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Use of volatile fingerprints for rapid screening of antifungal agents for efficacy against dermatophyte *Trichophyton* species

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Abstract

The potential of using an electronic nose (E-nose) as a rapid technique for screening the responses of dermatophytes to antifungal agents was studied. *In vitro*, the 50% and 90% effective concentration (EC) values of five antifungal agents including fungicides and antioxidant mixtures against *T. rubrum* and *T. mentagrophytes* were obtained by mycelial growth assays. The qualitative volatile production patterns of the growth responses of these fungi to the EC values incorporated into solid media were analysed after 96-120 hours incubation at 25°C using headspace analyses using 5 replicates per treatment. Overall, results, using principal components analysis and cluster analysis, demonstrated that it was possible to differentiate between various treatments within 96-120 hours of growth. The EC_{50} values were discriminated from the controls while the EC_{90} concentration treatments were often grouped with the agar blanks because of very slow growth. This study showed that potential exists for using qualitative volatile patterns as a rapid screening method for antifungal agents against microorganisms. This approach could significantly improve and facilitate the monitoring of antimicrobial drug activities and infection control programmes and perhaps also for monitoring of drug resistance build up in microbial populations.

Key words: *Trichophyton* species, volatile fingerprints, anti-fungal compounds, rapid screening

1. Introduction

Dermatophytoses are common among skin diseases worldwide. Even in Europe, infections such as tinea capitis are an increasing problem [5]. Although, resistance to antibiotics among dermatophyte species is very uncommon, determination of the *in vitro* susceptibility of dermatophytes, particularly for management of treatment failures, may prove helpful [1]. Many techniques, such as agar-based methods (dilution and diffusion) and broth dilution, have been used for antifungal susceptibility testing (AST) but there is no standard method for AST of dermatophytes [2, 9]. However, because dermatophytes grow slowly, agar-based methods have often been employed [14]. These methods are time-, labour-, and resource-intensive which make them unsuitable for rapid screening programmes. So, there is a need for novel and quick alternative laboratory approaches.

All micro-organisms produce by-products as a result of their normal metabolism [17]. Some of these metabolic by-products, including alcohols, aliphatic acids, ketones and terpens are volatile at low temperature and are known as volatile organic compounds (VOCs). Many VOCs have characteristic odours. Since the production patterns of VOCs are unique to certain micro-organisms (or disorders), they can potentially be used as biomarkers [19]. Quantitative analysis of VOCs has almost exclusively been based on gas chromatography-mass spectrometry which is time-consuming, relatively expensive, and required skilled operators [17, 19]. However, rapid qualitative analysis of volatile production patterns by means of electronic nose (E-nose) devices for early detection and discrimination of infections [3, 4, 7, 10, 11, 12, 18, 15, 16] or non-infectious diseases such as lung cancer and diabetes mellitus [4, 13, 19] has yielded promising results.

The aims of this work were to study the potential of using a hybrid sensor array as a rapid screening method for antifungal agents for controlling dermatophytes using the drugs and other antioxidants using volatile fingerprints. To achieve these objectives we (a)

determined the growth responses of two *Trichophyton* species, *T. rubrum* and *T. mentagrophytes,* against five antifungal agents (itraconazole, griseofulvin, butylated hydroxyanisole, octyl gallate and n-propyl-p- hydroxybenzoate), (b) calculated the effective doses for inhibition of 50 and 90% growth (EC_{50} and EC_{90}); (c) at these EC_{50} and EC_{90} concentrations, the effect on the volatile production patterns by these two fungi when grown for up to 96-120 hrs was evaluated and compared with negative and positive controls based on the responses of the hybrid sensor array system. PCA and CA were used to examine the potential for discriminating between antifungal treatments and the controls.

2. Materials and Methods

2.1 Fugal isolates

Two *Trichophyton* species, *T. rubrum* (No. 115) and *T. mentagrophytes* (No. 224), were used in this study. The *Trichophyton* species were human isolates and obtained from the National Collection of Pathogenic Fungi (NCPF), Bristol, UK.

2.2 Growth medium

Sabouraud's dextrose agar (SDA) medium was prepared in house by mixing 10 g l^{-1} mycological peptone (Amersham, Little Chalfont, UK; Oxoid, UK), 40 g l^{-1} glucose (Acros Chemicals, Belgium), and 15 g l^{-1} agar Technical No.3 (Oxoid, UK). At the end, 0.05 g l^{-1} chloramphenicol (Sigma, UK) was added.

2.3 Antifungal agents

Two antifungal drugs, itraconazole (Janssen Pharmaceuticals, Belgium) and griseofulvin (Darou Pakhsh Co., Iran), and three antioxidants including butylated hydroxyanisole (BHA; $C_{11}H_{16}O_2$) (Sigma, US), octyl gallate (O-G; $C_{15}H_{22}O_5$) (Fluka

Chemie GmbH, Germany) and n-propyl-p- hydroxybenzoate (P-P; $C_{10}H_{12}O_3$) (Sigma, US) were used in this research work. All antioxidants are considered as Generally Recognized As Safe (GRAS) compounds. For each drug, a stock solution with a concentration of 100 μg ml^{-1} was prepared by dissolving 2mg of anti-fungal agent in 20 ml of 99.5% dimethyl sulfoxide (DMSO) (Sigma, US). For antioxidants and based on the molecular weight of each preparation, a stock solution with the concentration of 1 mM in 20 ml of 99% ethanol was made. Stock solutions were stored at 4°C.

2.4 E.nose system

An AppliedSensor 3320 E-nose (AppliedSensor Group, Sweden) was employed in this study. The core sensor technology of this machine is based on a hybrid array of 10 metal-oxide-silicon field-effect-transistor (MOSFET) sensors and 12 metal oxide sensors (MOS), and a capacitance-based relative humidity sensor. These sensors are provided as standard in this specific system. The MOSFET sensors were sensitive to hydrogen, amines (MOSFET 101A); amines and esters (MOSFET 102A); aldhydes and alcohols (MOSFET 103A); hydrogen (MOSFET 104A); hydrogen and amines (MOSFET 105A); amines, aldehydes, esters, alcohols and ketones (MOSFET 101B); hydrogen, amines and alcohols (MOSFET 102B); amines, aromatics, aldehydes, esters, ketenes and alcohols (MOSFET 103B); hydrogen (MOSFET 104B) and amines, aldehydes, esters and ketones (MOSFET 105B). The sensor array was heated to 140^{o}C.

The 12 MOS sensors were specific for air contaminants such as hydrogen and carbon monoxide (MOS 101); hydrocarbons (MOS 102); alcohols, organic solvents (MOS 104); hydrocarbons (MOS 110); methane (MOS 111); propane and butane (MOS 112); hydrogen (MOS 113); organic solvents (MOS 114); alcohol (MOS 115); Freon (MOS 116); ammonia (MOS 117); organic solvents, alcohol and hydrogen (MOS 118).

The E.nose system used has a 12 vial auto-sampler system. The 30 ml volume vials were sealed and placed into the sample holders which were maintained at 30^{o}C. The computerized programme used consisted of 30 sec to obtain the base line, 30 sec to take a sample, 200 sec flushing, resulting in a total time of 4.20 mins. There are two gas filters connected to the inlet including a drying column of silica gel which acts as moisture trap, and a hydrocarbon filter which is in two parts, one is a moisture filter and the other is an active carbon adsorbent. The individual sample holders for the vials are temperature controlled at 37^{o}C to maintain stability of presentation of vials for head space analyses. Only when equilibration has been achieved does the system become operative. The air enters the system at a flow rate 60 ml min. The divergence response of each sensor was used in all analyses.

2.5 Determination of EC_{50} and EC_{90} values

Molten SDA media were serially diluted with stock solutions of itraconazole and griseofulvin so that 0.25, 0.50, 1.00 and 2.00 μg ml^{-1} treatments were obtained. SDA media were also amended with two different antioxidant combinations (BHA + P-P; O-G + P-P) to obtain final concentrations of 20 mM and 40 mM. Modified media were poured in 9cm plastic Petri plates and for each species, three replicate plates of each treatment and plain SDA (as control) were inoculated centrally with a 0.5 mm diameter mycelial agar plug taken from the margin of actively growing cultures of *T. rubrum* and *T. mentagrophytes* on SDA. Plates were incubated at 25°C in dark and mycelial extension diameters were daily measured in two perpendicular directions for 14 days. Tests were repeated with griseofulvin and antioxidants-amended plates over a narrower range of 0.025, 0.050, 0.100, 0.200 μg ml^{-1} concentrations. The EC_{50} and EC_{90} values of all antifungal agents involved in the study for *T. rubrum* and *T. mentagrophytes* were calculated by linear regression of the temporal

extension rates and then plotting the relative growth rates (mm day^{-1}) to compare treatments. The treatment list is shown in Table 1. All experiments were carried out with three replicates per treatment and carried out twice.

2.6 Procedure used for e.nose analyses

Subsequently, for each antifungal agent (single or combined, for each of the 5 treatment types) tested, molten SDA media were amended with the related calculated EC_{50} and EC_{90} values per species (*T.rubrum, T.mentographytes*) and poured into 9 cm plastic Petri plates. A total of 5 replicates per treatment for each time of analyses were prepared and these were inoculated with 0.25 ml of a 10^6 spore mL^{-1} suspension of each species and spread plate on the agar surface with a sterile bent Pasteur pipette. Negative and positive controls were used as additional treatments. These were incubated at 25°C in the dark for up to 120 hours. At each time point, four 2 cm diameter agar plugs from 5 replicate plates of each treatment were destructively sampled using a sterile cork borer and placed in sample vials (30 ml) which were sealed with a septum and screw-top lid. After one hour to allow sample equilibration the vial headspaces were analysed using the E-nose system. The headspace volume was approx. 25 ml and this was sampled automatically by a needle from the sample port which was then retracted into the e.nose. A second needle replaced the volume of headspace removed with filtered air. The headspace volatiles were automatically passed through the sensor chamber and over the heated sensor arrays. The air relative humidity over the sensor array was found to be in the range 15-30% based on the relative humidity sensor included in the array. The flow rate and times for each part of the cycle were detailed previously. Every few weeks we also included standards of specific volatiles of isopropanol, ethanol, acetone (1-2%) and de-ionised water to ensure that the results obtained from sensors were consistent over time when the responses were plotted using

PCA analyses and compared over time. Overall, separation of specific standards based on the responses are consistent over time. The cultures grown for 96-120 hours (five independent replicates per treatment at each time) were used. These were chosen as they represented times at which the early stages of microscopic and visible growth occur and when identification of these types of fungi is often difficult.

2.7 Data analysis

Mycelial growth assays data were analysed by Microsoft Excel. NSTSenstool software (an in-built software package in the AppliedSensor 3320 machine) was employed to perform principal components analysis (PCA) on the response parameter (mean-centred data) which also indicated the maximum peak response for the various sensors. These data were also analysed by cluster analysis (CA) using Statistica 8.

3. Results

3.1 Effective concentration values

Growth rates relative to the controls (data not shown) were used to determine the EC_{50} and EC_{90} values for antifungal agent concentrations (Figure 1; Table 1). Except for the antioxidant combinations of BHA + P-P where the actual EC_{90} values for *T. rubrum* and *T. mentagrophytes* were calculated, for other antifungal agents in the study the highest employed concentration in each experiment was equivalent to the EC_{90} values.

3.2 Early differentiation between inoculated antifungal treatments and controls

Figure 2 shows that there was very good reproducibility of the response of ten MOSFET sensors as an example, to five replicates of *T. mentagrophytes* growing on plain SDA. Figure 3 presents the PCA of the response of the hybrid sensor based E-nose to

inoculated treatments of amended SDA with the EC_{50} and EC_{90} values of itraconazole and the fungal species grown on antifungal-free SDA (as positive controls) together with blank SDA medium (as a negative control) and un-inoculated amended SDA at 2.00 μg ml^{-1} of itraconazole after 96 hours. There was a clear separation between the inoculated and un-inoculated itraconazole treatments, and the negative controls. Because the *Trichophyton* species did not grow at the EC_{90} values there was no separation after 96 hours based on volatile fingerprints. This however accounted for 97.6% of the data described by the first two principal components (PC). After 120 hours growth there was also distinct clusters of the two dermatophytes growing on un-amended SDA, with these treatments clearly differentiated from other treatments (Figure 4).

Figure 5 shows the CA after 96 hours, using Euclidean distances and Ward's method, for the treatments using griseofulvin as the test antifungal agent. This showed two tight clusters of the *T. rubrum* and *T. mentagrophytes* replicates growing on plain SDA, and clearly shows the *T.rubrum* EC_{50} replicates grouped separately. However, for the other treatments there was a mixed group with no clear differentiation after 96 hrs.

3.3. Effect of anti-oxidant treatments

Analysis of the data related to the response of the E-nose sensors to different anti-oxidant treatments (BHA + P-P treatments) by PCA after 96 hours showed three clusters of *T. rubrum* and *T. mentagrophytes* growing on SDA, and *T. mentagrophytes* growing on SDA at the EC_{50} concentration of the antioxidant combination discriminated from other treatments, which were all grouped together. This accounted for 94.9% of the data described by PCs 1, 2 and 9. After 96 hrs using the Euclidian distances, the dendrogram shows that to a large extent the positive controls of the two species (*T.rubrum*, *T.mentographites*) and EC_{50} concentration replicates could be effectively differentiated

(Figure 6). This was unclear for the EC_{90} treatments. After 120 hours, examination of the PCA map showed four distinct groups of dermatophyte species growing on SDA and that amended with the EC_{50} concentration of BHA + P-P per species, clearly separated from the other treatments (Figure 7).

4. Discussion

This is the first study to investigate the potential of using the E-nose as a qualitative screening tool for rapid assessment of the responses of dermatophytes to antifungal agents. Although growth on agar was used to determine the EC values of some antifungal agents, overall, the results showed that it was possible to discriminate between *T. rubrum* and *T. mentagrophytes* growing on un-amended solid media (SDA) from those inoculated on antifungal-modified media as early as 96 hours after incubation by analysing their volatile production patterns.

In the presence of a commonly used antifungal drug, itraconazole, the *Trichophyton* species, growing on control media and those amended with the EC_{50} and EC_{90} values of the drug were successfully differentiated after 96-120 hours. This supports previous studies with four *Trichophyton* species which showed differentiation within 96 hours using qualitative volatile fingerprints [15]. The results also revealed a similarity in the volatile production patterns of control media and those dermatophytes inoculated on antifungal modified media and hence the inhibition of growth by the antifungal drugs within the same time-point. Itraconazole is an azole agent which inhibits ergosterol synthesis in the fungal cell wall by inhibiting fungal cytochrome 14α- demethylase, and when given topically, may cause direct damage to the fungal cytoplasmic membrane [9].

The results of the griseofulvin test suggest that fungal age of cultures may affect the discrimination achieved. Differences were not very clear after 96 hrs. However, after 120

hrs clear differentiation between treatments of the EC_{90} values of griseofulvin and negative controls were similar to those in the itraconazole study. Griseofulvin, a fungistatic drug, acts in a different way, by inhibition of the formation of intracellular microtubules [6].

Detailed studies on potential alternative antioxidant compounds for the control of dermatophytes have not previously been investigated. Working with the experimental combination of BHA+P-P, the E-nose could successfully discriminate between three to four treatments of *Trichophyton* species growing on antifungal-free media and those modified with the EC_{50} values. The clear separation of negative controls and inoculated treatments at EC_{90} values from others within 96-120 hours indicated inhibition of growth similar to that for existing drugs such as itraconazole and griseofulvin. This also showed that the E-nose was able to differentiate between closely related treatments, notably those of the *Trichophyton* species growing on control media and on media amended to the EC_{50} value of the combination per species.

It was observed that the antifungal agents incorporated into the un-inoculated media did not affect the quality of the volatiles and therefore the process of discrimination. Previous studies have demonstrated that detection of micro-organisms in potable water in the presence of low quantities of heavy metals may modify their activity and thus the volatile production patterns enabling further separation to be made. Other studies have also shown that the nature of the culture media will influence the production of volatiles by micro-organisms [17, 19]. In contrast, Sahgal *et al.* [15] found that, regardless of medium the volatile fingerprints were could help in discrimination between four *Trichophyton* species.

To our knowledge, this is the first study that has employed qualitative volatile fingerprints for rapid screening of the susceptibility of dermatophytes to antifungal agents. The study shows the reproducibility of the method and the potential of using this approach

as a quick and simple way of qualitative screening of the responses of dermatphytoses to antifungal agents.

5. Conclusions

At the present, mould fungi, particularly dermatophytes, are not routinely tested for susceptibility [6, 14]. Most studies have focused on the selection of a medium that supports conidial formation in *T. rubrum* or susceptibility testing of dermatophytes isolated from patients by using the protocols of the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [1, 2, 14]. These methods are resource and time-consuming. In contrast, the detection of VOCs by the sensor array of an E-nose has many advantages [4]. The E-nose is non-invasive, sensitive, portable and relatively inexpensive and has a broad range of potential applications [4]. The current study demonstrated the potential for the rapid screening of novel antifungal compounds using volatile fingerprints and also offers the potential of use of this approach for monitoring the potential build up of resistance to specific anti-fungal or indeed anti-microbial drugs. By examining the PCA maps it would be possible to identify when poor control is being achieved by having both positive and negative controls on a regular basis for comparison.

Acknowledgments

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Table 1. The treatment values for 50% and 90% effective concentrations (EC_{50} and EC_{90}) for inhibition of mycelial growth of *Trichophyton* species which were used in this study.

Antifungal treatments	Species	EC_{50}[a]	EC_{90}[b]
Itraconazole	*T. rubrum*	0.18 (μg ml^{-1})	2.000 (μg ml^{-1})
	T. mentagrophytes	0.160 (μg ml^{-1}; M50)	2.000 (μg ml^{-1})
Griseofulvin	*T. rubrum*	0.010 (μg ml^{-1})	0.200 (μg ml^{-1})
	T. mentagrophytes	0.012 (μg ml^{-1})	0.200 (μg ml^{-1})
BHA[c] + P-P[d]	*T. rubrum*	0.137 (mM)	0.227 (mM)
	T. mentagrophytes	0.100 (mM)	0.215 (mM)
P-P + O-G[e]	*T. rubrum*	0.170 (mM)	0.250 (mM)
	T. mentagrophytes	0.110 (mM)	0.250 (mM)

[a] EC_{50} is the effective concentration of the antifungal drug at which mycelial growth was inhibited by 50%.; [b] EC_{90} is the effective concentration of the antifungal drug at which mycelial growth was inhibited by 90%; [c] Butylated hydroxyanisole; [d] n-Propyl-p-hydrohybenzoate; [e] Octyl gallate

Figure legends

Figure 1. Dose response curve for itraconazole concentrations and mean growth rate (mm day-1) of *Trichophyton rubrum (T.r.)* and *T.metagrophytes (T.m.)* used to calculate the EC_{50} and EC_{90} concentrations used.

Figure 2: Reproducibility of response of the sensor set to volatile produced by replicates of *T. mentagrophytes* after 96 hours growth on SDA at 25^{o}C. (*Key***:** M – *T. mentagrophytes*)

Figure 3: PCA score plot differentiating between *Trichophyton* species growing on plain SDA and various itraconazole treatments after 96 hours.(*Key*: B, blank SDA; ITZ, SDA amended at 2 μg ml^{-1} itraconazole; M, *T. mentagrophytes*; M50, *T. mentagrophytes* and EC_{50} itraconazole; M90, *T. mentagrophytes* and EC_{90} itraconazole; **R**, *T. rubrum*; R50, *T. rubrum* and EC_{50} itraconazole; R90, *T. rubrum* and EC_{90} itraconazole)

Figure 4: PCA score plot after 120 hours separating the positive controls from each other and from itraconazole and blank SDA treatments. *Key*: B, blank SDA; ITZ, SDA amended at 2 μg ml^{-1} itraconazole; M, *T.mentagrophytes*; M50, *T.mentagrophytes* and EC_{50} itraconazole; M90, *T.mentagrophytes* and EC_{90} itraconazole; **R**, *T.rubrum*; R50, *T.rubrum* and EC_{50} itraconazole; R90, *T.rubrum* and EC_{90} itraconazole.

Figure 5. Tree diagram of blank SDA, positive control, and griseofulvin treatment samples after 96 hours. *Key*: B – blank SDA; BP – un-inoculated SDA at 0.250 mM concentration of BHA + P-P; M – *T. mentagrophytes*; M50 – *T. mentagrophytes* and EC_{50} of BHA + P-P;

M90 – *T. mentagrophytes* and EC_{90} of BHA + P-P; *R* – *T. rubrum*; R50 – *T. rubrum* and EC_{50} of BHA + P-P; R90 – *T. rubrum* and EC_{90} of BHA + P-P.

Figure 6. Tree diagram showing the discrimination between the *T.rubrum* treatments, especially the *T.rubrum*, and *T.rubrum* EC_{50} and the blanks. *Key*: B – blank SDA; BP – un-inoculated SDA at 0.250 mM concentration of BHA + P-P; M – *T. mentagrophytes*; M50 – *T. mentagrophytes* and EC_{50} of BHA + P-P; M90 – *T. mentagrophytes* and EC_{90} of BHA + P-P; *R* – *T. rubrum*; R50 – *T. rubrum* and EC_{50} of BHA + P-P; R90 – *T. rubrum* and EC_{90} of BHA + P-P.

Figure 7. PCA score plot after 120 hours with clusters of *T. rubrum* and *T. mentagrophytes* growing on various treatments. *Key*: B, blank SDA; BP, un-inoculated SDA at 0.250 mM BHA+P-P; M, *T.mentagrophytes*; M50, *T.mentagrophytes* and EC_{50} of BHA+P-P; M90, *T.mentagrophytes* and EC_{90} of BHA+P-P; **R**, *T.rubrum*; R50, *T.rubrum* and EC_{50} of BHA+P-P; R90, *T.rubrum* and EC_{90} of BHA+P-P.

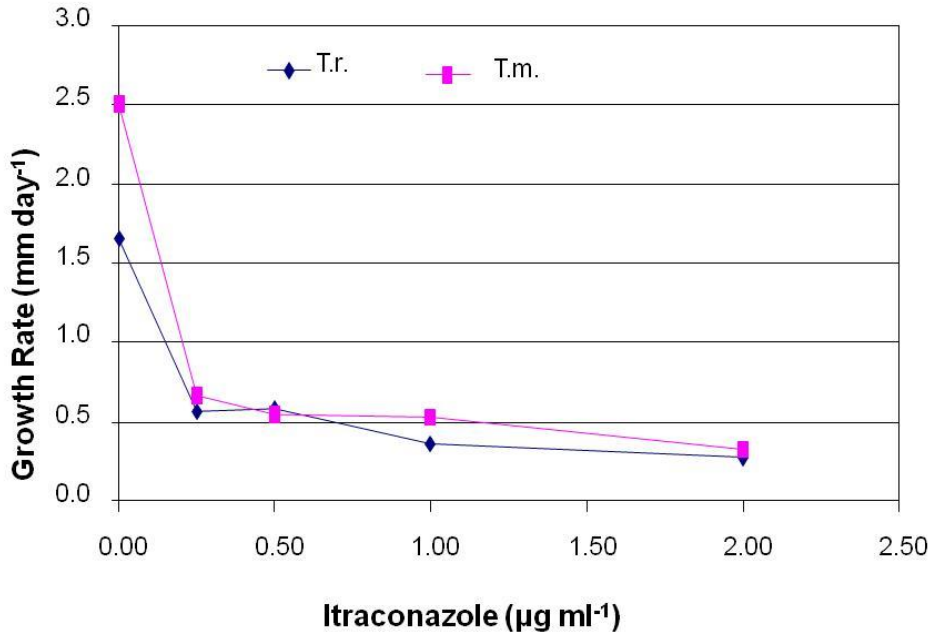


Figure 1.

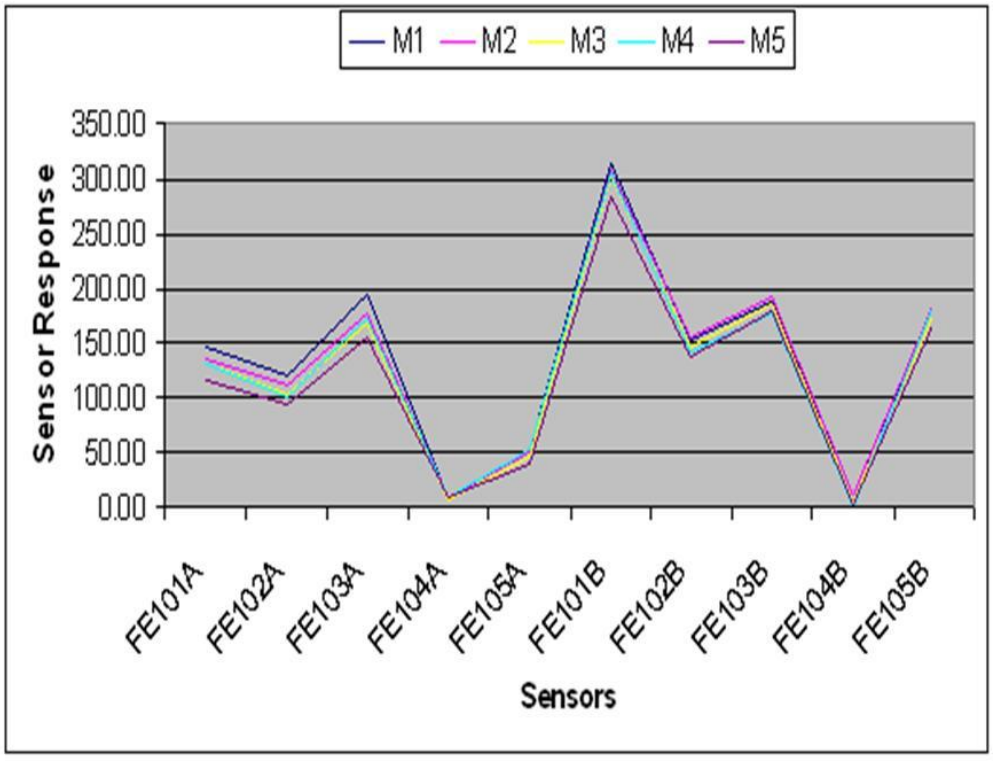
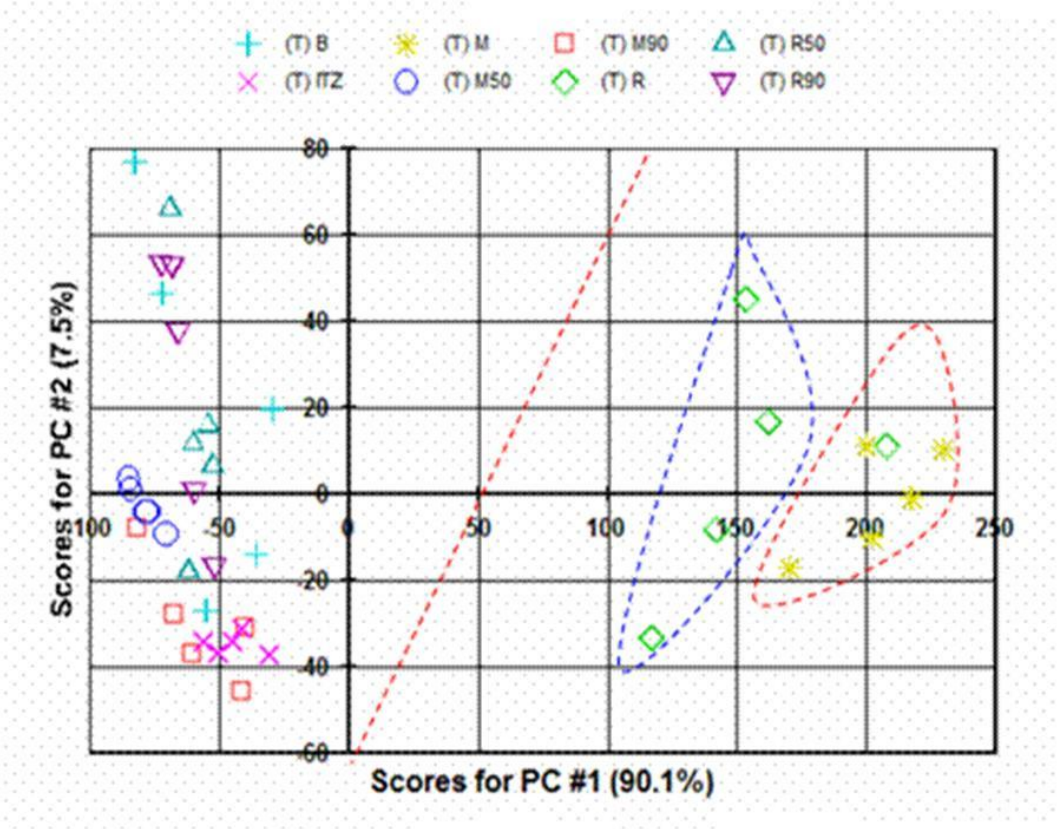


Figure 2.



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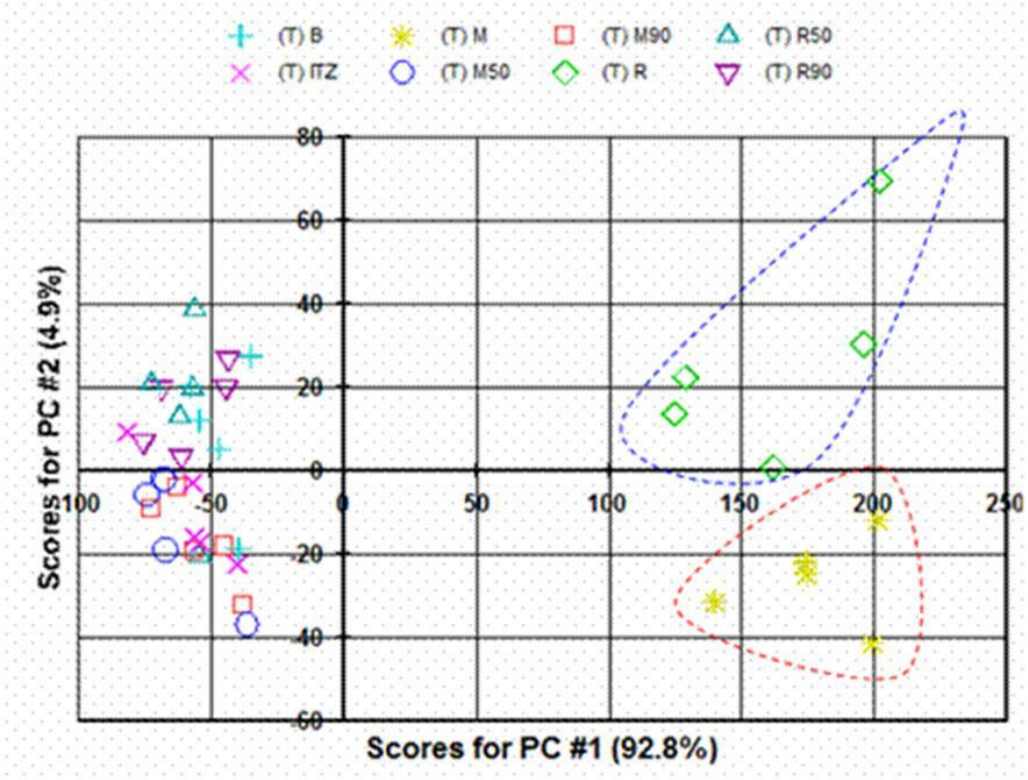

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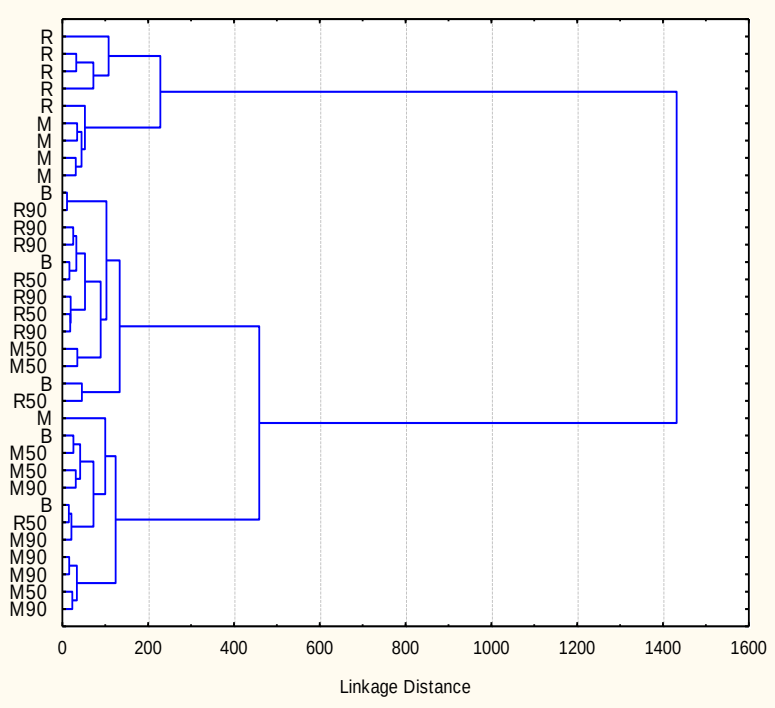

Figure 5.

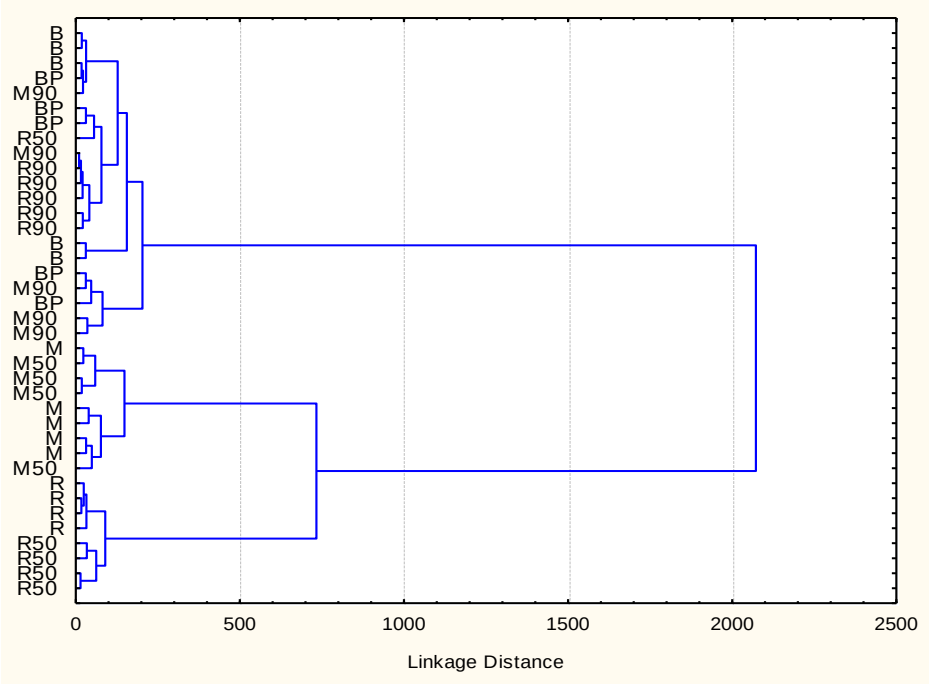

Figure 6.

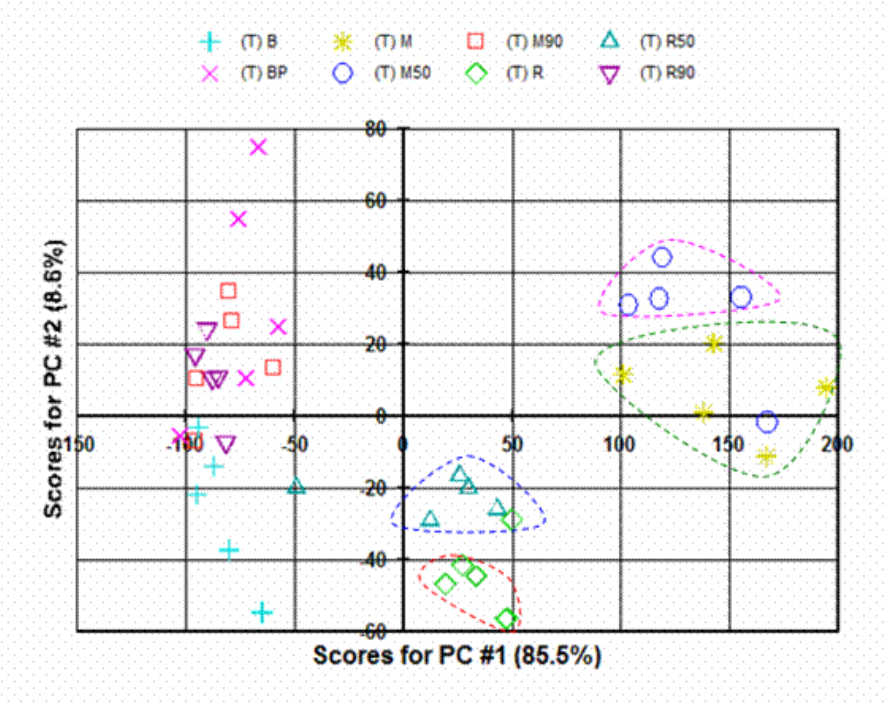
(T) B
(T) BP
(T) M
(T) M50
(T) M90
(T) R
(T) R50
(T) R90
Scores for PC #2 (8.6%)
Scores for PC #1 (85.5%)
80
60
40
20
0
-20
-40
-60
-150
-100
-50
0
50
100
150
200

Figure 7