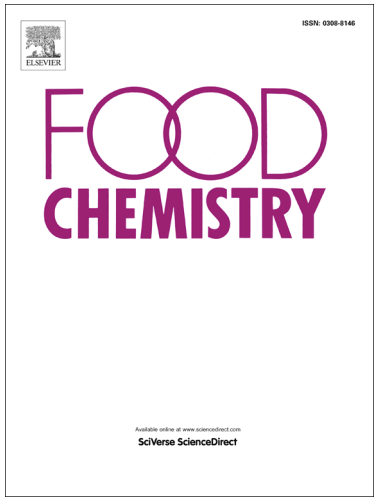

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Label free DNA-based Optical Biosensor as a potential system for Wine Authenticity

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ABSTRACT

The diversity found among the *Vitis vinifera* L. species allows the production of wines with very different characteristics. The development of platforms suitable for food composition analysis is currently an emerging area. Among these, DNA biosensors have been developed for a wide variety of applications, ranging from food safety to authenticity. The main aim of this work was to study the detection capacity of the DNA-based optical biosensor using different *V. vinifera* matrices (leaf, must and wine). Genomic DNA was extracted from leaf, must and wine of three *V. vinifera* varieties and was tested on the long-period grating (LPG) DNA-based biosensor developed within our group. The biosensor was able to distinguish the varieties even using DNA extracted from complex matrices, revealing its potential to be applied in wine authenticity.

Keywords: DNA-based biosensor; *Vitis vinifera* L.; authenticity; wine.

1. Introduction

The assessment of authenticity in food industry, particularly in added-value food products such as wine, has been a major concern that has challenged scientists to develop reliable and feasible technologies for such purpose (Arvanitoyannis, 2010). Wine quality is deeply influenced by the *Vitis vinifera* L. varieties used in winemaking, and therefore have a direct impact on wine's market price, particularly in referenced market segments such as Denomination of Origin (DO) wines. For that reason, some of these highly quoted wines are a preferential target for fraudulent practices (Pereira et al., 2012). The misleading addition of other grapevine varieties and/or replacement with other wines is often used as a mean to enhance the sensory characteristics of the final product and/or to decrease its production costs (Catalano, Moreno-Sanz, Lorenzi, & Grando, 2016). Since the incorrect labelling represents a commercial fraud, wine authenticity has become a subject of great concern (Fernandes et al., 2015). Therefore, the precise identification of grapevine varietal composition is one of the key points to combat fraudulent practices and to assure commercial fairness (Pereira et al., 2017).

There is currently a great range of combined techniques that can be used for wine authenticity assessment purposes (Arvanitoyannis, 2010). Nevertheless, DNA-based techniques have been applied in this field mainly due to their ability to correctly fingerprint the grapevine variety throughout the entire wine-chain, while being independent of the growing and production conditions (Fernandes et al., 2015). DNA is much more resistant to industrial processing than other molecules, such as RNA, proteins or secondary metabolites (Corrado, 2016). Thus, DNA-based detection methods are understandably valuable in the differentiation of grapevine varieties used in wine production (Işçi, Kalkan Yildirim, & Altindisli, 2014).

Regarding DNA-based techniques, the use of DNA markers allows the identification of variations in the nucleotide sequence of a genome, that can highlight inter and intra-species/varietal diversity, through their high information potential (Scarano & Rao, 2014). In *Vitis*, the identification of markers, such as single nucleotide polymorphisms (SNPs), for the development of molecular marker-based systems has recently boosted (Tomic, Stajner, & Javornik, 2013). On the other hand, the interest on SNPs as genetic marker for food authenticity has also been highlighted, due to several reasons: (1) their high abundancy in the genome; (2) their genetic stability and; (3) the possibility to be used in the amplification of small DNA fragments and the use of more sensitive techniques (Pereira et al., 2017; Pereira, Gomes, Barrias, Fernandes, & Martins-Lopes, 2018). Several DNA-based markers have been described to be suitable for grapevine varietal identification, such as the 9 Simple Sequence Repeats (SSRs) recommended by the International Organization for Vine and Wine (OIV, 2009) and the 48 Single Nucleotide Polymorphism (SNPs) set (Cabezas et al., 2011), which are based on alternative detection platforms. The set of SNPs detected within this grapevine varietal collection (Pereira et al., 2015; Gomes et al., 2018) constitute additional markers that have been applied to alternative detection platforms (Pereira et al., 2017; Gomes et al., 2018).

Generally, the DNA-based techniques used in combination with molecular marker systems are time consuming, expensive and require highly trained personnel, usually making them not adequate for a frequent/wide applied food monitoring system (Li, Yu, Li, & Wu, 2015).

The limitations of existing DNA-based detection methods have encouraged the development of new alternative technologies, especially to fulfill the current desirable features, such as low-cost equipment, portability, simplicity, rapid response and automation (Martín-Fernández, Manzanares-Palenzuela, Sánchez-Paniagua López, De-

los-Santos-Álvarez, & López-Ruiz, 2017). Thus, in the food industry, there is a great demand for alternative methods assuring objective and consistent measurement of food products, in a cost-effective manner. The development of biosensors in response to this is seemingly promising (Mehrotra, 2016).

A biosensor can be defined as an analytical device that combines a biological element with a physicochemical component generating a measurable signal that detects an analyte of biological importance (Patel, Nanda, Sahoo, & Mohapatra, 2016). This type of sensor employs biological molecules to recognize the target and uses output elements which can translate the biorecognition event into electrical, optical or mass-sensitive signal. As a sub-field of the biosensors, DNA-based biosensors use DNA strands as probes for sensing DNA targets (Chao, Zhu, Zhang, Wang, & Fan, 2016). These biosensors were developed based on the ability that single-strand nucleic acid molecules have to recognize and bind to their complementary strands in a sample (Mehrotra, 2016). Most recently, the development of new sensor systems based in optical fibers became a reality (Gonçalves et al., 2016; Moreira et al., 2016; Pospíšilová, Kuncová, & Trögl, 2015). These types of biosensors respond to alterations in the refractive index of the fiber's surrounding medium, generated by analyte binding. When the outer surface of the fiber is functionalized with specific biorecognition molecules, target analytes exposed to them will bind specifically to the modified surface, and these interactions will be detected (Sharma & Mutharasan, 2013).

The aim of this study was to develop quick and reliable assays using a long-period grating (LPG) DNA-based biosensor, suitable for *V. vinifera* varietal discrimination in leaf, must and wine gDNA samples based on SNP information.

2. Material and methods

2.1. Leaf, must and wine samples

Leaf, must and wine samples from the grapevine varieties Malvasia Fina (MF), Tinta Amarela (TA), and Tinta Roriz (TR) were collected. Grapevine leaf samples were obtained from certified vineyards (Sogrape Vinhos S.A. and Real Companhia Velha) and immediately frozen in liquid nitrogen. Monovarietal must and wine samples were produced at the National Institute for Agricultural and Veterinary Research (INIAV) in Dois Portos, Portugal, using freshly harvested grapes as described in Pereira et al. (2017). All samples were stored at -20ºC until DNA extraction was performed.

2.2. Genomic DNA extraction

Total genomic DNA was extracted from frozen young leaf samples using the described CTAB method (Doyle & Doyle, 1987). Must DNA extractions were performed using a modified CTAB protocol (Pereira et al., 2012). Wine genomic DNA extractions were performed according to the method described by Pereira, Guedes-Pinto, & Martins-Lopes (2011) (Figure 1). The DNA samples were eluted in 100 µL of ddH_2O and maintained at -20°C until use. The determination of the samples' purity and quantity were based on measurements performed using a Nanodrop™ 1000 Spectrophotometer. The concentration of all samples was adjusted to 0.25 µM.

2.3. DNA-based biosensor assays design

2.3.1. Probe and target design

The probes and targets used in this work were designed based on three of the SNPs detected within the *F3H* gene of *Vitis vinifera* L. (Gomes et al., 2018). The information regarding both oligonucleotides is presented in Table 1. Probe 35 was designed to target

the SNPs located at the positions 1,039 bp, 1,040 bp and 1,065 bp. A non-complementary target (NC Target) was designed with two mismatches with Probe 35, presenting one mismatch in each end of the sequence. A blastn (basic local alignment search tool nucleotide) using the NCBI genome database was performed to verify the specificity of the probe. Both oligonucleotides were acquired from Frilabo (www.frilabo.pt) and used at a concentration of 0.25 µM.

Besides these, genomic DNA from grapevine varieties Malvasia Fina, Tinta Amarela and Tinta Roriz were also tested (Table 2). Tinta Amarela DNA sequence is complementary to Probe 35; whereas Tinta Roriz DNA sequence differs from Tinta Amarela in one SNP targeted by Probe 35, at 1,065 bp position. Malvasia Fina DNA sequence is heterozygous for all the three SNP positions targeted by Probe 35.

2.3.2. Biosensor assays

Two different experiments were performed testing different DNA samples:

- Probe 35, NC Target, Malvasia Fina gDNA, Tinta Amarela gDNA;
- Probe 35, NC Target, Tinta Roriz gDNA, Tinta Amarela gDNA.

Each experiment was performed at least three times, in each type of sample (leaf, must and wine gDNA). During the experimental procedure, the LPG was maintained at constant strain and temperature (37 ºC). The optical data acquisition was performed using a fiber optic interrogation unit, the HBM FiberSensing (www.fibersensing.com), model BraggMeter FS2200SA. This sensing system (considering this specific optical detection scheme and this specific LPG) has a detection limit of 62±2 nM, a quantification limit of 209±7 nM and a sensitivity of 78±1 nM.RIU (Gonçalves et al., 2016).

The LPG surface was cleaned using a solution composed by ethanol 70% (v/v) and hydrochloric acid 1% (v/v) in a 1:1 ratio. In each assay, Poly-L-Lysine solution (PLL) was used as a bilinker and TE 1x buffer (Tris-HCl 100 mM, EDTA 1 mM pH = 8) was used after the PLL step, guaranteeing optimal pH conditions for PLL stabilization on the surface. In each cycle the following sequence was executed: water, PLL, TE 1x, Probe 35, NC Target (non-complementary) and gDNA samples. A water washing step was performed between the addition of each DNA sample, to assure that the differences observed were due to a chemical reaction and not just due to simple mass deposition effect. After each cycle, the LPG surface was cleaned using a diluted solution of nitric acid (HNO_3 1:3) to remove the different layers (PLL, Probe and complementary gDNA sample) attached to the fiber. All injected solution comprehended a 500 µL volume.

The LPG transmission spectra was recorded during 30 min with a 2-second interval in all the steps. Each spectrum was processed to determine the wavelength position of the LPG resonance. At constant temperature and strain, this wavelength is directly dependent of the effective refractive index of the observed cladding fiber mode, including the genetic material contribution that is adherent to the fiber's lateral surface. In these conditions, a change in the refractive index, reflects a chemical interaction between the biological materials on its surface. After data processing, the first 25 minutes of recordings were excluded (interval needed for each sample to establish thermal balance). The remaining data set was used to perform an arithmetic average and the resulting value was used as the measured resonance peak position for the protocol step. Then, the average measured resonance peak of each washing step performed after the gDNA samples was subtracted to the average value of the prior sample step and was used to calculate the wavelength shift obtained because of the chemical interactions between the sample and the probe. Finally, these values were compared to the average value obtained for the addition of the NC Target (reference value). Simultaneously, a

temperature sensor was positioned inside the reaction chamber near the LPG sensor and measurements were collected during all the experimental procedure to adjust the resonance wavelength, once it is directly influenced by temperature, using a correction algorithmic developed previously for the system (Gomes et al., 2018).

The values are given as mean ± SD as calculated from data from three independent experiences. All statistical treatments were performed regarding data from three replicates and compared to the NC Target values, through a T-student or one-way ANOVA test using the Statview software to test for significance ($p < 0.05$).

3. Results and Discussion

3.1. Genomic DNA extraction

All the genomic DNA samples were analyzed using the Nanodrop™ 1000 Spectrophotometer. The analyses were performed regarding the quantity and purity of the extracted DNA from leaf, must and wine samples of the grapevine varieties Malvasia Fina, Tinta Amarela and Tinta Roriz. The average results of the genomic DNA extraction with different starting materials are presented in Table 3.

The extraction was more efficient in leaf samples in comparison to must and wine samples. These results were in accordance with what was expected, as DNA extraction from must and wine samples is particularly difficult to perform (Işçi et al., 2014). This is mainly due to the high processing steps that theses samples are submitted to, considering maceration and the fermentation processes during vinification. These procedures have a direct effect on the DNA integrity and on the overall DNA recovery (Pereira et al., 2011; Pereira et al., 2018). However, our results show that DNA extraction was successfully accomplished.

Notoriously, a higher concentration was observed in Tinta Amarela wine samples in comparison to the must samples from this variety. Since the DNA obtained was only applied to the DNA-based biosensor and did not involve any amplification step with polymerase, the purity of the obtained DNA was not a primary concern. Although, the DNA extracted from leaf samples presented A_{260}/A_{280} and A_{260}/A_{230} ratios considered to be of good quality DNA samples, the same was not verified for the must and wine DNA samples, which presented ratios that revealed some sample contamination, that are likely to be with polyphenols for the red *Vitis vinifera* samples and of flavonoids and/or polysaccharides for the Malvasia Fina variety (white) (Pereira et al., 2012, 2011). However, these samples were applied in the assays and the biosensor was able to detect the DNA present in all of them, independently of the purity ratio.

3.2. Biosensor assays

The assays performed with the biosensor used the three sample types to test for its suitability to detect SNP events in differentiated matrices. Additionally, the hybridization behaviour process when using samples that differed in terms of SNP position and genotype were also investigated.

The results of both assays, performed in leaf, must and wine samples, are presented in Figures 2 and 3. In the first set of experiences (Figure 2) the gDNA samples differed in terms of the studied SNPs, being Malvasia Fina (MF) heterozygotic in the three positions in relation to the probe and Tinta Amarela (TA) being complementary. When leaf gDNA samples were tested (Figure 2.a), the wavelength shift generated after the TA sample was added was significantly higher than the shift observed when variety MF was added, indicating that the sensor was able to detect the complementary DNA present in variety TA.

When must gDNA samples (Figure 2.b) were used, the wavelength shift observed in sample TA was only slightly higher than the shift observed for the variety MF, however it was still statistical significant ($p < 0.05$). The shift observed between the variety MF and the probe was also statistically significant, suggesting the occurrence of hybridization with this variety (MF) and the tested probe. This can be explained by the fact that this variety is heterozygotic for the three targeted positions within the probe and, although the number of DNA sequences present in a heterozygotic variety is theoretically present in half the concentration, it is still enough to hybridize with the probe and to produce a hybridization signal. Nevertheless, with the must gDNA samples, detection of the complementary grapevine variety (TA) was still possible meaning that the probe was not fully saturated with MF DNA sample, as all the experiments were performed in a sequential mode.

As for the wine gDNA samples (Figure 2.c), the variety MF presented a similar behavior to the must gDNA samples, again suggesting some hybridization between the DNA present in this sample and the probe. Still, detection of the complementary DNA (TA) was achieved, since the wavelength shift obtained after the addition of the variety TA sample was significantly higher than the one verified with variety MF.

Generally, the results suggest that it is necessary to consider the heterozygote existence when designing probes, knowing that some hybridization may occur, and that the quantity of sample required for it to happen is low. Despite the occurrence of partial hybridization between the heterozygotic sample and the probe, the biosensor was able to detect the complementary sequence, in the TA DNA obtained from all the three types of sample matrices.

In the second set of experiences, the two grapevine varieties used only differed by a single SNP at the 1,069 bp position, result of a tranversion mutation (C/A; grapevine variety TA/TR, respectively). The remaining positions were equal in both grapevine

varieties. Leaf gDNA samples resulted in wavelength shifts that were not statistically significant for TA variety (Figure 3.a). As for the must gDNA samples, when TR variety was added there was a significant wavelength shift, suggesting the ocurrence of hybridization between this variety and the probe. Although TR variety is not complementary to the probe sequence, it only differs from the complementary variety in one basepair, suggesting or partial hybridization between the sample and the probe, capable of generating a signal detected by the biosensor, or a slight deposition of gDNA on the LPG surface, generating a slight signal shift.

Finally, the results from the wine gDNA samples (Figure 3.c) showed that the biosensor detected the DNA from the complementary TA variety, with a statistically significant wavelength shift occuring after the TA sample addition. In this type of gDNA sample, the shift obtained from the addition of the TR sample was not significant and was pratically inexistent, suggesting that no hybridization ocurred between this DNA sample and the probe.

Regarding the results when analysing the three sample types, it is visible that the biosensor was able to detect the complementary sequence, in the TA DNA samples obtained from must and wine matrices, nevertheless it was not as successful when leaf samples were used. This result was quite surprising, but if we consider that before each DNA sample insertion, into the biosensor chamber, a denaturation step is always performed to assure that DNA is available as single strand, so it can hybridize with the probe. The absence of additional wavelength shift in relation to the addition of the TA leaf DNA could be explained by the possibility of an unsuccessful denaturation process that failed to assure the total denaturation of the double-stranded DNA, hence it would not be available to hybridize with the probe.

This biosensor was able to detect DNA obtained from complex matrices, which are known to be challenging starting materials when performing DNA extraction protocols.

Although a lot of progress was made in this area (Bigliazzi, Scali, Paolucci, Cresti, & Vignani, 2012; Pereira et al., 2012, 2011) it is still sometimes difficult to obtain non-contaminated DNA in a good quantity from must and wine samples, as the product of the extraction is typically contaminated with several organic compounds naturally present in the samples, and also highly degraded after the processing that it is submitted during the winemaking process (Pereira, Gomes, & Martins-Lopes, 2016). This can represent an obstacle for the application of other sensitive DNA-based methodologies besides biosensors, especially the ones that resort to amplification reactions. Nevertheless, these can still be applied successfully for the detection of grapevine DNA extracted from different matrices (Fernandes et al., 2015; Pereira et al., 2012, 2017; Pereira & Martins-Lopes, 2015). The utilization of molecular marker systems like SSRs, aiming for the detection of *V. vinifera* DNA is still a current practice. However, the reproducibility of this methodology can be hampered when dealing with highly degraded DNA samples. To prevent this situation and achieve more reliable results, SSRs with lower allele size need to be applied (Martins-Lopes, Gomes, Pereira, & Guedes-Pinto, 2013; Pereira et al., 2018). Marker systems using SNPs may overcome this issue, due to their small size and simplicity. Many High Resolution Melting (HRM) assays were developed using SNPs detected in many varieties of *V. vinifera*, and were successfully applied in leaf, must and wine DNA samples. For instance, in Pereira et al. (2017), three different HRM assays were designed and applied in DNA from different matrices. These assays differed in fragment size analysed, with assays designed to target long fragments (i.e. 705 bp) being capable of distinguishing *V. vinifera* varieties in must DNA samples. The profile obtained in this assay was consistent with the one obtained in a previous study, where the same assay was applied to *V. vinifera* leaf DNA samples (Pereira & Martins-Lopes, 2015). The remaining two HRM assays, targeting smaller fragments (i.e. 375 and 119 bp), produced good results in the DNA extracted from leaf,

must and wine samples (Pereira et al., 2017). The success of these assays relies on three main factors: i) the utilization of an efficient DNA extraction protocol, ii) the use of smaller DNA fragments as targets (success was higher when 119 bp assay was used) and iii) the usage of various SNPs so the discrimination of several varieties can take place with only one assay.

In comparison, the biosensor used in this work was able to detect DNA from the three types of matrices, using a probe with a small size of 35 bp and targeting a total of three SNPs.

When discussing the advantages and disadvantages between the biosensor and other molecular-based approaches, it is important to consider that they are currently standing in different stages of development. While most of the molecular-based approaches are already well established, our biosensor is still being developed and improved. Comparing to the application of marker systems, such as Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphism (AFLPs) and SSRs, as well as to HRM and other PCR-based approaches, our biosensor presents some disadvantages. At the moment, the time required for temperature stabilization of the system hampers the quickness of the analysis. The protocol used is not a single-step, closed-tube method and it only detects a single genotype at a time for each specific probe, opposite to the HRM assays (Pereira et al., 2017).

Nevertheless, the main advantage of our biosensor in comparison to the other mentioned molecular approaches is the capability to successfully analyze samples with high level of degradation and contamination. In contrary to what occurs in the PCR-based methods, the inhibition of DNA polymerase and the inability for primers to attach to degraded DNA are not a problem in our biosensor, because it does not require any amplification steps. It also does not need specialized users, and the data obtained is simpler to analyze based on the algorithms developed for the purpose. Furthermore, the

biosensor does not require the use of fluorescent dyes or enzymes and the probe utilized can be reused, making this system much cheaper than other approaches (Gomes et al., 2018).

In terms of sensitivity, the biosensor developed by our group has previously shown to be comparable to other molecular-based approaches, revealing to be highly sensitive and specific, with the ability to detect differences of a single nucleotide (Gonçalves et al., 2016; Moreira et al., 2016). To summarize, this method represents a much simpler and cheaper alternative for the detection of specific DNA sequences, dealing with degraded and contaminated DNA without the need to use any amplification steps. Thus, it can be applied in a broader usage targeting defined *V. vinifera* varieties.

There is a current trend to apply biosensors in the food processing field (Thakur & Ragavan, 2013). Thus, other DNA-based biosensors have been developed for many applications as for the detection of food banned compounds (Ensafi, Jamei, Heydari-Bafrooei, & Rezaei, 2014), the detection of allergens (Sun et al., 2015), multiple DNA components in GMO products (Huang et al., 2015) and the detection of pathogenic bacteria (Abdalhai et al., 2015; Amouzadeh Tabrizi & Shamsipur, 2015). Most of these biosensors use some sort of labeling and various techniques to detect the target analyte. The need for labeled compounds usually implicates higher costs and longer assay times, compared with most label-free methods. Furthermore, the labels can interfere with the analyte binding and lead to distorted results (Rapp, Gruhl, & Länge, 2010). To our knowledge, the DNA-based biosensor used in this work is the first capable of detecting DNA extracted from *V. vinifera* complex matrices, in a straightforward and label-free manner being the first showing potential to be applied for wine authenticity purposes.

4. Conclusion

The LPG DNA-based biosensor proved to be able to distinguish specific grapevine varieties, even in the presence of heterozygotic material, with the occurrence of partial hybridization, using gDNA extracted from leaf, must and wine samples. This reveals the potential of this label-free and PCR-less technology to be applied to grapevine varietal fingerprint throughout the wine-chain, analyzing DNA with different levels of contamination, from various matrices subjected to different processing levels.

Compliance with ethics requirements

This study does not contain any studies with human or animal subjects.

Conflict of interest

None.

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List of Tables:

Table 1
Nucleotide sequence and size regarding Probe 35 and NC Target.

Code	*Nucleotide sequence (5'→ 3')*	*Size (bp)*
Probe 35	GCG A**AA** GGC TGA AGC TAA TCT TTT CTT TGT **C**TT TG	35
NC Target	CA AA<u>T</u> ACA AAG AAA AGA TTA GCT TCA GCC TT<u>A</u> CGC	35

Note: In **bold** are presented the SNP positions targeted by Probe 35, while the mismatches between NC Target and Probe 35 are presented <u>underlined</u>.

Table 2
The three SNPs targeted by Probe 35, identified in *F3H* gene across the three grapevine varieties used in this work (adapted from Gomes et al., 2018).

Grapevine variety	Nucleotide position (bp)		
	1,039	1,040	1,065
Tinta Amarela	A	A	C
Tinta Roriz	A	A	A
Malvasia Fina	W	W	M

Nucleotide code: A – adenine; C – cytosine; T – thymine; M – A or C; W – A or T.

Table 3
Average values obtained from the Nanodrop reads relatively to the three grapevine varieties used in this work, in several sample types (leaf, must and wine) considering DNA concentration (ng/µL), absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}) and yield (ng).

Sample type	Grapevine variety	Concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}	Yield (ng)
Leaf	Malvasia Fina	650.26	1.90	1.85	65,026 a)
	Tinta Amarela	551.89	1.87	1.58	55,189
	Tinta Roriz	452.82	1.80	1.68	45,282
Must	Malvasia Fina	199.05	1.51	0.83	19,905 b)
	Tinta Amarela	61.44	1.25	0.39	6,144
	Tinta Roriz	112.88	1.36	0.42	11,288
Wine	Malvasia Fina	49.81	1.33	0.70	4,981 c)
	Tinta Amarela	155.77	1.33	0.86	15,577
	Tinta Roriz	115.16	1.32	0.59	11,516

a) 100 mg of leaf; b) 2 mL of must; c) 10 mL of monovarietal wine.

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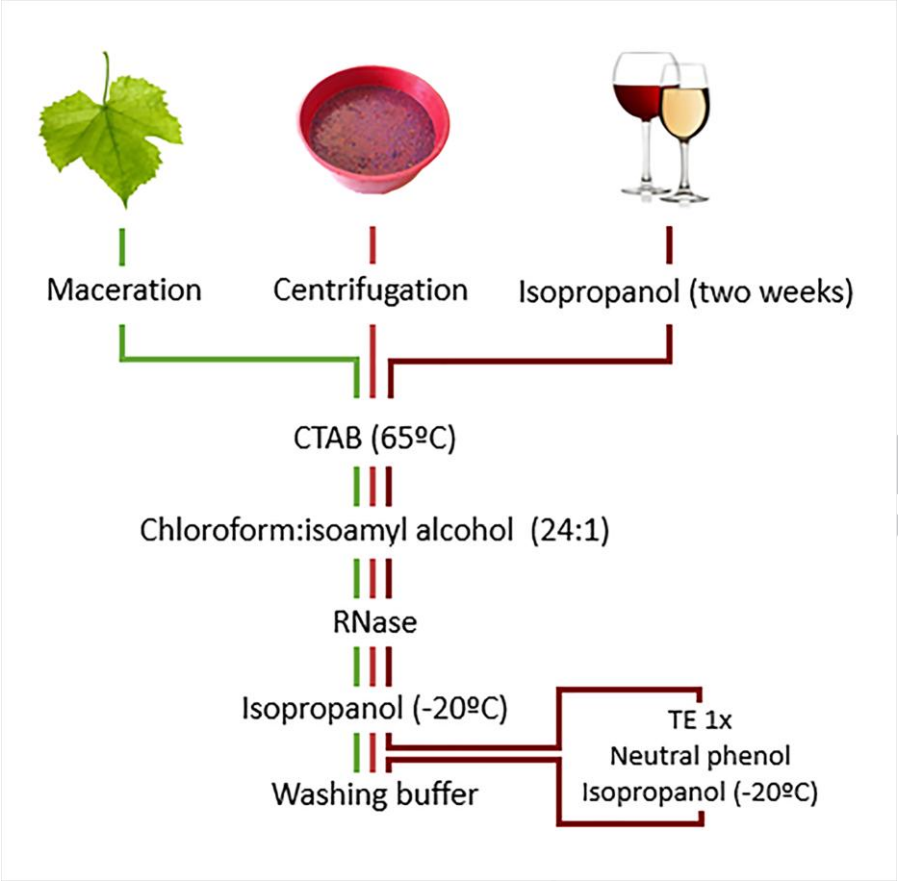

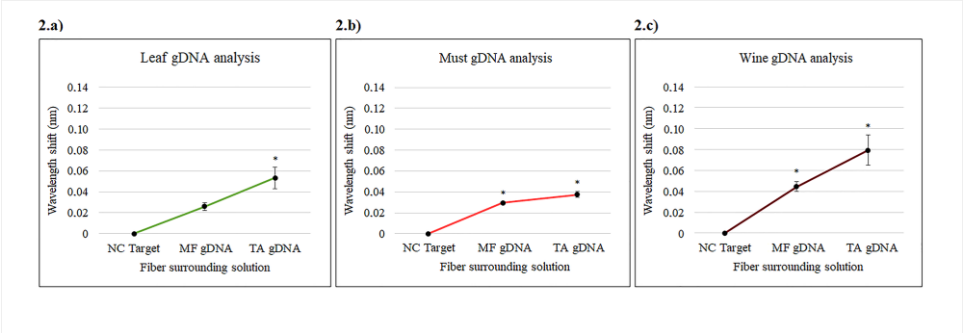

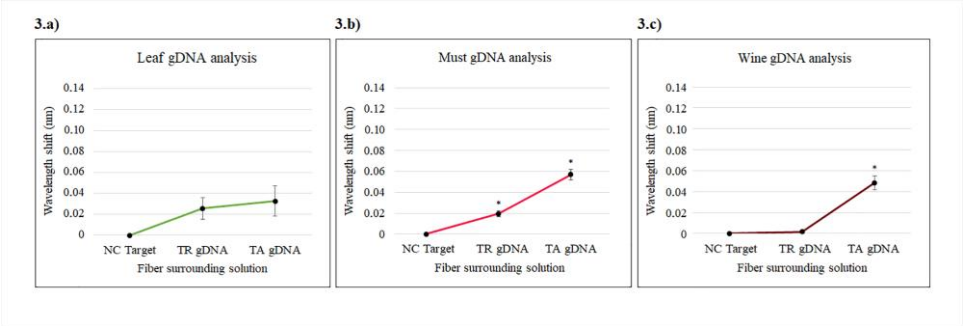

Highlights

- Label-free DNA-based biosensor applied to authenticity
- Varietal identification in different matrices (leaf, must and wine samples)
- PCR-free detection