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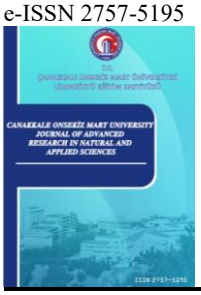

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Detection and Validation of A2 Milk Suitable for Consumers Having Milk Intolerance by ELISA Method

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Research Article

Abstract − Casein proteins, which make up 80% of the total proteins in cow's milk, consist of mainly A1 and A2 genetic types which differ by a mutation that causes conversion from proline to histidine. Histidine-containing A1 protein undergoes proteolytic degradation in the gastrointestinal system, while this is not observed during the digestion of A2 protein. A1 milk consumption causes bloating, gas, discomfort and symptoms confused with lactose intolerance. Studies showed that A1 milk consumption may cause diabetes, coronary heart disease, arteriosclerosis, sudden infant death, and is associated with autism and schizophrenia. With an increasing trend in the world, A2A2 milk (milk without A1 protein) production is becoming widespread with consumer preferences; and, A2 milk takes its place on the market shelves. With the onset of this trend, the need for a new analysis on food safety became evident. It will be required by food control laboratories to test the absence of A1 protein in milk to be labeled as A2 milk. In this study, the quantitative analysis and validation of β-casein A1 and A2 proteins in cow's milk by Enzyme-Linked ImmunoSorbent Analysis (ELISA) method was investigated. The methods have detection limits of 1.8 and 0.8 ppm, and quantitation limits of 17 and 2.4 ppm for A1 and A2, respectively.

Keywords *− β-casein A2 protein, cow milk, ELISA, food safety, method validation*

1. Introduction

Casein proteins (α_{S1}-, α_{S2}-, β- and κ-casein) constitute approximately 80% of the total proteins in cow's milk (Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008); and, β-caseins constitute approximately 30% of these proteins. There are two main genetic types of β-caseins as A1 and A2. The distinguishing feature between these two forms of β-caseins is due to a mutation at position 67 of this 209 amino acid long protein. With this point mutation, A2 was thought to have undergone a point mutation that converts proline (Pro^{67}) to a histidine (His^{67}) in A1 in ancestors from European-type cows such as Holstein-Friesian, Ayrshire and Red (Kamiński, Cieslińska, & Kostyra, 2007). Therefore, although cow's milk produced in many countries is a mixture of A1 and A2 β-casein variants, the His^{67} mutation is not found in purebred Asian and African cows (Kamiński et al., 2007). Similarly, a histidine mutation at the equivalent position has not been found in other mammals, including humans, or a similar mutation has been observed very rarely (De Noni et al., 2009; Pal, Woodford, Kukuljan, & Ho, 2015).

Goat, sheep, camel, buffalo, yak and donkey milk consists of only A2 type milk, while most of the Holstein, Friesian, Ayshire and British Storthorn cows produce either A1 or A1A2 milk, which are considered to be produced with higher yield (Boro, Naha, Saikia, & Prakash, 2016). Because of this feature, cow breeds with β-casein A1 mutation are thought to become widespread due to commercial concerns. Consumption of A1 milk creates a feeling of bloating, gas and discomfort. There are also studies showing that problems caused by A1 milk consumption are confused with lactose intolerance (Suchy et al., 2010). Studies have shown that A1 milk can cause type 1 diabetes, coronary heart disease, arteriosclerosis and sudden infant death (Sodhi,

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Mukesh, Kataria, Mishra, & Joshii, 2012). In addition, A1 milk consumption is associated with diseases such as autism and schizophrenia (Woodford, 2006). All these studies bring along the increasing consumer demand for anti-A1 dairy. Many doctors recommend that consumers, especially those with celiac disease and stomach ulcers, and infants under one year old, should strictly avoid A1 milk.

A1 protein with His^{67} mutation is quickly affected by proteolytic degradation, while A2 with Pro^{67} is resistant. Therefore, A1 proteins cause the release of short β-casomorphin (BCM) opioid peptides during gastrointestinal digestion. Opioid is used to describe chemicals that have morphine-like effects in the body (Kamiński et al., 2007). Studies have shown that BCMs are released from β-casein A1 protein during gastrointestinal digestion, while BCM release after A2 protein digestion is minor (Boutrou et al., 2013; Kamiński et al., 2007). It has been proven that A1 slows down the opioid-induced gastrointestinal transit. Longer gastrointestinal transit times are thought to lead to increased effects of lactose fermentation and other dietary components such as FODMAPs, which, together with genetic predisposition, is hypothesized to be consistent with clinical and sub-clinical outcomes including digestive distress and pro-inflammatory effects (De Noni et al., 2009).

In recent years, A1-free (A2A2) cow's milk has been among the commercially sold milks in many countries such as Australia, England, the United States, New Zealand and the Netherlands, and these milks are advertised as suitable for the use of consumers with milk intolerance (Brooke-Taylor, Dwyer, Woodford, & Kost, 2017). Recently, Vietnam's largest dairy company, Vinamilk, has started A2A2 milk production by importing a type of cow that produces A1-free milk from New Zealand (Hang, 2018). In addition, baby foods produced with β-casein that do not contain A1 are sold in China and Australia, and there have been advertisements indicating that these foods are light and suitable for the baby's digestive system (Brooke-Taylor et al., 2017). A2 milk has been preferred among consumers at an increasing rate all over the world, and it is rapidly becoming widespread. Industry and governments have to regulate the food policies and make food safety and control laboratories perform food analysis that aims to characterize food products in terms of chemical composition, traceability, safety, quality, sensory perception and nutritional value (Jain & Gupta, 2005; Nestle, 2002; Silver & Bassett, 2008; Kudlejova & Risticevic, 2012; Tang, Vasas, Hatzakis, & Spyros, 2019). Therefore, the prevalence of A2 milk brings an obligation of a new test to be made by food safety and control laboratories in order to control the content of cow's milk so that the consumers are not misled. The products marketed as A2 dairy are required to be tested for the presence of A2 β-casein protein and non-presence of A1 β-casein protein by food control laboratories.

There are various methods in the literature that allow the detection of A1 and A2 β-casein proteins in cow's milk. Capillary zone electrophoresis (De Noni, 2008), reverse-phase high-performance liquid chromatography (RP-HPLC) (Bonfatti et al., 2008), isoelectric focusing electrophoresis (Anna, Salvatore, Omar, & Eugenio, 2016), and urea-polyacrylamide gel electrophoresis (Duarte-Vázquez et al., 2018) are the main methods used to distinguish β-casein variants. More powerful methods such as liquid chromatography coupled mass spectrometry (LC-MS/MS) (Asledottir et al., 2017) or high resolution mass spectrometry (LC-HRMS) (Givens, Aikman, Gibson, & Brown, 2013) have also been successfully applied for the determination of protein types in milk and quantitative studies.

In this study, it was aimed to introduce and validate a new method to be used by food safety and control laboratories for the traceability and authenticity of A2 milk products. For the purpose of quantitative detection of β-casein A1 and A2 proteins in cow's milk, ELISA methodology was presented. Considering the method application by prevalent food control laboratories, ELISA is not only an easier assay to perform compared to aforementioned methods, but also accurate, sensitive and widely applicable. The applied method for quantitative detection of β-casein A1 and A2 proteins by ELISA had a limit of detection of 1.8 ppm and 0.8 ppm, and limit of the quantitation of 17 ppm and 2.4 ppm for A1 and A2 proteins, respectively.

2. Materials and Methods

2.1. Chemicals and Devices

The milk of genotypically tested cows of A1A1, A1A2 and A2A2 were obtained from Uluova Dairy Company (Çanakkale). "A1 ELISA Kit and A2 ELISA Kit" (GeneTel Laboratories LLC, USA) was used for the detection of A1 and A2 proteins by ELISA method. Kits contain ELISA microtiter strips coated with rabbit

anti-β-casein polyclonal antibodies (pAbs), A1 β-casein or A2 β-casein protein standard solutions, chicken anti-A1 or anti-A2 β-casein specific pAbs, rabbit anti-chicken IgY HRP conjugate, BSA for antibody dilution buffer, 10X Tris buffered saline (TBS) buffer, 50% solution of Tween-20 (Polyoxyethylene-Sorbitan Monolaurate) and 3, 3', 5, 5'-tetramethylbenzidine substrate (TMBS). Microcentrifuge tubes (Isolab, Turkey) and 50 mL sterile tubes (Falcon-Corning, USA) are also used for this analysis. The devices used in the analysis are micropipettes (Mettler Toledo Rainin Piper-Lite XLS, USA), vortex mixer (VWR International, USA), plate shaker/mixer (Bioer, China) and ELISA reader (Thermo Scientific Varioskan ® Flash, USA).

2.2. Sample Preparation

Solutions were prepared according to the manufacturer's instructions (GeneTel Laboratories LLC, A1 & A2 ELISA Kit Protocols). For the preparation of seven A1-containing standard solutions, seven 1.5 mL tubes are used. 10 µL of 40 µg/mL A1 standard and 990 µL of antibody dilution buffer are added to the first tube to obtain 400 ng/mL A1 standard. 2X serial dilutions are made with antibody dilution buffer for the other standards, resulting in standard solution concentration of 400 ng/mL in the 1st tube, 200 ng/mL in the 2nd tube, 100 ng/mL in the 3rd tube, 50 ng/mL in the 4th tube, 25 ng/mL in the 5th tube, 12.5 ng/mL in the 6th tube and 6.25 ng/mL in the 7th tube.

Milk samples are prepared by diluting in the antibody dilution buffer. In general, casein proteins are not soluble at neutral pH and need a raise in pH. Therefore, in order to obtain soluble casein proteins, the first dilution is made with 0.5 M NaOH at a ratio of 1:100 (10 µL milk + 990 µL NaOH). Subsequent dilutions are made with the antibody dilution buffer. A1A1 or A2A2 milk should be diluted in the range of 1:10.000 – 1:100.000. A1/A2 milk should be diluted in the range of 1:10 – 1:1000. In a regular assay, for the samples which are expected to be negative for A1 β-casein or A2 β-casein, 1:100 dilution is used. However, since NaOH prevents the reaction, it is sufficient to dilute these milk samples only with the antibody dilution buffer.

2.3. ELISA

The protective tape on the microtiter strips is removed and the wells are washed 3 times with TBST. 100 µL of standard solutions and diluted milk samples are added to the wells. The plate is mixed on a plate shaker at 400 rpm for 2 hours at room temperature. The wells are washed 3 times with TBST. A1 and A2 specific chicken IgY pAbs are prepared at 1:2000 and 1:300 dilutions, respectively in antibody dilution buffer, and 100 µL of dilutions are added to each well and incubated while shaking for 2 hours at room temperature on a plate shaker. The wells are washed 3 times with TBST. Rabbit anti-chicken IgY HRP conjugate is prepared at a 1:1000 dilution in antibody dilution buffer and 100 µL is added to each well and incubated while shaking for 1 hour at room temperature on a plate shaker. The wells are washed 5 times with TBST. 50 µL of substrate (TMBS) is added to each well and incubated for 4-8 minutes for a color change (it is recommended to wait 8 minutes after the solution is added to the first well). The reaction is terminated with 50 µL of 1 M HCl. An absorbance reading is taken at 450 nm immediately.

2.4. Evaluation of ELISA Results

For absorbance readings at 450 nm, Thermo Scientific Varioskan ® Flash device was used. For this purpose, the SkanIt RE for Varioskan Flash 2.4.3 software is used for the evaluation of the results. After defining the plate with respect to the positions of the standards and samples, as well as the concentrations of standards and dilutions of the samples, absorbance readings are taken at 450 nm. After the reading, the "Result" tab in the upper list is selected, and then, "Photometric 1" tab in the lower list is selected. From the calculation tools on the left side, "Quantitative CurveFit3" is clicked. On the page that opens, under the "Parameters" tab, as the "Fit Type", "Quadratic Polynomial" and as the "Transformation", "Conc. Logarithmic" and "Meas. Logarithmic" is selected. The software automatically lists the concentrations of the samples and draws the standard graphs. The analysis must be repeated, if any of the "blank" samples or the A2A2 milk sample in the A1 assay or the A1A1 milk sample in the A2 assay is calculated to be higher than the detection limit. Similarly, if the standard graph differs from the graph provided in the kit validation and quality assurance certificate, the analysis should be repeated. In addition, if the amount of protein calculated in the samples does not fall within the range determined in the standard graph, the analysis should be repeated by changing the dilution factor applied to the samples.

2.5. Method Validation

For the validation of ELISA, a) dynamic range, b) limit of detection (LOD), c) limit of quantification (LOQ), d) repeatability and e) measurement uncertainty parameters were considered (National Institute of Standards and Technology, 1995).

a) In order to determine the dynamic range of the methods, 8 different standard concentrations were prepared by using A1 and A2 standards included in the kit content. The prepared standards were analyzed on 3 different days, re-prepared each time, and the dynamic range was determined.
b) LOD was calculated for A1 assay by using milk with no β-casein A1, and for A2 assay by using milk with no β-casein A2. LOD was calculated as the mean of the sample + 3*(standard deviation).
c) LOQ was calculated as the mean of the sample without β-casein A2 (or A1) + 10*(standard deviation).
d) Repeatability study was performed by repeating the assay on 3 different days with standard samples. Within-assay repeatability was calculated by using standard curve plots and intra-assay repeatability was calculated by studies performed on two different days using A1A1 and A2A2 milk samples.
e) Uncertainty of measurement was calculated by combining the uncertainty from automated pipettes, ELISA reader and repeatability. For each measurement, relative standard deviation (RSD) is calculated by dividing the standard deviation to the mean value. The combined standard deviation ($RSD_{combined}$) was calculated by taking the square root of the sum of the squares of all RSD values.

3. Results and Discussion

Method validation is an essential part of the process that regulates the introduction of new products into the market (ISO/CASCO, 2018). When implementing the new method, the laboratory has to verify and validate that the method can be used for its intended purpose (FDA ORA, 2020). In this context, a study for method validation was conducted to detect β-casein A1 and A2 proteins in cow's milk by ELISA method. Within the scope of this method validation, a) dynamic range, b) limit of detection (LOD), c) limit of quantification (LOQ), d) repeatability and e) measurement uncertainty parameters were considered (National Institute of Standards and Technology, 1995).

In order to define the highest and lowest measurable concentrations, dynamic ranges of the ELISA methods were calculated. The dynamic range of the methods were determined (FDA ORA, 2020) by using 8 different concentrations of A1 and A2 standards included in the kit content (GeneTel Laboratories LLC, A1 & A2 ELISA Kit Protocols). The method provided a non-linear correlation in the form of quadratic polynomial between the measurement and the amount of the target (Plikaytis et al., 1994). The quadratic polynomical standard graph of the assays were plotted by using the concentration of standards (ppm) against the absorbance values obtained. The results are given in Figure 1. The equations of the standard graphs are given above the plots. The standard graphs were used to calculate the β-casein protein concentrations of the non-standard milk samples.

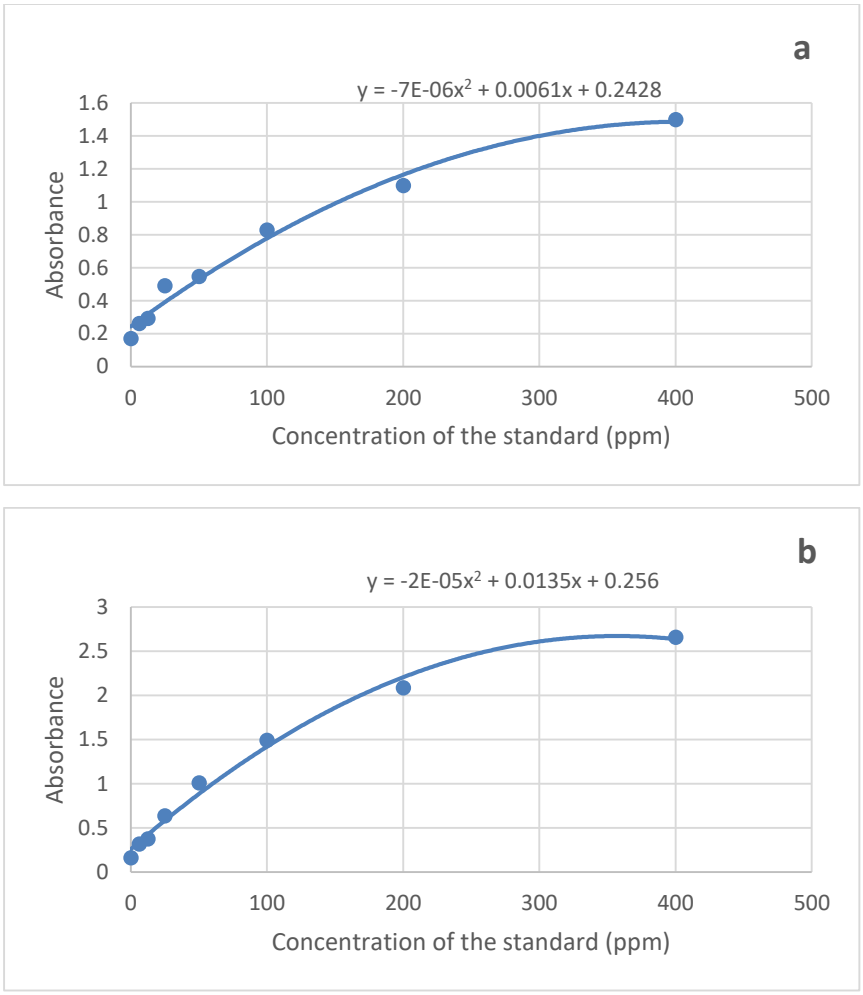

Figure 1. Standard graphs obtained for the ELISA methods of a) β-casein A1 and b) β-casein A2 for the dynamic range determination.

LOD was calculated based on the standard deviation of the blank sample which is the lowest concentration distinguishable (FDA ORA, 2020). LOD was calculated as the mean of the sample + 3*(standard deviation). This calculation indicates the presence of the analyte, but does not allow for quantitative measurement. In order to show that there was no cross-reaction, as the sample without β-casein A1, A2A2 milk and as the sample without β-casein A2, A1A1 milk was used in 10 independent studies, and the mean and standard deviation of the measurements were calculated. The results are given in Table 1.

From the mean absorbance values measured, the LOD of the A1 test was calculated as 0.147 + 3*0.0172 = 0.198, the LOD of the A2 test was calculated as 0.177 + 3*0.024 = 0.250. By using the equations of the standard graphs, the corresponding concentration for an absorbance of 0.198 is 1.8 ppm, and an 0.250 is 0.8 ppm. Therefore, the detection limits of the methods were determined as 1.8 ppm for the A1 assay and 0.8 ppm for the A2 assay.

Table 1
Determination of LOD

Sample no	Absorbances for A2A2 milk samples	Absorbances for A1A1 milk samples
1	0.123	0.152
2	0.138	0.159
3	0.158	0.169
4	0.172	0.155
5	0.148	0.148
6	0.162	0.196
7	0.124	0.207
8	0.153	0.215
9	0.130	0.194
10	0.160	0.173
mean	0.147	0.177
Standard deviation	0.0172	0.024

LOQ is the lowest concentration at which the analytical method operates with acceptable precision and repeatability (FDA ORA, 2020). According to the calculation made using the results shown in Table 1, the detection limit for the A1 assay was 0.147 + 10*0.0172 = 0.318, and the detection limit for the A2 assay was 0.177 + 10*0.024 = 0.421. From the equation of the standard graph, an absorbance of 0.318 corresponds to 2.3 ppm, and an absorbance of 0.421 corresponds to 4.0 ppm. Therefore, the LOQ of the methods were determined as 2.3 ppm for the A1 assay and 4.0 ppm for the A2 assay.

Due to the fact that the standard graphs of β-casein A1 and A2 ELISA tests are created with a quadratic rather than a linear slope, the absorbance values of standards with lower-concentration are very close to each other and may cause calculation errors (Plikaytis et al., 1994). For this reason, it was anticipated that inconveniences may be encountered in verifying the LOQ as calculated above; and therefore, instead of applying a routinely used LOQ calculation method, 4 different approaches were tested and verified for LOQ determination. The approaches used for LOQ determination were (Taverniers, De Loose, Van Bockstaele, 2004):

a. Mean + 10*(standard deviation)
b. 3*LOD
c. 10*LOD
d. 1.5* the lowest calibration standard (150% of the target level for the analyte)

For each LOQ determination approach above, the calculated LOQ values for the A1 test were:

a. 2.3 ppm
b. 5.4 ppm
c. 18 ppm
d. 9.4 ppm

for the A2 test were:

a. 4.0 ppm
b. 2.4 ppm
c. 8 ppm
d. 9.4 ppm

The prepared four different LOQ samples were attempted to be detected with 4 parallel studies by A1 and A2 assays, and among the detectable LOQ values, the smallest value was accepted as the LOQ of the assay (Taverniers, De Loose, Van Bockstaele, 2004). The results are as shown in Table 2.

Among the different LOQ calculation approaches, 10*LOD (18 ppm) and 3*LOD (2.4 ppm) approaches for A1 and for A2 test, respectively were verified as the smallest values. The verification with 4 parallel studies was repeated by performing 10 parallel studies for the selected approach, and the verifiable LOQ value was found to be 17.10 ppm for the A1 test and 2.4 ppm for the A2 test. Therefore, the LOQ value of the methods were accepted as 17 ppm for the A1 test and 2.4 ppm for the A2 test.

Table 2

Results of LOQ verification with 4 different approaches. Each sample was run in 4 parallels. The concentrations were calculated from absorbance values by using the standard graph equation. The concentrations that cannot be calculated from the equation as a positive value are indicated as "< 6.25".

	A1 test			A2 test		
LOQ calculation method	Expected concentration	Observed absorbance (mean)	Observed concentration	Expected concentration	Observed Absorbance (mean)	Observed concentration
Mean + 10*SD	2.3 ppm	0.224	14.01 ppm	4.0 ppm	0.244	4.46 ppm
3*LOD	5.4 ppm	0.211	< 6.25ppm	2.4 ppm	0.193	2.38 ppm
10*LOD	18 ppm	0.229	17.01 ppm	8 ppm	0.276	6.20 ppm
1,5* the lowest calibration standard	9.4 ppm	0.216	< 6.25ppm	9.4 ppm	0.336	10.32 ppm

LOQ: Limit of Quantification

LOD: Limit of Detection

Repeatability of the assay defines the precision under the same operating conditions over a short period of time. The precision is described by statistical methods such as coefficient of variation or the confidence limits; and, it expresses within-laboratory variations, such as different days, different analysts, and different equipment (FDA ORA, 2020). In this study, for the calculation of repeatability parameter, 8 different concentration of standard samples were tested and the assay was repeated on 3 different days. The average of the coefficients of variation calculated for each standard value, given as percentage, was taken, and the mean variation value for the A1 test was calculated as 6.1%, and the variation value for the A2 test was calculated as 6.9%. Therefore, within-assay repeatability values calculated using standard curve plots were 6.1% for the A1 test and 6.9% for the A2 test. Intra-assay repeatability value was also calculated by studies performed on two different days using A1A1 and A2A2 milk samples. The results were given in Table 3.

The average of the coefficients of variation calculated for each milk sample, given as %, was taken, and the average variation values for the A1 and A2 tests were calculated as 10.3% and 2.9%, respectively. Therefore,

within-assay repeatability values calculated using milk samples were found to be 10.3% for the A1 test and 2.9% for the A2 test.

Table 3
Intra-assay reproducibility of β-casein A1 and A2 protein analyzes by ELISA method with milk samples. A1A1 milk samples were studied with A1 ELISA test, A2A2 milk samples were studied with A2 ELISA test.

	Concentration (mean, mg/mL)	Standard deviation	Variation (%)
A1A1 milk sample -1	65.5	3.1	4.8
A1A1 milk sample -2	47.2	7.5	15.9
A2A2 milk sample -1	1348.75	33.85	2.5
A2A2 milk sample -2	895.73	28.66	3.2

The objective of the validation of an analytical method is to ensure that every subsequent measurement made during routine analysis will be accurate enough to generate the desired robustness that the unknown true value can be approximated with minimum error for the sample's contents (Biswas & Saha, 2015; Ellison & Williams, 2007; EURACHEM/CITAC, 2001; ISO, 1995). This approximation is determined by the confidence level of the assays, which is calculated as uncertainty of measurement (FDA ORA, 2020). In order to calculate the uncertainty of measurement, ELISA method was primarily divided into two steps as "antigen-antibody reaction" and "HRP-substrate reaction" (Biswas & Saha, 2015) since both steps are time sensitive and small changes in volumes at μL levels thought to affect the result of the assay. For each step, the total uncertainty was calculated, consisting of the uncertainty from the automated pipettes (Table 4); and the combined RSD was calculated by combining these numbers with the uncertainty from the ELISA reader, and the uncertainty from repeatability. For each part, RSD was calculated separately, and $RSD_{combined}$ was calculated by taking the square root of the sum of the squares of all RSD values (Biswas & Saha, 2015).

For the calculation of uncertainty coming from automated pipettes (Blues, Bayliss & Buckley, 2004), the pipettes that were used during the ELISA protocol were taken into account. Therefore, the standard uncertainties given in the calibration certificate of the 10 μL, 100 μL and 1000 μL measuring automated pipettes were checked and noted (Table 4). Relative standard uncertainties were calculated by dividing standard uncertainty over the volume measured by the automated pipette. Then, RSDs for both "antigen-antibody reaction" and "HRP-substrate reaction" steps were calculated by taking the square root of the sum of the squares of relative standard uncertainties for each pipette used (equations 3.1 and 3.2)

Table 4
Uncertainty of measurement from automated pipettes.

Automated pipettes	Standard uncertainty (from the certificate)	Relative standard uncertainty
10 µL	0.03	0.0030
100 µL	2.78	0.0278
1000 µL	2.78	0.0028

$$RSD_{(antigen\text{-}antibody\ reaction)} = \sqrt{(0.003)^2 + (0.0278)^2 + (0.0278)^2} = 0.028 \quad (3.1)$$

$$RSD_{(HRP\text{-}substrate\ reaction)} = \sqrt{(0.003)^2 + (0.0278)^2 + (0.0278)^2} = 0.028 \quad (3.2)$$

For the uncertainty calculation from the ELISA reader, as manufacturer's declaration, linearity was 0.02, repeatability was 0.05. Therefore, the uncertainty from the device during absorbance reading was calculated as the square root of the sum of the squares of each variable over the number of variables (equation 3.3)

$$RSD_{(device)} = \frac{\sqrt{(0.02)^2+(0.05)^2}}{\sqrt{2}} = 0.03808 \quad (3.3)$$

After the calculation of the uncertainty coming from "antigen-antibody reaction" step, "HRP-substrate reaction" step and the "device", RSD for routine analytical uncertainty was calculated by only taking one-time performance of the assay into account (equation 3.4). Then, this number was also combined with the uncertainty coming from the performance of the assay on different days as the repeatability (equation 3.5). RSDs were calculated again by the square root of the sum of the squares of each variable.

$$RSD_{(routine\ analytical\ uncertainty)} = \sqrt{RSD^2_{(antigen-antibody\ reaction)} + RSD^2_{(HRP-substrate\ reaction)} + RSD^2_{(device)}}$$

$$=\sqrt{0.028^2 + 0.028^2 + 0.03808^2} = 0.055 \quad (3.4)$$

Until this stage, the calculations for both A1 and A2 tests were the same. Since the repeatability values of the A1 and A2 tests were different, the calculated value for the $RSD_{combined}$ differed for each test.

$$RSD_{combined} = \sqrt{RSD^2_{(repeatability)} + RSD^2_{(routine\ analytical\ uncertainty)}} \quad (3.5)$$

For A1 test $RSD_{combined} = \sqrt{0.103^2 + 0.055^2} = 0.117$ (3.6a)

For A2 test $RSD_{combined} = \sqrt{0.029^2 + 0.055^2} = 0.062$ (3.6b)

The total measurement uncertainty of the A1 test was found as 11.7% and that of the A2 test was found as 6.2%.

4. Conclusion

There is an increasing consumer demand for A2 milk because of health issues or personal choices as A2 milk has also recently become widespread in many countries. With this demand, industry and governments become responsible for the control of the new product released into the market (Hawkes, 2007; Nestle, 2002; Silver and Bassett, 2008). Therefore, A2 milk and dairy products derived from A2 milk has to be checked for their authenticity. For this reason, food safety and control laboratories all around the world must follow and validate an acceptable assay preferentially in an accredited laboratory. This study will set an example for the quality control tests that should be applied for A2 milk and related dairy products in the market. By using the same test kits and following a similar validation and analysis system, many food control laboratories will be able to check the authenticity of the products declared to be from A2 milk in the market shelves; and therefore, consumers will be prevented from being deceived.

This study introduces a new test to be used for the determination of the authenticity of A2 milk, and the protocol to follow for its validation. The selection of ELISA methodology for this application was intended to make the assay easy to adapt to any food safety and control laboratories, since ELISA reader has been a widespread device and the methodology has been used extensively over the years. The immunological tests are simple, rapid, and requires only a small quantity of samples. Yet, there are substatial limitations to this study, which are mainly because of the discrimination of one amino acid difference between the two β-casein proteins. In the developed kits by the manufacturer, the antibodies used for the detection of A1 or A2 β-casein were to detect and differentiate one amino acid difference between the two proteins, which might cause false positive results because of the high similarity between the two proteins. Therefore, the ELISA protocol followed had some drawbacks that were dealed during the validation process with the aforementioned implementations. The drawbacks were the lower-concentrated standards giving absorbance values very close to each other, blank samples giving non-zero absorbances; and hence, the routine LOQ calculation for ELISA did not let the verification of the calculated LOQ value. These drawbacks were overcome by plotting a non-linear standard graph and the LOQ determination approach mentioned above. With the validation study, the applicability of the method of quantitative determination of β-casein A1 and A2 proteins by ELISA method with real milk samples in an accredited food control laboratory has been demonstrated. The non-linearity of the standard graph of the ELISA kit used distinguishes this method from the ELISA methods that are routinely applied in food control laboratories; and, necessitated the standard curve generation in second-order polynomial form. Similarly, the approach applied in LOQ determination is different from routine ELISA validation protocols. Therefore, this validation study will set an example for many food control laboratories both for the analysis of A1-A2 ELISA tests and other new methods that would require a non-linear standard graph calculation and a discrete LOQ calculation approach.

Validated ELISA method was well suited for screening and quantification of β-casein A1 and A2 proteins in milk. The introduced methods can reliably detect β-casein A1 and A2 proteins quantitatively. Yet, for the authentication of A2 milk, the presence of A2 β-casein and non-presence of A1 β-casein are sufficient to test; therefore, qualitative interpretation of the assays are admissible. Nevertheless, it has been foreseen that similar to the problems experienced in determination of GMO contaminations for legal purposes, when A2 milk sector becomes more available, there will be A1-contamination problems that will lead laboratories to test for the amount of A1 protein present in the dairy product. Afterwards, the quantitative application of ELISA is expected to be applied more.

In summary, this validation study on an ELISA for the quantification of β-casein A1 and A2 proteins from milk samples revealed difficulties due to the high similarity between the two proteins to be differentiated. Even though an acceptable validation was achieved, for future studies, an inter-laboratory comparison test should

be arranged. Considering the increase in demand and supply in A2 milk sector all around world, for the authentication of A2 milk dairy in terms of food safety and regulations, food safety and control laboratories may reliably start to use the β-casein A1 and A2 detection assays validated by this study.

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Author Contributions

Mediha Esra Altuntop Yayla: The author of this study conceived and designed the analysis, collected data, performed statistical analysis and wrote the paper.

Conflicts of Interest

The author declares no conflict of interest.

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