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A novel and rapid approach to yeast differentiation using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

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Abstract. Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS) was investigated as a method for the rapid identification of yeast cells. Following pretreatment of yeast samples with a cell wall digesting enzyme (lyticase), distinct and reproducible mass spectra over the m/z range 2,000 to 16,000 were obtained by MALDI-TOF-MS. Using an optimised procedure, characteristic mass spectra that distinguished between *Candida* spp. and between strains of *Saccharomyces cerevisiae* were produced. The approach offers the potential for rapid differentiation of yeasts in clinical diagnosis and in the fermentation industries.

1. Introduction

There is a need for rapid and accurate methods of yeast differentiation in clinical diagnosis and in the beverage industries. Morphological or biochemical parameters have traditionally been used to distinguish yeast species and individual strains [4]. These methods, however, are time consuming and often unreliable. Yeasts are currently identified by molecular biological methods, such as restriction fragment length polymorphism analysis (RFLP) [14] and techniques based on polymerase chain reaction (PCR) technology, for example random amplification of polymorphic DNA (RAPD) [17,21]. These methods are slow, labour-intensive and require considerable technical expertise. Several processing steps involving expensive reagents are involved, any one of which can introduce errors that invalidate the assay. For the brewing industry, a further problem has been the need to obtain high-purity DNA from yeasts since fermentation cultures contain inhibitors that influence the activity of *Taq* polymerase [16]. There exists a need therefore to develop more rapid and simplified methods for the accurate differentiation and identification of yeasts. With recent developments in analytical instrumentation, whole-organism fingerprinting by physicochemical spectroscopic methods has become possible. Pyrolysis-mass spectrometry (Py-MS), UV resonance Raman spectroscopy and Fourier transform-infrared spectroscopy (FT-IR) are the most common techniques used for this application [5,15,18]. Cumbersome data analysis and the need for skilled interpretation of spectra are general limitations of the whole-organism fingerprinting approach for rapid identification of microorganisms. When applied to differentiation of *Candida* spp. for example, Py-MS and FT-IR spectra showed very little qualitative difference between species and analysis by multivariate statistical methods was necessary to differentiate species [18].

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Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) is emerging as a powerful tool for identification of bacteria [19,12]. Intact bacterial cells analysed by MALDI-TOF-MS have been shown to give reproducible and repeatable spectra that are simple to interpret visually. The technique detects differences in cell wall constitution through ionisation of surface molecules. Identification of an unknown organism is then based on assignment of genus, species and/or strain specific biomarkers. Intact cell MALDI-TOF-MS has been successfully used for the rapid identification of bacterial cells [1,3,6,7,13]. Although this approach has recently been used to characterise fungal spores [20] and filamentous fungi [2], its application to the differentiation and identification of genera, species and strains of yeasts has not been demonstrated.

In the work reported on here, we investigated the possibility of differentiating yeasts by intact cell MALDI-TOF-MS. The aim of the work was to develop a method for generating genus, species or strain specific biomarkers from yeast cell wall components.

2. Materials and methods

2.1. Organisms and sample preparation

Characterised yeasts were obtained from the National Collection of Yeast Cultures (Colney, Norwich, UK) [*Candida albicans* NCYC 597, *Candida boidinii* NCYC 1513, *Candida tropicalis* NCYC 1503, *Saccharomyces cerevisiae* NCYC 19201, NCYC 22118] or from the culture collection at De Montfort University, Leicester, UK (*Saccharomyces cerevisiae* RR1). Organisms were maintained on malt extract agar slopes at 4°C. Cells for analysis were cultivated in 50 ml malt extract broth (Oxoid, Basingstoke, UK) incubated at 30°C and 150 rev $\min^{-1}$ for 24–48 hours. Cells were harvested by centrifugation at $10\,000 \times g$ for 2 minutes and washed once in 0.05 mol l^{-1} potassium phosphate buffer (pH 7.2). Cells (1–5 mg wet weight) were enzymatically treated by resuspending in 0.05 mol l^{-1} potassium phosphate buffer (100 μl) containing 5–100 units of lyticase from *Arthrobacter luteus* (Sigma-Aldrich, Poole, UK) and incubating at 30°C for 5–30 minutes. For untreated cells, this step was omitted. Cells were subsequently harvested by centrifugation at $10\,000 \times g$ for 2 minutes and washed once in 0.1% trifluoroacetic acid, once in sterile distilled water and resuspended in 100 μl of methanol. Matrix chemical [dihydroxybenzoic acid, 2-(4-hydroxyphenylazo)benzoic acid or 3,5-dimethoxy-4-hydroxycinnamic acid] dissolved in solvent [sterile distilled water, methanol, formic acid : isopropyl alcohol : water (17 : 33 : 50), acetonitrile : water (33 : 67) or trifluoroacetic acid : acetonitrile : water (0.067 : 33 : 67)] was added to the cell suspension to give a ratio of cell suspension to matrix solution of between 1 : 1 and 1 : 20. An aliquot (1 μl) of the mixture was applied to a stainless steel MALDI probe and evaporated to dryness at ambient temperature.

2.2. MALDI-TOF-MS analysis

Positive ion spectra were acquired with a Lasermat 2000 linear time-of-flight mass spectrometer (Finnegan Mat Ltd., Hemel Hempstead, UK) with an acceleration voltage of 20 kV. Ionisation of molecules was induced using a nitrogen laser at a wavelength of 337 nm. Mass spectra were averaged over 12–24 individual laser shots using dedicated Lasermat 2000 software. Averaged spectra were baseline corrected and smoothed using the proprietary software.

3. Results and discussion

Initial experiments focused on obtaining characteristic MALDI-TOF-MS spectra from yeast cells in the absence of enzymatic pretreatment of cells. The experimental parameters investigated were matrix chemical, matrix solvent and the ratio of sample to matrix. Distinct peaks were not generated by MALDI-TOF-MS analysis of untreated yeast cells under the range of conditions used.

Many yeast cell wall proteins are covalently bound to glucan and chitin in the cell wall. They are therefore more securely attached to the yeast cell wall than are bacterial cell wall proteins [18]. Pretreatment of yeast samples with lyticase (a $\beta 1,3$-glucanase preparation) was introduced into the method in an attempt to partially digest the glucan cell wall leading to release cell wall components that may then be ionised by MALDI. Following lyticase treatment of yeast samples, distinct peaks were present in positive ion spectra from MALDI-TOF-MS analysis that were not present in those from untreated cells nor in those from no-cell controls (Figs 1 and 2). These spectra indicate that lyticase treatment of samples gave rise to ionisable molecules from yeast cells that could be detected by MALDI-TOF-MS. The lyticase treatment of yeast samples was optimised by adjusting enzyme concentration and time of treatment. Ionisation of molecules by MALDI was optimised through (1) altering the matrix, (2) matrix solvent and (3) ratio of sample to matrix, as described above. The optimised procedure most suited to generation of distinct peaks in MALDI-TOF-MS analysis of lyticase treated yeast cells was: 100 units lyticase treatment for 15 minutes; 3,5-dimethoxy-4-hydrocinnamic acid as matrix in an aqueous solvent containing trifluoroacetic acid (0.067%) and acetonitrile (33%); sample to matrix ratio of 1 : 9 to 1 : 12 (v/v).

Using the optimised procedure, distinct peaks ranging from m/z 2000 to 16 000 were obtained from each of the yeasts. Analysis of replicate cultures of *Saccharomyces cerevisiae* NCYC 22118 (Fig. 3) and *Candida albicans* NCYC 597 (Fig. 4) gave simple and consistent mass spectral profiles within this range. Comparison between different strains of *S. cerevisiae* (Fig. 5) and different *Candida* spp. (Fig. 6) showed that a distinctive mass spectral profile was generated for each of the yeasts analysed, which may be used for the purposes of identification. For example, *S. cerevisiae* NCYC 19201 displayed a strong multiplet of mass ions in the range m/z 2400 to 3400 and a highest mass ion at m/z 6700. Conversely, *S. cerevisiae* NCYC 22118 displayed only one high-mass ion in the m/z 2400–3400 range (the peak at m/z 3,195 was common to all three *S. cerevisiae* strains), but released ions detectable at m/z values of 8800–8900 and 11 700. Similarly, the *Candida* species all released ions with approximate m/z values of 3700 and 7000–7400, whilst also releasing ions specific to the individual species under analysis.

As has been reported for fungal spores [20], the spectra generated by analysis of lyticase-treated yeast cells displayed greater peak width than those generated for bacteria (e.g., [1,3,12]). This may be related to the properties of the yeast cell wall and to the nature of enzymatic pretreatment. Cell wall proteins are covalently linked to β-1,6-glucan, which is in turn linked to β-1,3-glucan and to a lesser extent to chitin [8–11]. Since lyticase is a β-1,3-glucanase with residual β-1,6-glucanase (due to the use of a partially purified enzyme), the majority of proteins exposed to ionisation will be β-1,6-glycosylated [8,9,11]. Fractionation of yeast cell wall proteins following β-1,3-glucanase digestion has been reported to yield large heterogeneous molecules, whereas relatively discrete molecules are obtained following digestion with β-1,6-glucanase [8]. Our future work will investigate the influence of other yeast cell wall digesting enzymes and enzyme combinations on the generation of species and strain specific biomarkers by MALDI-TOF-MS.

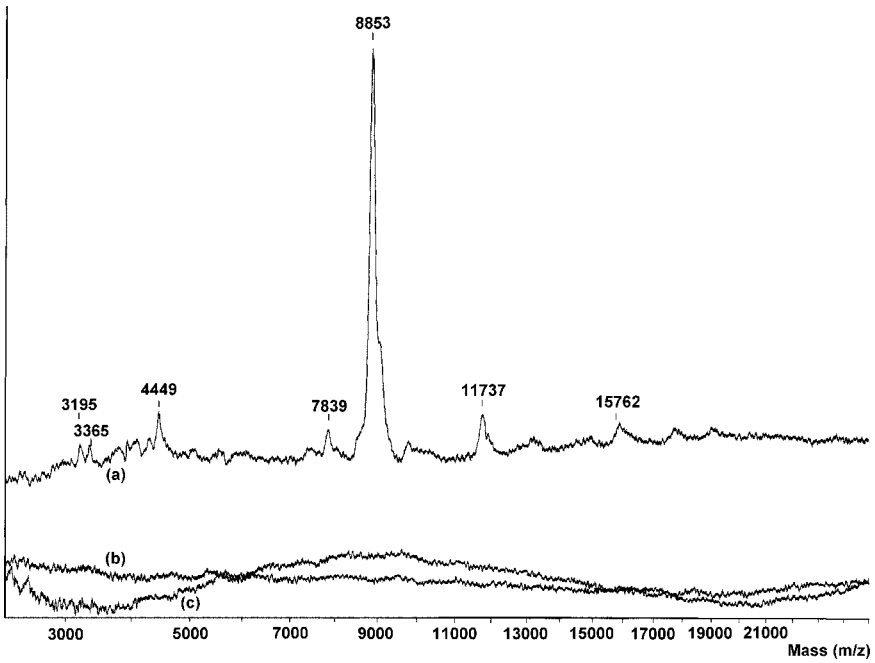

Fig. 1. MALDI-TOF mass spectra obtained from *S. cerevisiae* NCYC 22118. Spectra are from lyticase treated cells (a), untreated cells (b) and no-cell control (c).

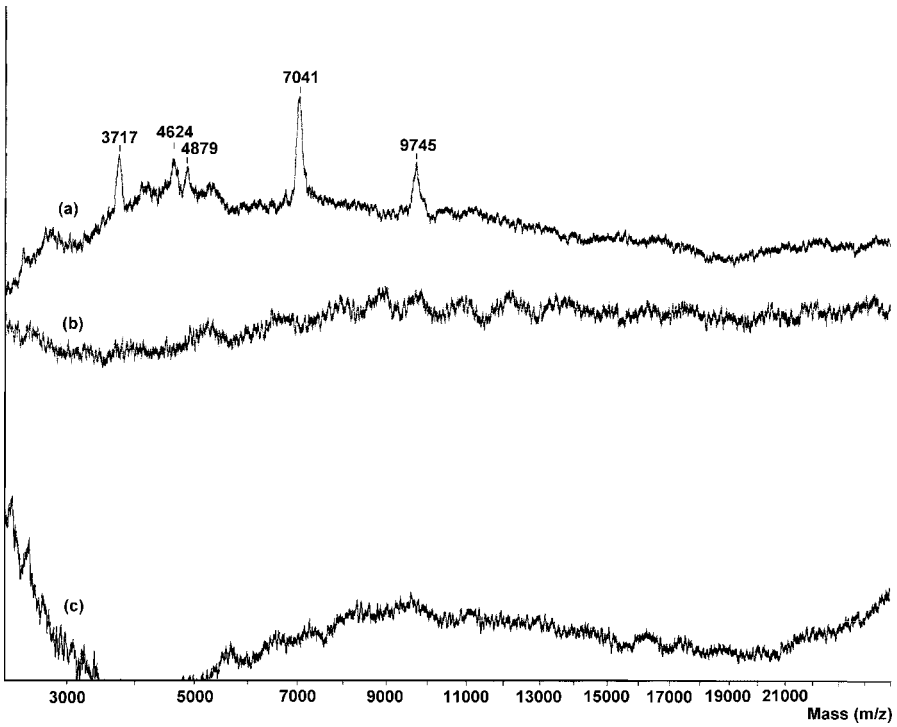

Fig. 2. MALDI-TOF mass spectra obtained from *C. albicans* NCYC 597. Spectra are from lyticase treated cells (a), untreated cells (b) and no-cell control (c).

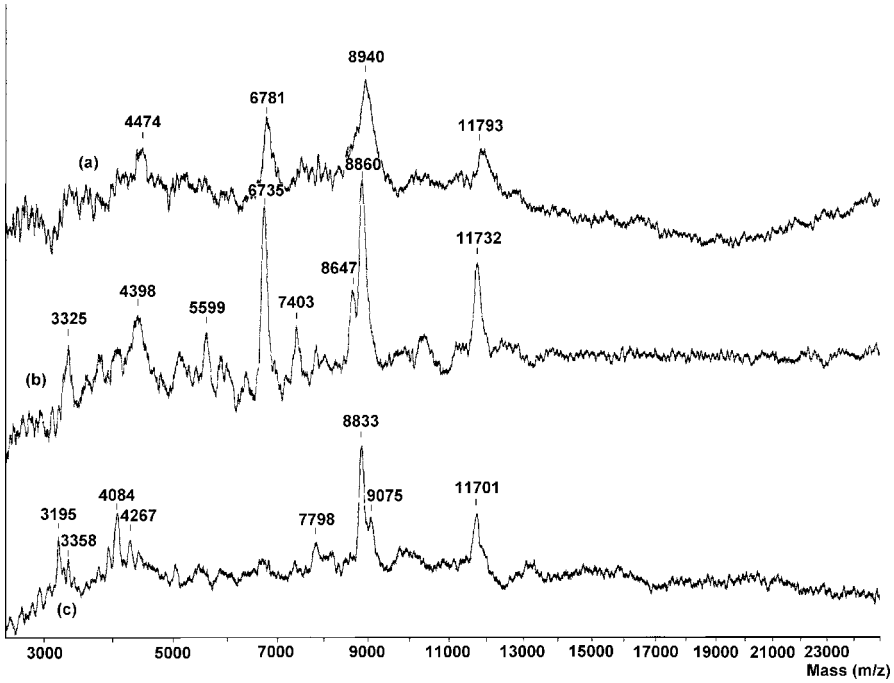

Fig. 3. MALDI-TOF mass spectra obtained from three replicate cultures of *S. cerevisiae* NCYC 22118 following lyticase treatment of cells.

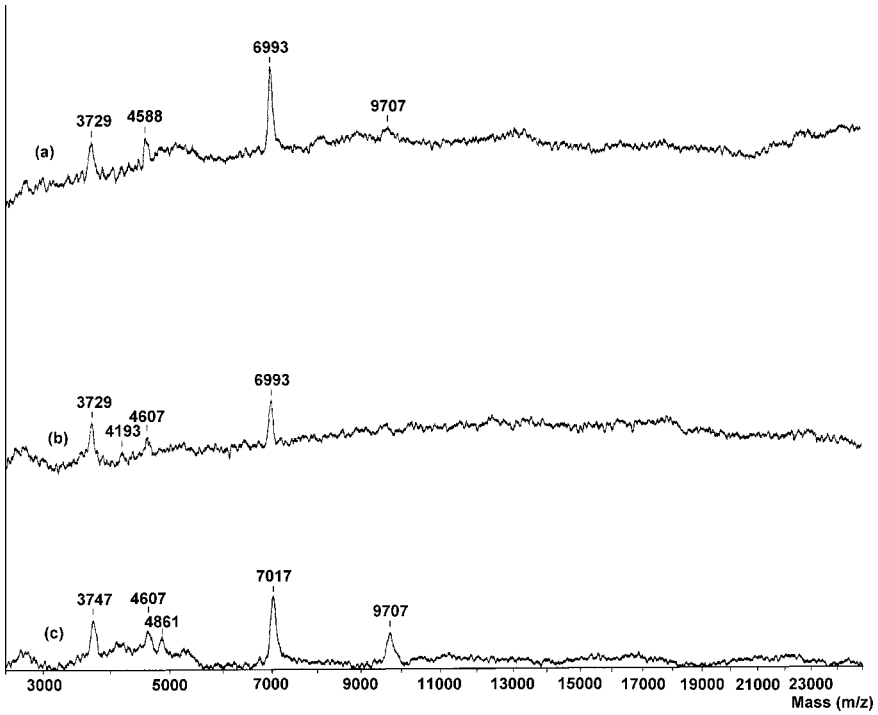

Fig. 4. MALDI-TOF mass spectra obtained from three replicate cultures of *C. albicans* NCYC 597 following lyticase treatment of cells.

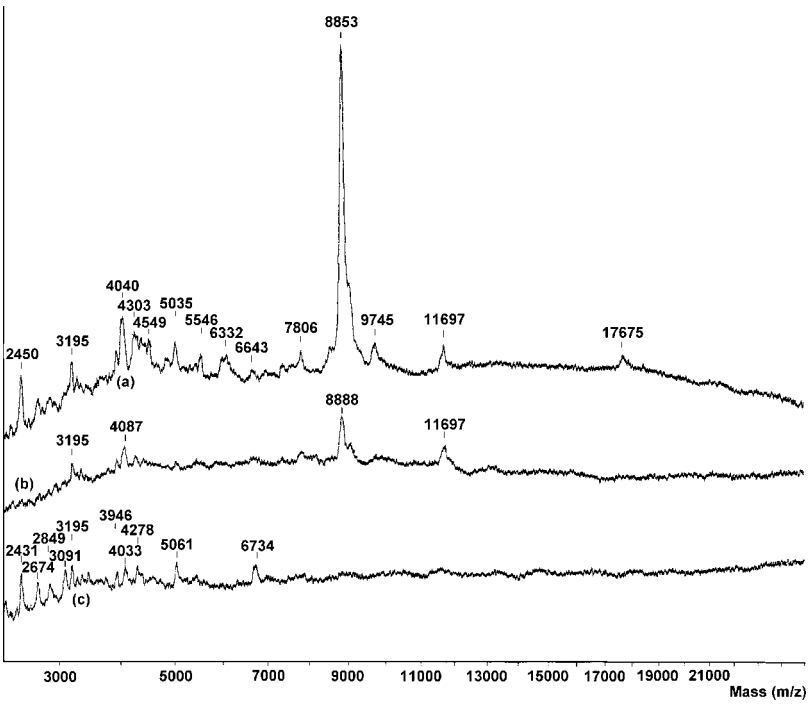

Fig. 5. MALDI-TOF mass spectra obtained from strains of *S. cerevisiae* following lyticase treatment. Spectra are from strain RR1 (a), NCYC 22118 (b) and NCYC 19201 (c).

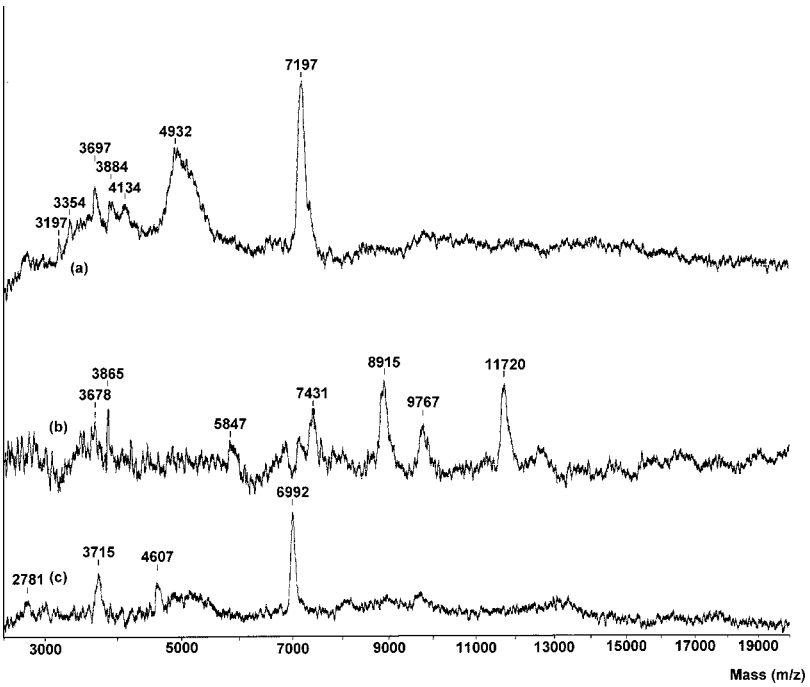

Fig. 6. MALDI-TOF mass spectra obtained from *Candida* spp. following lyticase treatment. Spectra are from *C. tropicalis* (a), *C. boidinii* (b) and *C. albicans* (c).

4. Conclusion

We have shown that following enzymatic treatment of yeast samples characteristic and reproducible spectra can be generated by MALDI-TOF-MS. The analysis can distinguish between *Candida* spp. and between strains of *S. cerevisiae*, offering the potential for rapid differentiation and identification of yeasts in clinical diagnosis and in the fermentation industries.

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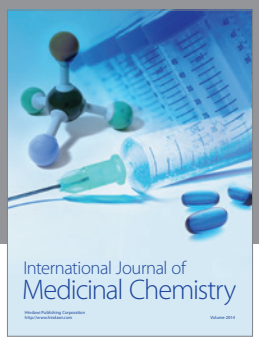

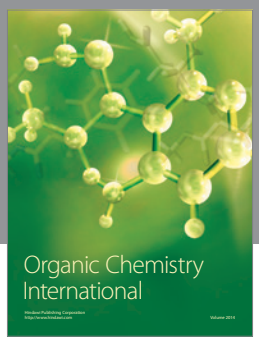

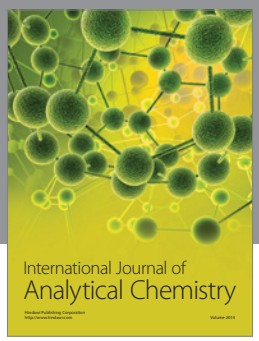

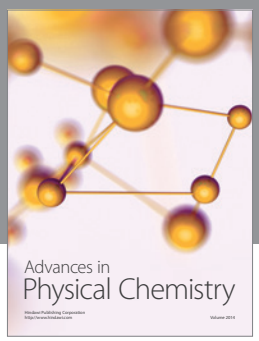

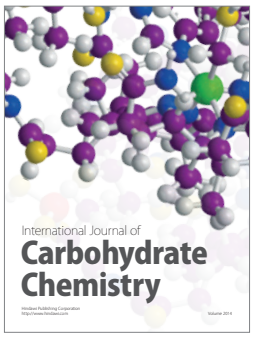

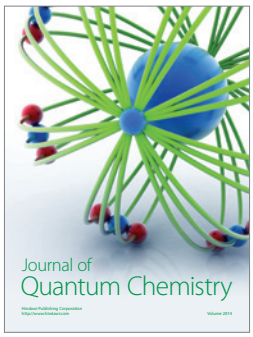

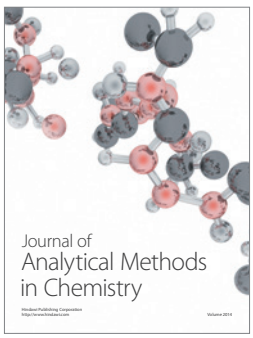

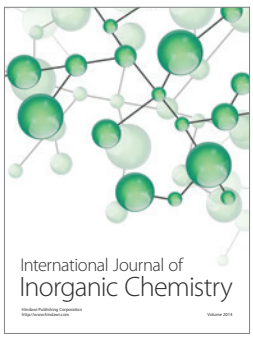

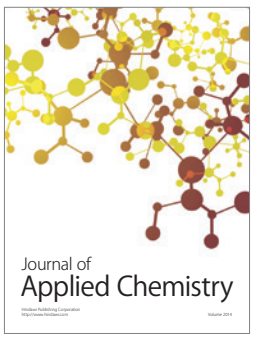

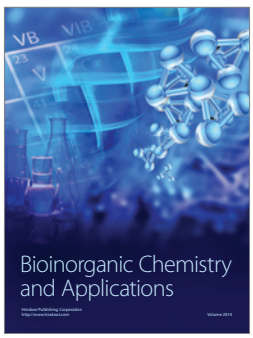

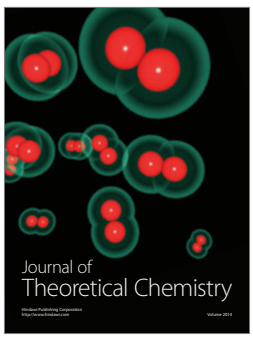

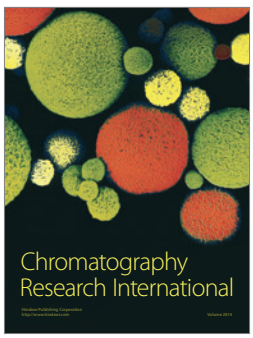

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