

Research Article

Traceability of PDO Olive Oil “Terra di Bari” Using High Resolution Melting

Cinzia Montemurro,^{1,2} Monica Marilena Miazzi,¹ Antonella Pasqualone,³
Valentina Fanelli,¹ Wilma Sabetta,⁴ and Valentina di Rienzo^{1,2}

¹Department of Soil, Plant and Food Sciences, Section of Genetics and Breeding, University of Bari Aldo Moro, Via Amendola 165/A, 70126 Bari, Italy

²Spin Off Sinagri s.r.l., University of Bari Aldo Moro, Via Amendola 165/A, 70126 Bari, Italy

³Department of Soil, Plant and Food Sciences, Section of Food Science Technology, University of Bari Aldo Moro, Via Amendola 165A, 70126 Bari, Italy

⁴Institute of Biosciences and Bioresources, CNR, Via Amendola 165/A, 70126 Bari, Italy

Correspondence should be addressed to Cinzia Montemurro; cinzia.montemurro@uniba.it

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The aim of the research was to verify the applicability of microsatellite (SSR) markers in High Resolution Melting (HRM) analysis for the identification of the olive cultivars used in the “Terra di Bari” PDO extra virgin olive oil. A panel of nine cultivars, widespread in Apulia region, was tested with seventeen SSR primer pairs and the PCR products were at first analysed with a Genetic Analyzer automatic sequencer. An identification key was obtained for the nine cultivars, which showed an unambiguous discrimination among the varieties constituting the “Terra di Bari” PDO extra virgin olive oil: Cima di Bitonto, Coratina, and Ogliarola. Subsequently, an SSR based method was set up with the DCA18 marker, coupled with HRM analysis for the distinction of the Terra di Bari olive oil from non-Terra di Bari olive oil using different mixtures. Thus, this analysis enabled the distinction and identification of the PDO mixtures. Hence, this assay provided a flexible, cost-effective, and closed-tube microsatellite genotyping method, well suited to varietal identification and authentication analysis in olive oil.

1. Introduction

The average worldwide production of olive oil has grown steadily in the last years, mainly due to the recommendation of doctors and nutritionists about the benefit of Mediterranean diet, in which olive oil is a key element. Despite the fact that Italy remains one of the main producers of this sector, the total production and consumption of olive oil have undergone a considerable decrease [1]. In this scenario, Apulia is the main oil producing region in Italy [2]. Due to their authenticity, most of the Apulian olive oils obtained the quality marks of Protected Denomination of Origin (PDO) by the European Community, according to the EC Regulation 2081/92. This certification implies the use of specific local cultivars and peculiar sensory features of oil (Official Journal of the European Communities, 1992).

Consequently, monitoring the origin of raw material and industrial process becomes of primary importance during the production of a high value PDO product.

The verification of the cultivars used to produce olive oil is becoming of particular interest in the last decade. This fact assumes a strong commercial appeal especially in the protection of high-quality olive oils, such as PDO, which might be adulterated with other low-quality oil using minor or less expensive cultivars [3]. In fact, the quality of olive oil strictly depends on the variety employed for its production and there is a strong link between the cultivar and the territory of cultivation. Several analytical approaches have been developed to help the identification of olive oil cultivars, constituents, and possible adulterants. The compositional markers monitored for traceability purposes are different, such as triglycerides, sterols, fatty acids, phenolic compounds, volatile compounds,

pigments, hydrocarbons, and tocopherols [4–7]. However, all these compositional markers can be severely affected by the environmental conditions during the plant growth, which might cause ambiguous or erroneous results [3]. Since the chemical analyses are not enough for themselves to verify the olive oil authenticity or its varietal identification, other traceability markers based on DNA analysis have been used to identify olive cultivars. In fact DNA, being less influenced by environment and food processing, is the best resource for comparison of different genetic material [8–10].

In this contest, molecular markers represent an ideal tool to accurately and exclusively characterize olive cultivars by detection of DNA polymorphisms and the establishment of an identification key. To this purpose, microsatellites or Simple Sequence Repeats (SSR) are among the most suitable molecular markers, since they are characterized by a high polymorphism level, due to variations of the repeats number. Moreover, SSR analysis is easy to perform, just implying an amplification with species-specific primer pairs and the subsequent electrophoresis on agarose or acrylamide gels. In particular, microsatellites have been preferred by several authors for variability studies, germplasm characterization, and varietal fingerprinting of numerous species such as wheat [11], rice [12], grape [13], tomato [14], and olive [15–18]. SSR markers can allow to determine an unambiguously identification key of the most common Italian monovarietal oils, also thanks to their capacity to detect any “alien” allele [9, 19].

The extra virgin olive oil “Terra di Bari” is one of the most important Italian PDO oils, whose spread on the national market is equal to 13.1% [20]. According to the Ministerial Decree of 4 September 1998, the EC Regulation 2325/97, and the article 17 of EEC Regulation 2081/92, the disciplinary regulations about the production of “Terra di Bari” oil, with “Bitonto” as additional geographical mention, expect that this oil is obtained from the following olive cultivars, in the minimum quantity of 80%, alone or mixed together: Cima di Bitonto or Ogliarola Barese and Coratina (UG n. 227 of 29 September 1998). Thereby, the main aim of the present study was to test the applicability of microsatellite markers for the identification of cultivars constituting the “Terra di Bari” PDO oil.

2. Material and Methods

2.1. Plant Material and DNA Extraction. Nine olive cultivars, diffused in the Apulia Region (Italy), were sampled at the Olive Pre-multiplication Centre field “Conca d’Oro”, Palagiano (Taranto, Italy): Cima di Bitonto, Ogliarola, Coratina, Toscanina, Maiatica, Cellina di Nardò, Nociara, Cima di Mola, and Simone. These cultivars were previously tested for the genetic identity and are considered as certified varieties. The DNA was extracted both from leaves and 9 monovarietal olive oils. The experimental material was enriched with two commercial PDO olive oils used for DNA extraction and HRM analyses. Genomic DNA from leaves was extracted according to Li protocol [21] modified as reported by Sabetta et al., 2011 [22], starting from 30 mg of lyophilized leaves. DNA from monovarietal and commercial olive oils was

extracted by means of Gene Elute Plant Kit (Sigma, St. Louis, MO) following the manufacturer’s instructions and the modifications reported in Pasqualone et al. [9]. Cellular residuals were obtained by centrifuging 250 mL of oil at 10,000 rpm for 5 min. The extracted DNA was checked in terms of quality and quantity by means both of 0.8% agarose gel electrophoresis and spectrophotometer (Nanodrop 1000, Thermo Scientific, Waltham, MA, USA) at 260 nm. DNA from lyophilized leaves resulted to have optimal quality and a concentration of 100 ng/ μ L, whereas DNA extracted from oil had lower concentration (5 ng/ μ L) and was partially degraded [23].

2.2. SSR Markers Analysis and Genetic Relationship. Seventeen microsatellite primer pairs DCA03, DCA04, DCA18, DCA05, DCA09, DCA13, DCA14, DCA15, DCA16, DCA17 [15] and GAPU103a, GAPU71b, GAPU101, GAPU45 [24], EMOL, EMO90 [25], and UDO43 [26] (Table 1) labelled with FAM or HEX fluorochromes were used in the analysis and the amplification reactions were carried out in a final volume of 25 μ L containing the following: 50 ng DNA, 1X PCR buffer, 0.25 mM dNTP, 0.25 μ M of each primer, 2.5 mM MgCl₂, and 0.06 U Taq Polymerase (Euroclone). The PCRs were carried out in a C1000 thermal cycler (Bio-Rad) and the conditions were set as follows: 5 min at 95°C; 35 cycles consisting of 30 sec at 95°C, 30 sec at the specific annealing temperature, 30 sec at 72°C; 60 min at 72°C for final elongation. The amplification products were separated by capillary electrophoresis on an automated sequencer ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems) and the obtained electropherograms were analysed by the GeneMapper 3.7 software (Applied Biosystems).

The amplified fragments were used to get a binary matrix, in which amplicons were marked with 1 for presence and 0 for the absence of a fragment to a certain molecular weight. Genetic similarity among the olive cultivars was calculated by Jaccard coefficient and the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) was performed for cluster analysis with NTSYS-PC 2.0 [27].

2.3. DNA Mixtures and SSR Genotyping by High Resolution Melting (HRM) Analysis. Reference DNAs of cultivars Coratina, Cima di Bitonto, and Ogliarola were used alone or combined in order to get mixtures simulating Terra di Bari oil, which comprises a 20% of unspecified local varieties widely spread in the area. The local varieties, indicated as “other cultivars,” were Simone, Toscanina, Cima di Mola, Nociara, Maiatica, and Cellina di Nardò. In addition, we analysed two commercial olive oils Terra di Bari and an extra-European monovarietal oil obtained from the cultivar Aeleh (Algeria) in order to consider a possible introduction of unlabelled extra community olive oil (Table 2). Progressive adulteration of a PDO oil sample (monovarietal Coratina olive oil) was simulated by adding a crescent proportion of cultivar Aeleh from 10% to 50%.

We performed a High Resolution Melting (HRM) analysis choosing the SSR marker DCA18, on the basis of its different allelic profiles in the cultivars Coratina, Cima di Bitonto, and Ogliarola Barese obtained by capillary electrophoresis.

TABLE 1: Locus name, repeat motif, annealing temperature, primer sequences referred to SSR markers, and allelic profiles of the cultivars included in the PDO Terra di Bari olive oil.

Locus	Repeat motif	Primer sequence (5'-3')	Annealing temperature	Allelic profiles (bp)		
				Cima di Bitonto	Ogliarola Barese	Coratina
DCA03	(GA) ₁₉	ccaagcggaggtgtatattgttac tgcttttggtttgagatgttg	50°C	237	256	239
DCA04	(GA) ₁₆	cttaactttgtgcttccatattcc agtgacaaaagcaaaagactaaagc	55°C	130	186	162
DCA05	(GA) ₁₅	aacaaatcccatacgaactgcc cgtggtgctggaagaaaatcg	50°C	202	202	194
DCA09	(GA) ₂₃	aatcaaagtcttctctcatttcg gatccttccaaaagtataacctctc	55°C	178	170	180
DCA13	(CA) ₁₅	gatcagattaatgaagattggg aactgaacctgtgtatcttgcatcc	55°C	116	116	153
DCA14	(CA) ₁₈ A ₆ (TAA) ₇	aatttttaatgactataatttac ttgaggtctctatatctcccagggg	50°C	179	170	187
DCA15	(CA) ₃ G(AC) ₁₄	gatcttgtctgtatattccac tatacctttccatcttgacgc	50°C	264	268	245
DCA16	(GT) ₁₃ (GA) ₂₉	ttaggtgggattctgtagatgggtg tttaggtgagttcatagaattagc	50°C	153	148	171
DCA17	(GT) ₉ (AT) ₇ AGATA(GA) ₃₈	gatcaaatctaccaaaaatata taatttttggcacgtagtattgg	50°C	113	140	111
DCA18	(CA) ₄ CT(CA) ₃ (GA) ₁₉	aagaaaagaaaaggcagaattaagc gttttcgtctctacataaagtgc	50°C	173	169	176
GAPU101	(GA) ₈ (G) ₃ (AG) ₃	catgaaaggagggggacata ggcacttgtgtgcagattg	57–60°C	197	183	217
GAPU103a	(TC) ₂₆	tgaatttaactttaaccacaca gcatcgctcgattttatcc	57–60°C	208	157	132
GAPU71b	GA(AG) ₆ (AAG) ₈	gatcaaaggaagaaggggataaa acaacaaatccgtacgcttg	57–60°C	120	120	120
GAPU45	(AG) ₇	atcgggaggatgtgatgta catcgcatcgctgtaata	57–60°C	181	183	183
EMOL	(GA) ₁₂	cttccaatattgggctctcg atggcacttacgggaaaaa	50°C	192	198	198
EMO90	(CA) ₁₀	catccggatttcttctttt agcgaatgtagctttgcatgt	50°C	183	189	183–189
UDO43	(GT) ₁₂	tcggctttacaaccatttc tgccaattatggggctaact	57°C	216	176	198

TABLE 2: Composition of the mixtures of DNA olive oil used in HRM analysis.

Cultivar	DNA mixtures (v/v in %)																		
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11*	M12*	M13	M14	M15	M16	M17	M18	M19
Coratina	100						26.6	40	40						90	80	70	60	50
Ogliarola		100			80		26.6	40		40									
Cima di Bitonto			100			80	26.6		40	40									
Nociara				80									50						
Toscana		80											50						
Aeleh			80											100	10	20	30	40	50
Other cultivars**	26.6	26.6	26.6	20	20	20	20	20	20	20									

* On the bases of PDO “Terra di Bari” procedure rules, these commercial samples should be made by 80% of Coratina, Ogliarola Barese, and/or Cima di Bitonto alone or in mixture and a 20% of other cultivars.

** Mixture in equal proportion of cultivars: Simone, Cima di Mola, Nociara, and Toscana.

HRM reaction was performed in a final volume of 10 μ L consisting of 50 ng of genomic DNA, 1 \times Sso Fast EvaGreen Master mix (Bio-Rad, Hercules, CA), and 0.25 μ M of each primer (Sigma-Aldrich, St. Louis, MO). A No Template Control (NTC) was included in each run [28].

Amplification and HRM analysis were performed on CFX96 Touch Real Time PCR Detection System (Bio-Rad, Hercules, CA) and the cycling program consisted of a touchdown protocol: 2 min of initial denaturation at 98°C, followed by 5 cycles of denaturation at 98°C for 8 sec, annealing at 56°C for 8 sec (with decrement of 0.5°C per cycle), and extension at 72°C for 12 sec. The annealing temperature was maintained at 54°C for the successive 40 steps and denaturation temperature was decreased to 95°C, acquiring fluorescent data at the end of each cycle. The amplification protocol was immediately followed by the High Resolution Melting steps of 95°C for 10 sec, cooling to 58°C for 30 sec, and raising the temperature from 65°C to 95°C with increasing of 0.2°C every 10 sec with fluorescence acquisition.

After verification of robust amplification curves, the melting curve stage was further analysed by CFX Manager software (Bio-Rad, Hercules, CA). The melt curve was normalized along the temperature axis (temperature shifting) to permit easy differentiation of DNA curve.

3. Results and Discussion

Among the different types of genetic markers available for varietal identification purposes, the nuclear microsatellite or SSR (Simple Sequence Repeat) is the marker of choice largely used and the only one accepted for forensic applications [29]. This is essentially due to the numerous advantages intrinsic of such marker: codominant nature, high polymorphism, wide distribution across the genome, and automated detection. As SSR can be used to distinguish olive varieties when DNA is extracted directly from olive oils and based on our previous works [30] we identified the most informative and effective markers (DCA04 and DCA18) to genotype the selected cultivars showing a different allelic profiles (Table 1).

In this work SSR markers were directly applied to DNA extracted from olive oil, which is highly degraded and poor in quantity. The best approach is to select SSR markers providing a simple and reproducible pattern, whilst they maintain their informativeness and efficiency. The SSR markers used in this work were selected for their high polymorphism and for the clear allelic profile.

All 17 SSR markers showed a very high value of PD (Power Discrimination) [31], with the maximum value of 0.88 scored for DCA18.

Figure 1 reports the genetic similarity dendrogram obtained with 17 SSR markers. The cultivars Toscanina and Nociara showed the highest degree of diversity, whereas the others are separated in two subclusters. In the first group there are Cima di Mola, Ogliarola, Maiatica, and Simone, and in the second one there are Cellina di Nardò, Coratina, Cima di Bitonto, and Toscanina. All the cultivars showed small sized fruits, characteristic of olive oil attitude cultivars, with Cima di Mola and Ogliarola, and Coratina and Cima

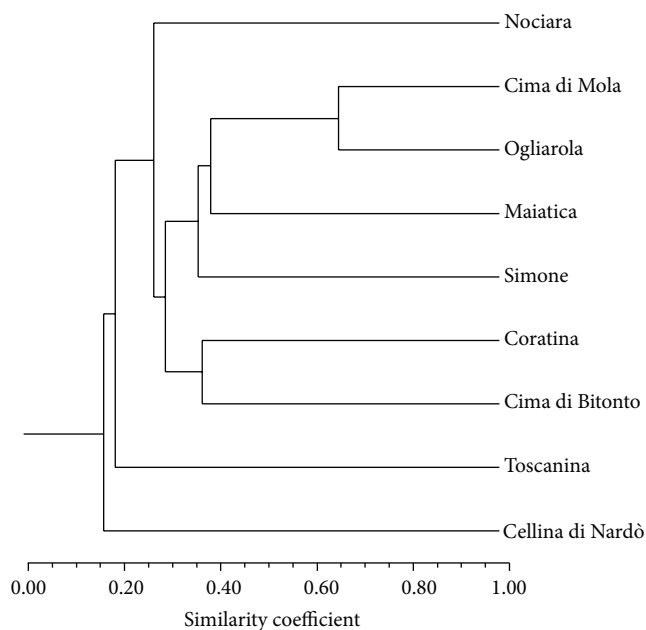


FIGURE 1: Genetic similarity dendrogram of nine olive cultivars obtained by the analysis of seventeen microsatellite markers.

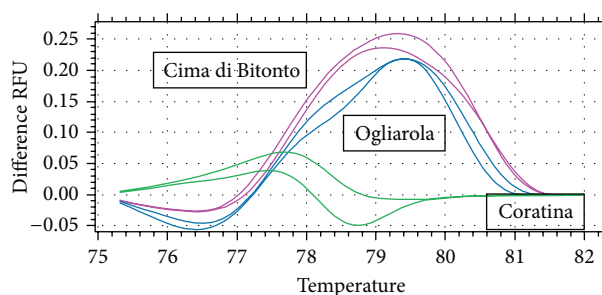


FIGURE 2: Normalized difference curve of the three cultivars Coratina, Cima di Bitonto, and Ogliarola obtained with the DCA18 marker.

di Bitonto that are very similar in the morphological traits. Ogliarola and Cima di Bitonto are commonly considered as synonymous referred to the same variety, and also the disciplinary of “Terra di Bari” olive oil production induces in an ambiguous appellation of them, not clarifying that are two different cultivars. The results of our analyses define Cima di Bitonto and Ogliarola as two distinct cultivars, as confirmed by capillary electrophoresis and HRM analysis (Figure 2).

Among the seventeen markers, the DCA18 was the most polymorphic SSR able to discriminate all samples, and for this reason it was chosen to realize an identification key (Figure 3). This marker revealed 10 different amplicons that are combined in 9 unique genotypes and for this reason was selected for the HRM application. In recent years different authors reported the use of HRM for the varietal identification [32, 33], genotyping [28, 34], and food traceability [35]. The advantages of this technique that was originally conceived for the human diagnostic [36], such as absence of manipulation after PCR, cost effectiveness, closed-tube

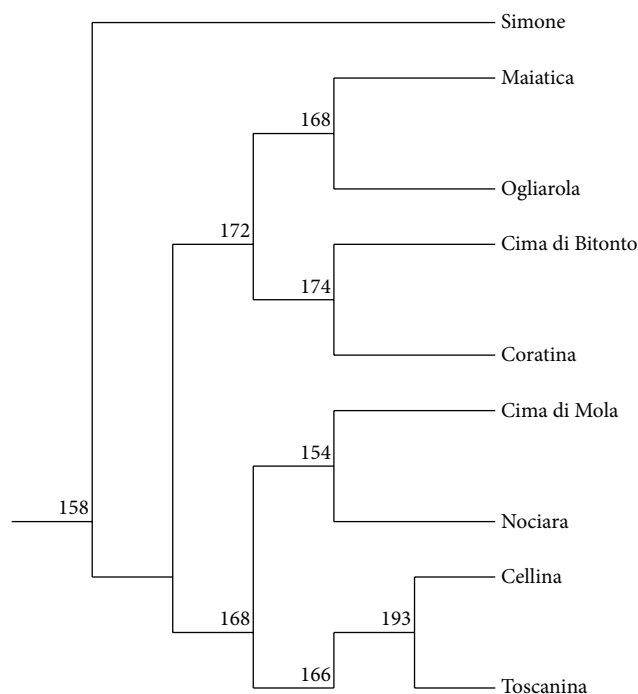


FIGURE 3: Identification key of nine olive cultivars obtained by the analysis of DCA18 microsatellite markers.

analysis, and results obtainable in less than 3 hours, are nowadays emerging in plant and food sector. All the HRM experiments were realized with the marker DCA18 and in Figure 4 is reported the difference curve of some Terra di Bari experimental samples compared to commercial Terra di Bari olive oil and one experimental olive oil made up of Nociara and Toscanina. The plot well established the difference among the Terra di Bari group (mixes 3, 6, 7, 9, 10, 11, 12) and non-Terra di Bari group (mix 13). The preparation of different mixtures (Table 2) is essential to constitute a dataset of melting curve profiles in order to perform a quick preliminary analysis and define the belonging of an unknown sample to the Terra di Bari group, specified and declared as well in the label. This analysis might be useful as first check of the varietal composition of olive oil, especially when it is necessary to work with large number of samples. The high efficiency of discrimination of the technique, already tested by Vietina et al. [37] and Ganopoulos et al. [38] in adulteration of olive oil by the addition of cheaper oils obtained from other plants (i.e., maize, sunflower, and soybean), is proved also in the varietal identification if a cultivar is required and specified by production disciplinary. A further application of HRM method is to quantify the presence of adulterants; in literature are reported several studies regarding the identification of adulterants (i.e., different species or botanical varieties) and their quantification [35, 38, 39]. Figure 5 showed the set-up of quantification of increasing addition of the Algerian cultivar Aeleh to a monovarietal Coratina olive oil. It is possible to appreciate the clear distinction between the monovarietal oil of Coratina and Aeleh, showing different melting temperature, and the placement of the different

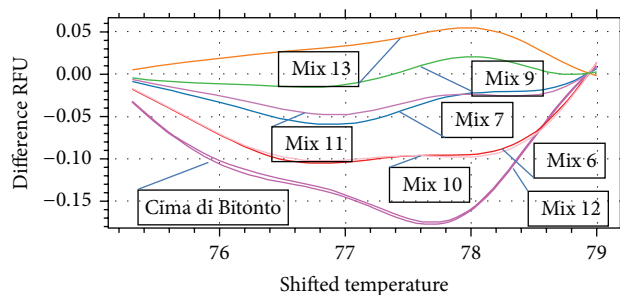


FIGURE 4: Normalized and temperature-shifted difference curve of different commercial samples (mixes 11 and 12), Terra di Bari oils (monovarietal Cima di Bitonto oil and mixes 6, 7, 9, 10), and non-Terra di Bari olive oil (mix 13).

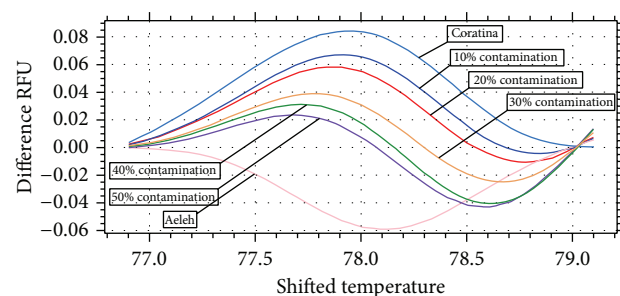


FIGURE 5: Temperature-shifted difference curve obtained with the addition of increasing quantity of cultivar Aeleh in Coratina monovarietal oil.

level of adulteration (10–20–30–40–50%) between them. The obtained results confirmed the possibility to use the HRM technique not only for a qualitative application but also for a quantitative detection of the addition of different amounts of olive oils produced by cultivars not allowed in the PDO disciplinary production.

4. Conclusion

This molecular analysis allowed the distinction of the cultivars included in the “Terra di Bari” PDO disciplinary with respect to those widely diffused in the Apulian region. In addition, SSR markers were able to provide a specific profile for Coratina, Ogliarola Barese, and Cima di Bitonto cultivars. The production rules of “Terra di Bari” olive oil consider the cultivar Cima di Bitonto and Ogliarola as two synonymous referred to the same genotypes. The allelic profiles obtained with capillary electrophoresis analysis clarify unambiguously that Cima di Bitonto and Ogliarola are two distinct varieties, and this aspect should be taken into account for an accurate review of the disciplinary.

These results should be useful in the agrofood compartment, where the application of molecular techniques could lead to the identification of raw materials and derived products allowing to trace origin and identity of cultivars used for the obtainment of typical products.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

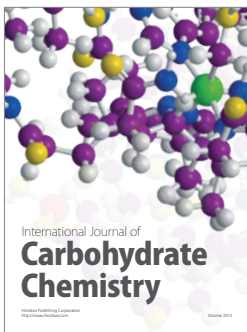
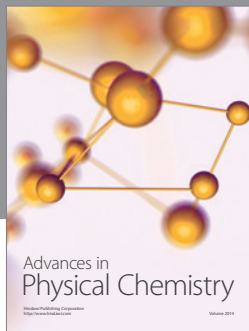
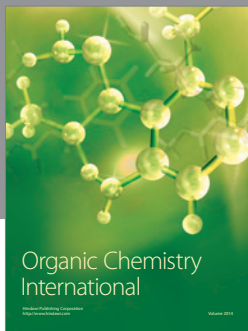
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