaclavik, Kryntisky and Rader, 2014).

A further method of analysis is liquid chromatography tandem mass spectrometry (LC-MS/MS), in which a further mass spectrometric analysis is performed to confirm the compound detected. This is one of the most

commonly used techniques for the detection of adulteration of THM's; it is used due to its specificity, selectivity, sensitivity, less sample preparation and because it provides quantitative results (Haneef *et al.*, 2013).

In 2005, an LC-MS-MS method was developed that was successful in detecting synthetic drugs in herbal medicines, but was not used to detect Sibutramine (Liang *et al.*, 2006). In 2006, Bogusz and coworkers performed an LC-ESI-MS-MS technique by the use of a TSQ quantum LC/MS/MS together with a Surveyor AS Autosampler and quaternary LC pump; this method that detected 20 weight reducing adulterants in THM's; however, Sibutramine was not detected using this method as well (Bogusz *et al.*, 2006).

Therefore, a specific and accurate HPLC-ESI-MS method was developed in 2008 by Wang and coworkers, that targeted the detection of Sibutramine and its metabolites; separation by chromatography was done using a C_8 reversed-phase column and solvents of acetonitrile and ammonium formate were used (Figure 7). The LOD ranged from 0.0018- 0.73 µg g^{-1}; three of the six samples analyzed were found to contain either Sibutramine or N-mono-desmethylsibutramine at levels of 0.212- 96.2 mg g^{-1} (Wang, Chen and Yao, 2008).

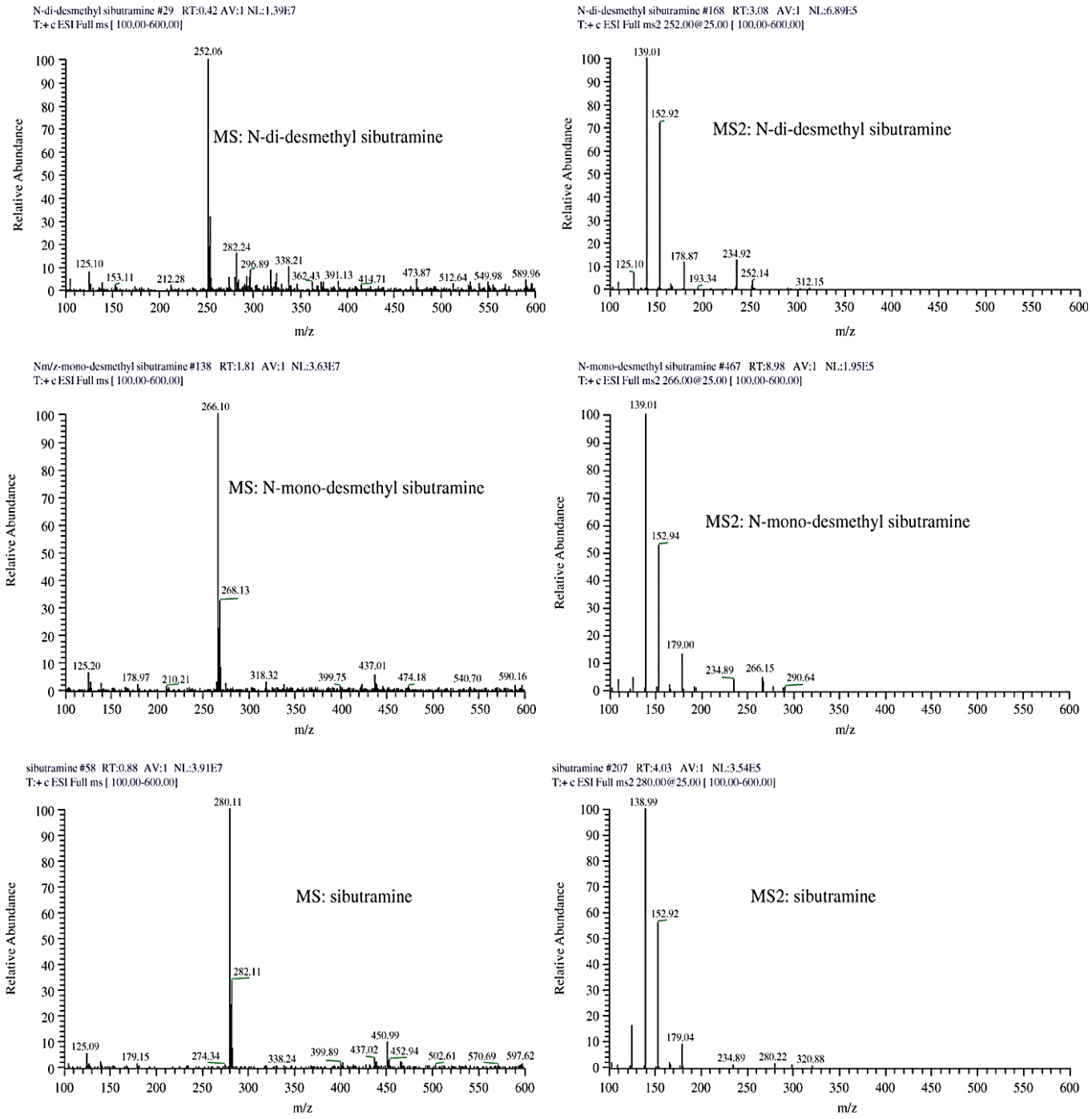

Figure 7: MS^2 spectrum of N-di-desmethyl sibutramine, N-mono-desmethyl sibutramine, sibutramine. The mass spectrometer used was a Finnigan LCQ Advantage MAX (Thermo, USA). ESI+; sheath gas flow rate: 40 arb; aux/sweep gas flow rate: 5 rab; spray voltage: 3 kV; capillary temperature: 260°C; capillary voltage: 30 V; tube lens offset: 30 V; collision energy: 25%. (Wang, Chen and Yao, 2008)

Furthermore, an HPLC-ESI-MS/MS method was established by Shi and coworkers in 2011, using a Hypersil Gold column and solvents of formic acid-ammonium formate buffer and methanol; Sibutramine was detected and the LOD was 0.03mg/kg and the LOQ value was 0.10mg/kg, with a mean recovery of 89.9% (Shi *et al.*, 2011).

A newer method of LC-MS-MS through MRM and EPI mode, plus library searching method was developed in 2009 by Chen and coworkers, which successfully detected Sibutramine in 8 botanical dietary supplements; the LOD value was found to be satisfactory to screen for adulterants. The method used 3200 QTRAP Hybrid Quadrupole-Linear Ion Trap Mass (Chen *et al.*, 2009). In 2013, an LC-ESI-MS/MS method was developed using a reverse phase column and solvents of acetonitrile and water with 0.1% formic acid; analysis was then performed using multiple reaction monitoring (MRM) ratios. Sibutramine was detected in concentrations of 0.000184 - 4.81 mg/capsule, the LOD was 1.3ng/ml and the LOQ was 4.0 ng/ml. This method was accurate, easy to perform, precise and sensitive (Yano *et al.*, 2013).

2.3.3 Quadrupole Time of Flight Liquid Chromatography Mass spectrometry (QTOF LC/MS)

The QTOF analyzer functions by acceleration of ions through a tube by the use of a high voltage; molecules are then detected, and the time taken to reach the detector depends on the compound's respective m/z values. It is a newer technique and is starting to be used more often because of its advantages such as high resolving power, high mass accuracy, and high sensitivity (Pitt, 2009).

In 2007, Zou and coworkers were the first to report detection of two active metabolites along with a new analogue of Sibutramine in a THM, by the employment of TOF-LC/MS. 1g of the samples were required and equipment used were an Agilent 1200 series LC system coupled with an Agilent 6210 TOF mass spectrometer; TOF-LC/MS determined and thereby suggested molecular formulae of $C_{15}H_{22}NCl$, $C_{16}H_{24}NCl$ and $C_{17}H_{26}NCl$. Therefore, the two metabolites were confirmed as N-didesmethylsibutramine and N-desmethylsibutramine, whereas the analogue detected was termed Homosibutramine (Figure 8) (Zou *et al.*, 2007).

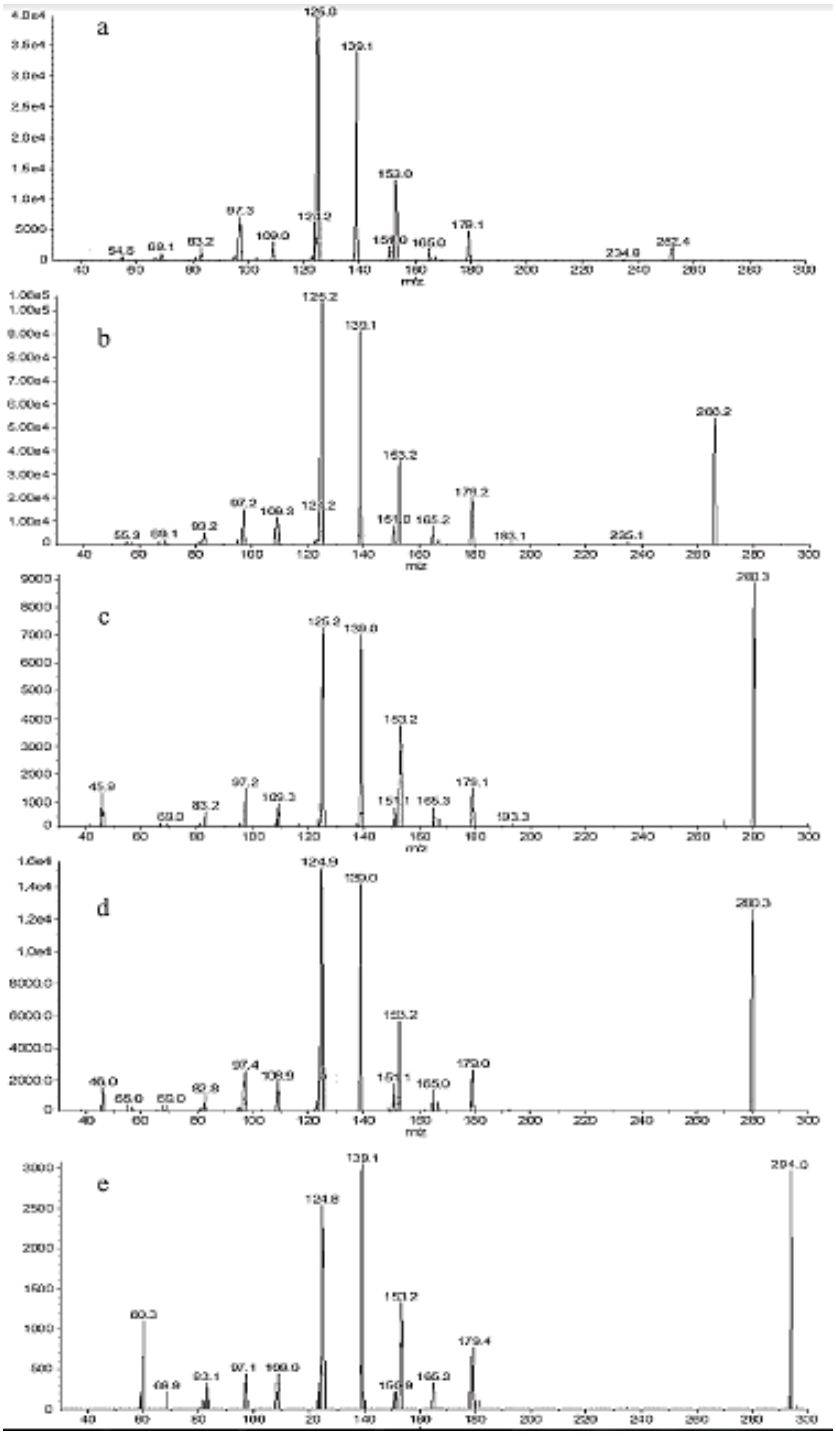

Figure 8: MS/MS product spectra of $[M+H]^+$ ions of compunds (a) N-didesmethylsibutramine, (b) N-desmethylsibutramine, (c) sibutramine, (d) isomer of sibutramine and (e) homosibutramine (Zou *et al.*, 2007)

In 2011, a UPLC/Q-TOF MS (Figure 9) was implemented to detect Sibutramine and its 2 major metabolites; a waters ACQUITY UPLC system coupled to a Bruker micrOTOF-q II mass spectrometer with a waters ACQUITY chromatographic column was used; solvents used were formic acid, acetonitrile. UPLC was used here due to its short analysis time and good repeatability during analysis. The test detected sibutramine and its analogues, specifically N-desmethylsibutramine (9.4 mg); the LOD and the LOQ were from 0.4- 2.0 $\mu g\ kg^{-1}$ and from 1.3- 6.0 $\mu g\ kg^{-1}$ respectively (Roh *et al.*, 2011). UPLC/Q-TOF/MS is considered a powerful technique to

detect components that are low in abundance in the drugs due to its features of high resolution, reproducibility and selectivity (Li *et al.*, 2011).

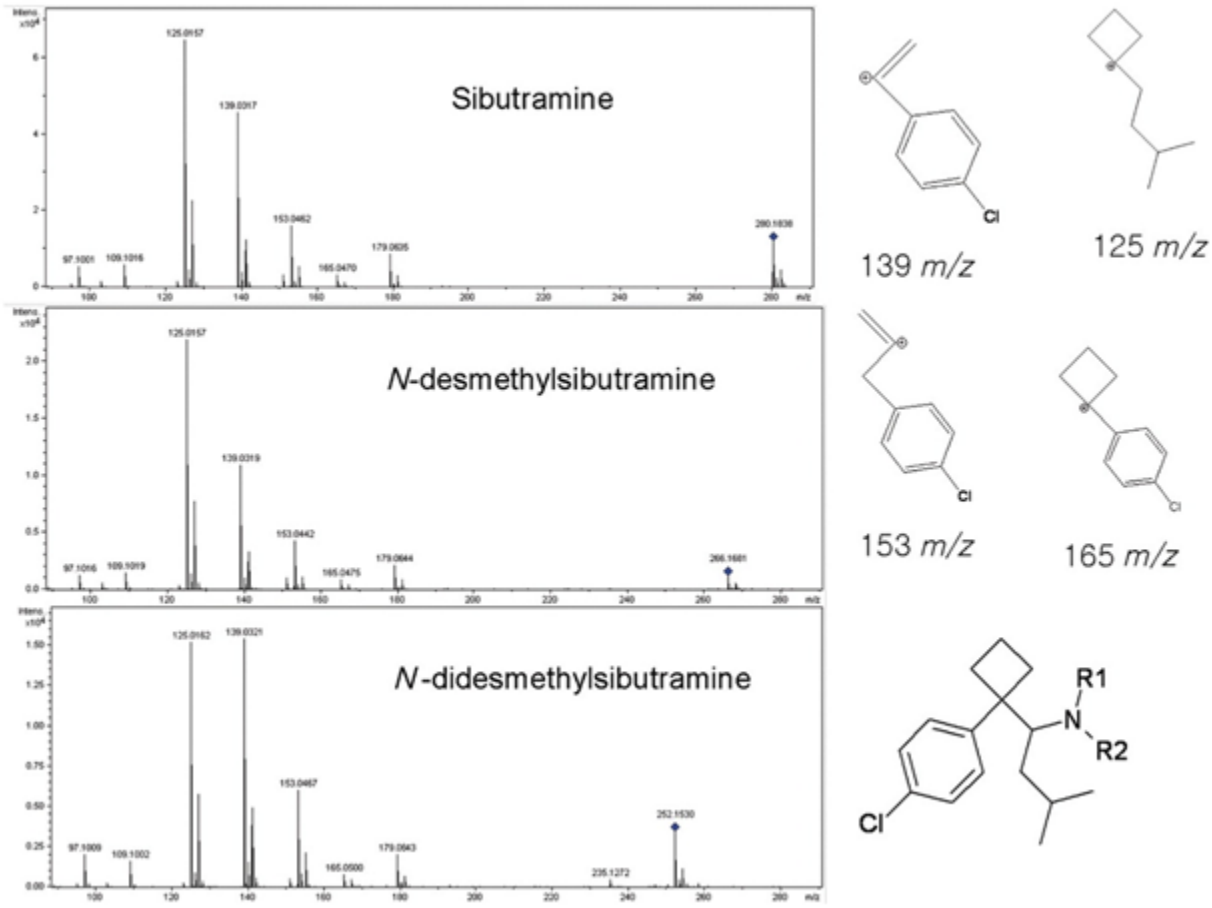

Figure 9: Mass fragmentation pattern and fragment compounds of the compound Sibutramine and its analogues. (Roh *et al.*, 2011)

Methods involving LC coupled with various detectors (usually MS), have the advantage of high sensitivity; however, they also have disadvantages of being laborious, time consuming and the fact that they are too selective and cannot provide global information in detecting a particular component, because compositions of drugs vary among different brands (Monakhova *et al.*, 2012). These methods were also considered to have inadequate reliability for screening because herbal medicines contain a complex mixture of many components which may interfere with chromatographic and mass spectroscopic assays (Calahan *et al.*, 2016). Furthermore, chromatographic methods can yield false-positive results; therefore, these methods cannot be ideal for screening of adulterated drugs in THMs (Vaysse *et al.*, 2010).

Therefore, recently, techniques related to coupling with spectroscopic techniques have been developed to analyze for Sibutramine adulteration.

2.4 Spectroscopic methods

The chromatographic methods can be coupled with spectroscopic techniques such as NMR spectroscopy, Fourier transform infrared (FTIR) spectroscopy with attenuated total reflectance (ATR), portable ion mobility spectrometer (IMS), Raman Spectroscopy and X-ray powder diffractometry, to analyze for Sibutramine adulteration (Nnaji *et al.*, 2016). The use of chromatographic techniques paired with other detection methods is not the ideal method of detection because these techniques maybe too selective sometimes and not provide

global information; furthermore, false-positive results may also occur (Vaysse *et al.*, 2010). Moreover, to spare the time loss of using chromatographic methods, more spectroscopic-related techniques have been developed (Nnaji *et al.*, 2016).

One particular method developed was the use of an LC-QTOF-MS method combined with NMR spectroscopy, using the basic principle of generating a peak with the initial LC-QTOF-MS, where MS identifies the compound; the identity is then confirmed by using NMR and quantified using qNMR. This method detected Sibutramine along with other adulterants (Figure 10); the concentration of Sibutramine detected in the capsule LIPO8 was 1mg. A measurement uncertainty of 1.5% was reported, with an S/N ratio of 150:1 or more; these two values were then applied as the LOQ (Johansson *et al.*, 2014).

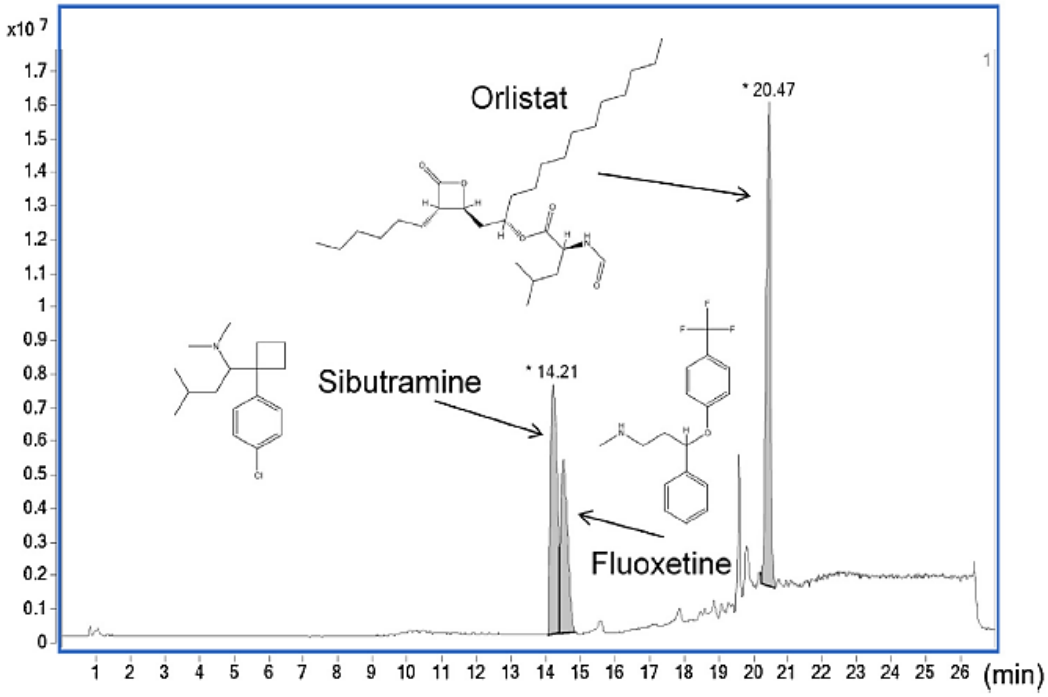

Figure 10: Chromatogram of LIPO8, a food supplement containing undeclared active pharmaceutical ingredients. Slimming product seized by Swedish customs. Sibutramine detected at 1 mg/capsule. Detector: MS TOF; scan m/z 110-1000 scan rate 1s; centroid (Johansson *et al.*, 2014)

2.5 Future prospects

To detect chemical compounds adulterated in a herbal sample by spectroscopy, the compound must be present in large doses with no masking effects from the other components in the sample; else, the spectroscopic result alone is not reliable; therefore, it is best to combine the spectroscopic techniques with separation techniques (Deconinck *et al.*, 2013; Lu *et al.*, 2007). Therefore, research is still being performed in combining more spectroscopic techniques other than MS with liquid chromatography methods, to work and develop spectroscopic techniques that overcome these disadvantages and use them to detect the adulteration of Sibutramine in THM's.

3.0 Summary

Table 2 summarizes the main techniques described in this review.

Table 2: Summary of the techniques including TLC, GC-MS, HPLC, LC/MS/MS and QTOF-LC/MS

Method	Equipment	LOD	LOQ	Sibutramine concentration detected	Reference
TLC-image analysis	Silica gel 60 F_{254} TLC plate, toluene-*n*-hexane-diethylamine (9:1:0.3, v/v/v) + Dragendorff reagent	190ng/spot	634 ng/spot	6- 24 mg	Phattanawasin *et al.*, 2012
GC/MS	Agilent 7000, Triple Quaed, GC7890A) + capillary column	-	5 ng/mL	6-78 mg	Khazan *et al.*, 2014
HPLC	Varian HPLC system + Prostar 240SDM quaternary high-pressure pump, diode-array detector + CN Nucleosil column	1.34-2.34 µg/mL	4.47-7.81 µg/mL	24.71 mg	Ancuceanu, Dinu and Arama, 2013
LC-ESI-MS/MS	Reverse phase column + solvents of acetonitrile and water with 0.1% formic acid. Analysis by multiple reaction monitoring (MRM) ratios	1.3ng/ml	4.0 ng/ml	0.000184 - 4.81 mg/capsule	Yano *et al.*, 2013
QTOF-LC/MS	Waters ACQUITY UPLC system coupled to a Bruker micrOTOF-q II mass spectrometer + solvents of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B)	0.4-2.0 µg kg^{-1}	1.3-6.0 µg kg^{-1}	9.4 mg	Roh *et al.*, 2011
LC-QTOF-MS + NMR	LC–QTOF-MS of 1290 Infinity UHPLC + diode array detector +	-	S/N ratio of 150:1	1mg/capsule	Johansson *et al.*, 2014

	a reversed phase column, Aqcuity UPLC C18 + NMR
	Bruker Avance spectrometer

In summary, the detection of Sibutramine adulteration THM's is very important due to the side effects it possesses, and despite it being banned and withdrawn in markets, it is still been found to be detected. Out of all the techniques, QTOF-LC/MS detected concentrations of sibutramine adulterated in 9.4 mg and had LOD and LOQ values of 0.4- 2.0 $\mu g\,kg^{-1}$ and 1.3- 6.0 $\mu g\,kg^{-1}$ respectively, meaning that it has the ability to detect adulterated Sibutramine even if it was present in 1 µg within 1 kilogram. LC/MS/MS is also used rather frequently as it is also a highly developed, sensitive technique, but QTOF-LC/MS is the best technique compared to all the other techniques as it has advantages of higher sensitivity, accuracy of mass and high resolution. However, research is currently undergoing to combine spectroscopic techniques other than MS, to overcome disadvantages such as lack of global information, false-positive results, time and labor consumption to detect Sibutramine adulteration in the future.

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