MÜRÜVVET SEZEY

VALIDATION OF MOHR TITRATION METHOD TO DETERMINE SALT (NaCl) IN OLIVE AND OLIVE BRINE

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A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCE OF NEAR EAST UNIVERSITY

By
MÜRÜVVET SEZEY

In Partial Fulfillment of the Requirements for The Degree of Master of Science in Food Engineering

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not orginal to this work.

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Mürüvvet SEZEY: VALIDATION OF MOHR TITRATION METHOD TO DETERMINE SALT (NaCl) IN OLIVE AND OLIVE BRINE

Approval of the Graduate School of Applied Science

Prof. Dr. Nadire ÇAVUŞ

We certify this thesis is satisfactory for the award of the degree of Master of Science in Food Engineering

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ABSTRACT

Method validation is necessary for all laboratory analysis. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

The aim of this study was to test that we have achieved the desired result for routine salt analysis in the production phase of olives and applying internal quality check that method provides the desired performance in the routine use. All 3 fortified samples and olive brine samples taken during the olive fermentation period were analyzed for the salt content by Mohr titration method.

The method was validated using 3 fortified samples (Fortified of water with salt solutions, cracked olive's brine and olive) matrices spiked at 3, 5 and 7% salt concentration at three different times. Overall recovery of the method was 107% for all three commodities over the validation range with a relative standard deviation of 5%.

Keywords: method validation; salt analysis; fortified sample

ÖZET

Tüm laboratuvar analizler için metot validasyon gereklidir. Metot validasyonu sonuçları, analitik sonuçların kalitesini, güvenilirliğini ve tutarlılığını yargılamak için kullanılabilir; Herhangi bir iyi analitik uygulamanın ayrılmaz bir parçasıdır.

Bu çalışmanın amacı, zeytinlerin üretim aşamasında rutin tuz analizi için istenen sonuca ulaştığınızı ve bu yöntemin rutin kullanımında istenilen performansı sağladığını kontrol etmenin test edilmesidir. Zeytin fermantasyon dönemi boyunca alınan 3 adet tuz eklenerek hazırlanan örnek ve zeytin salamura numuneleri titrasyon ile tuz içeriği için analiz edilmiştir (Mohr Metodu).

Tuz takviyesi yapılan su, kırık zeytin salamurası ve zeytinde uygulanan metod valide edilmiştir, %3, 5 ve 7 tuz konsantrasyondaki matriksler metod validasyon ile doğrulanmıştır. Tüm kosantrasyonların geri kazanım ortalaması %107 ve tekrar üretilebilirliği %5 tir.

Anahtar Kelimeler: metod validasyon; tuz analizi; takviye edilmiş su

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LIST OF ABBREVIATIONS

AOAC :	Association of the Official Analytical Chemists
AgCl :	Silver chloride
ARA-II :	Angiotensin-II-receptor antagonist
AMV :	Analytical method validation
CaCl2:	Calcium chloride
CO2 :	Carbon dioxide
COX :	Cyclooxygenase
DAD:	Diode array detection
FPD:	Flame photometric detector
FDA:	U.S. Food and Drug Administration
FeCN :	Ferrocyanide ions
GLP:	Good Laboratory Practice
GC:	Gas chromatography
HDL:	High density lipoprotein
HPLC:	High Performance Liquid Chromatography
ISO:	International Organization for Standardization
ICH :	Internatıonal Councıl for Harmonısatıon
IOOC:	International Olive Oil Council
ISE:	Ion-selective electrode
ISA :	Ionic strength adjuster
KCl :	Potasyum klorür
K2CrO4 :	Potassium chromate
LGC:	Laboratory of the Government Chemist
LAB:	Lactic acid bacteria
LDL :	Low density lipoprotein
LLE:	Liquid–liquid extraction
LC:	Liquid chromatography
LOD:	The limit of detection

LOQ :	The limit of quantitation
MgCl :	Magnesium chloride
mV:	millivolt
MDL:	Method detection limit
MS :	Mass spectrometry
NaCl:	Sodyum Klorür
NSAİD :	Non-Steroidal Anti-İnflammatory Drug
N :	Plate number
NFA:	National Food Administration
NaOH :	Sodium hydroxide
OCP:	Organochlorine pesti-cide
pH:	Potential of hydrogen
PT:	Profession Test
PCA:	Principal components analysis
RSD :	Relative standard deviation
R s :	Resolution
R&D :	Research and Development
RID:	Refractive index detection
SD:	Standard deviation
SFE:	Supercritical fluid extraction
T F:	Tailing factor
t R :	Retention time
QC:	Quality Control
QA:	Quality assurance
QMS:	Quadruple mass spectrophotometer
VIM :	International vocabulary of metrology

CHAPTER 1
INTRODUCTION

Historically, the olive tree (*Olea europaea L.)* is an important crop grown throughout the Mediterranean basin (Zohary and Spiegel-Roy, 1975). It is widely cultivated for the production of both oil and table olives and very significant because of its economic value (Uyuşer V. and Yıldız G.,2014).

"Table olives are the sound fruit of varieties of the cultivated olive trees (Olea europaea L.) that are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh taste, firmness and ease of detachment from the stone make them particularly suitable for processing; treated to remove its bitterness and preserved by natural fermentation; or by heat treatment, with or without the addition of preservatives; packed with or without covering liquid" (IOOC, 2004).

Table olives are classified according to the degree of ripeness of the fresh fruits i.e. green olives, olives turning colour and black olives, trade preparations (treated olives, natural olives, dehydrated and/or shrivelled olives, olives darkened by oxidation, specialities), and styles (whole, pitted, stuffed, salad and other styles).

Çakıstes is one of most typical table olive products of Northern Cyprus prepared by cracking of pink or green domestic olives. Domat, Memecik and Yamalak varieties are also used to prepare çakıstes. Soon after the olives are transported to the plant, they are size-graded, sorted and scratched on 2 or 3 sides and put into water. The water is changed every other day to obtain the deserved taste.The olives transferred to the fermentation tanks. The brine salt ratio is increased progressively and reaches to 5-6%. After the fermentation, the olives, are ready for consumption and packaging (''Present and future of the Mediterranean olive sector'', 2013).

During the fermentation process anaerobic conditions must be maintained; temperature, salt, acidity controls, yeast and mold cleaning on the upper surface of the brine must be done (‘’Sofralık Zeytin Fermantasyonu’’, 2011).

1.1 Table Olive Preservation Methods

The object of any food preservation system is to treat the fresh product in some way to prevent it from deteriorating. If done correctly, preserving renders the product safe for human consumption over a longer time. Various methods of food preservation have been developed over thousands of years and some of them are still used today, although newer and more sophisticated methods are now more common. In general most methods involve changing the environment of the product to eliminate or inhibit spoilage microorganisms.

The production of table olives involves the use of one or more of the following preservation methods: (‘‘Food Safety Requirements for Table Olives and Infused Olive Oil’’, 2007)

a) Addition of salt
b) Reduction of pH by fermentation or by acid addition
c) Elimination of fermentable material
d) Lowering of water activity
e) Addition of preservatives
f) Application of heat
g) Removal of oxygen
h) Prevention of oxygen access to the product
i) Addition of oxygen to encourage aerobic fermentation to use up fermentable material
j) Refrigeration and freezing

1.2 Importance of Salt for Table Olives

A parameter which strongly influences the storage and quality of table olives is salt (sodium chloride- NaCl) concentration. Its level is important for achieving stability of the products because it prevents spoilage and growth of pathogens. During recent years, consumers have developed an attitude on low sodium intake principally because a diet rich in sodium leads to higher blood pressure. So, several scientific studies (Arroyo-López et

al., 2008b; Romeo et al., 2009; Bautista-Gallego et al., 2010; Bautista-Gallego et al., 2011; Panagou et al., 2011) have focused on the viability, application and consequences of replacement of sodium with calcium or potassium in table olive fermentation. Apparently, NaCl may be substituted in diverse proportions with KCl or CaCl2 without substantially altering the usual fermentation profiles and producing good sensorial characteristics. In particular, a mixture of NaCl, CaCl2 showed the ability to reduce both bacterial and yeast growth, while KCl showed similar effect of NaCl. Moreover, using different mixed salts, Tsapatsaris and Kotzekidou (2004) showed that the replacement of NaCl by KCl in Kalamon olives resulted in a strong synergy between calcium lactate and calcium acetate with higher growth rates of starter cultures of *Lactobacillus plantarum and Debaryomyces hansenii*. The replacement of NaCl with other chlorides could be important in those productions traditionally processed in a high salt concentration, such as Greek-style olives, because this action could lower the NaCl concentration without reaching the lowest limits necessary to obtain a safe product. Therefore, besides the pH decrease and the NaCl concentration, several actions have been proposed in order to overcome all the fermentation problems such as pasteurization, addition of sugars (glucose and sucrose), extra salt addition and use of starter cultures (Flora Valeria Romeo, 2012).

Salt, chemically known as sodium chloride, is commonly used in table olive processing and packaging of table olive products. Coarse, dry salt is used for processing salt-dried olives, while coarse salt in water (salt brine) is used in fermentations and packaging brines. Food grade salt with no additives must be used for all table olive operations. Nonconforming salt can cause the following problems and should be avoided in olive processing:

• anticaking agents (as in table salt) make brines cloudy;
• lime impurities can reduce the acidity of final products;
• iron can darken olive products;
• magnesium impurities can impart a bitter taste;
• carbonates can alter texture, causing softening;
• iodised salt may darken olives and possibly give the olives a chemical taste (Stan Kailis and David Harris, 2007).

1.3 Impact of Different Density of Brine on Table Olive Product

Fermentation occurs rapid in low salt concentration, slow in high salt concentration. If the salt concentration is too low, unwanted microorganisms may be possible to develop so there is a risk of deterioration of product. Low salt content, causes softening in the olive while the high salt concentration causes the wrinkles, wrinkles, and debris on the deck. Therefore, the salt ratio of the olive should be proper to variety, method and the time of supply to the product market.

The salt in the brine is concentrated in the bottom of the tank after a while. In this case the olives in the upper region of the tank are deteriorated and taste change is observed. Salt concentration at the top and the bottom of the tank must be the same. The salt concentration level will prevent developing or growth of undesirable microorganisms and allow the development of lactic acid bacteria.

When the olives are placed in the brine, the osmosis occurs between olives and brine. The soluble substances in the olive are passing brine, salt of brine passes to olives, so salt level of brine decreases until the osmotic pressure being in equilibrium. For this reason, the salt concentration should be checked ragularly and to adjust salt concentration reduced amount of salt should be added to brine.

When salt concentration is high at the beginning of fermentation, the growth of lactic acid bacteria is inhibited and some of the undesirable microorganisms will have the opportunity to develop in high salt concentration. For example; while *Debaryomyces* and *Saccharomyces* yeast species develop in 20% of salt concentration, *Pichia* species develop in 15% and Candida species develop in 10% salt concentration.

In the first days, the salt concentration will decrease rapidly with the effect of osmosis. For this reason, the salt concentration should be measured initially every 2-3 days, then weekly and later in monthly periods. The amount of reduced salt should be added to brine. The balance between olives with brine is established within 1-1.5 months. When salt concentration is balanced the optimum green olive's salt concentration is 7-8 bome, while the black olive's salt cocentration is 10-13 bome(''Sofralık Zeytin Fermantasyonu'', 2011)

1.4 Methods of Salt Analysis

Selection of a method of analysis to determine salt content in any food is a significant decision to make, when designing a quality assurance plan. There are several different technologies and methods available for determining salt content of food; each method has their own advantages and limitations. Some of the most obvious advantages and limitations include the cost of investment, accuracy, and turnaround time for each test. However, simplicity cost effetiveness, and the need for technical expertise required to perform each analysis are often significant concerns. Based on these parameters, QC departments typically use standard methods for assessing salt content such as refractometry, ion-selective electrodes, and titration (Masulli, 2015).

When a method is applied for the first time in a laboratory, when a new method is developed for an analysis, when the method is modified, when a validated method is used in another laboratory or when a different person use the method or when different methods are compared, and after the quality control tests, when it is understood that there is a change in the performance of the method over time, method validation should be applied according to Iso 17025 (Karaman and Akalın, 2008).

1.5 Method Validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

The confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled. Analytical methods need to be validated or revalidated:

before their introduction into routine use;

- whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix); and
- whenever the method is changed and the change is outside the original scope of the method (Taylor & Francis, 31 Oct 1998).

1.5.1 Why is method validation necessary ?

•Ethical

-Establish fitness-for-purpose on customer's behalf

-Good science

•Commercial

-''due care'' in product liability

•Regulatory/regulatory

-Legal requirements

-Consistent application of method

-Comparability between analysts / laboratories / countries

From ethical point of view, method validation is important because the customer employs the expertise of the laboratory to do an analysis which it could not do by itself. It trusts the laboratory to use a fit for purpose method. The laboratory in turn should apply all aspects of good science to the problem - this includes appropriate validation of the methods used. It is good commercial sense to have some assurance that a measurement will be correct before it is carried out. Validation helps to provide that assurance. The unsatisfactory alternative is to carry the measurement out, detect errors and have to repeat the measurement. It is better to prevent problems from happening rather than have to correct them afterwards. In a production environment, the producer has a duty to have taken all reasonable care to ensure the quality of a product before releasing it to the consumer. Validation provides part of the minimum liability. In some areas, the validation of methods is a regulatory requirement. Compliance with Good Laboratory Practice (GLP), a legal requirement for certain types of study, requires technically valid operating procedures. Likewise methods accredited to the ISO/IEC 17025 standard must be validated. Evaluation of method performance parameters during the validation process yields data that show which parts of the method are stable and which can cause problems in overall performance. Thus validation helps in the design and implementation of suitable quality control procedures. Method validation data provide information which enables the comparability of results from samples analysed in different laboratories and using different.

1.5.2 When do you validate a method ?

•During method development

•Before using any method for samples

-verify own ability to match published data

-verify suitability for analiytical requirement

•Change of application / working environment / analyst

•Following period of non-use

Validation usually begins during the method development stage when some performance parameters are evaluated approximately to determine whether the method capabilities are in line with the levels required. Once the method is deemed good enough, the development phase finishes, giving way to more formal validation studies. Published methods may not necessarily be properly validated. The analyst is always advised to check the level of validation again that required and add further validation as needed. The analyst who uses the method routinely will not necessarily be the same with the one who has carried out the validation. Methods are sometimes validated in one part of a laboratory and then transferred to other parts for routine use. Whether the validated method is published or has been developed in-house, the analyst who will actually use it to analyse samples should first confirm that the validation data and subsequent fitness for purpose applies to the method when they are using it. This is sometimes known as verification. A change of use of the method, or use after a period of non-use, requires the validation to be checked. Extending the use of the method to different sample types, or analyte levels, will require the performance to be checked using the new type of samples. The effect of changes to other parameters such as analyst, instrument, or laboratory environment should also be checked.

1.5.3 Who validates a method ?

•The analyst

- in house development and validation of new methods
- verification of the performance of previously validated methods

•The laboratory

- method development and validation section

•Sectoral / professional / standardiation body

- validation of methods via interlaboratory study

It is principally the analyst who validates methods although they may work to a standard laboratory protocol to do so.

It should be recognised that methods may also be validated by groups of laboratories co-operating in a collaborative trial. Validated methods may be published by sectoral, professional or standardisation bodies.

Large laboratories may have a central development section which develops and validates methods before passing them on to other sections for routine use.

1.5.4 How do you validate a method ?

•Decide analytical requirements

•Plan and carry out experiments to evaluate performance

•Use data to assess fitness for purpose

•Statement of validation

Method validation is not an accidental activity, it should be both deliberate and planned.

The first stage is to examine the problem presented by the customer. Look at the reasons behind carrying out the analysis and find out what it is that the customer hopes to establish from having the work carried out. From this it should be possible to decide which method performance parameters are relevant to the work and what sort of target values are required. From this, a suite able of experiment can be designed which can be used to evaluate the performance of candidate methods.

The plan will include details on what is going to be analysed at each stage, what degree of replication is required. It is possible that several parameters may be examined in one set of experiments in which case, the order in which things are done can be important.
Once the plan is finalised the method parameters are evaluated and the data used to decide whether the method is fit-for-purpose. The statement of validation is the positive assertion of fitness-for-purpose.

1.5.5 Ensuring Results are Correct

Method validation is an essential part of the process of ensuring that measurement results reported to customers are correct. However, it is important to have other aspects under control such as satisfactory laboratory design, stable environmental conditions, suitable quality control (QC) procedures (all of these fall under the general heading of quality assurance (QA)). Well trained analysts are also essential to assess the analytical problem (understand the customer needs) and consequently determine the required method performance parameters.

1.5.6 Method Performence Parameters

Confirmation of identity/ Specificity/ Selectivity
Trueness(bias)
Precision (repeatability, reproducibility)
Limit of defection, limit of quantitation
Working range (Linearity)
Ruggedness/robustness
Sensitivity

Different method performance parameters will be important in different situations. Trueness has at most importance for calculating absolute values of properties or analytes. It should be noted that the term ‘accuracy’ is often used in documents/standards referring to method validation. Under current ISO definitions, accuracy is defined as the closeness of agreement between a measured value and a true value, and therefore includes the effect of both precision and trueness. Precision is important for all measurements but particularly in comparative studies. Working range will be of interest in most cases. For trace level work,

limits of detection and quantitation may be relevant. For planning calibration strategies it may be useful to know over the range where the response is linear. The meaning of “sensitivity” depends on the sector in which it is used. In an instrument sense it refers to the rate of change of response with analyte concentration/property value. Medical and clinical chemists often use it as an alternative to limit of detection. Ruggedness studies, carried out mainly during method development will indicate which parameters need to be controlled in order to preserve performance. This in turn enables suitable quality control strategies to be devised (‘‘Method Validation Course’’, 2015).

The aim of this study is to validate Mohr method to determine salt amount in olive and olive brine and to evaluate accuracy and presicion of the method and to estimate measurment uncertainty. Method performance will also be checked in routine through çakıstes production season by a quality control technique.

CHAPTER 2
THEORETICAL FRAMEWORK

2.1 Methods for Curing Olives

When olives are picked off, they contain a very bitter compound called oleuropein. Harvested olives must be ‘cured’ to remove the bitterness in order to make them palatable. The most common curing processes use brine, dry salt, water, or lye treatment. During these curing processes the water-soluble oleuropein compound is leached out of the olive flesh.

The flavor and texture of each style of olive depends partly on the curing process used. Lye-curing is the most rapid and efficient process for de-bittering, but many people think that lye-cured olives are less aromatic than other styles of olives. Brined olives undergo a natural fermentation unlike that is used for traditional dill pickles and sauerkraut. Acids are produced in the fermentation process by lactic acid bacteria that are naturally present on the fruit give these olives a distinctive flavor and aroma. Brined olives tend to be saltier than lye-cured olives. Water curing does not change the flavor of the olives as much as other curing methods.

Table 2.1: Suitable preservation methods for the olive styles (‘‘Olives: Safe Methods for Home Pickling’’, 2007).

	Suitable preservation methods				
Olive style	Brine	Refrigeration	Freezing	Drying	Pressure
Water-cured					
Kalamata sytle olives	+	+			
Mediterranean					
style cracked olives	+	+			
Brine cured					
Greek style					
black olives in brine	+	+			
Sicilian style					
green olives in brine	+	+			
Dry salt cured		+	+		
Lye-cured					
Green olives	+	+	+	+	+
Dark ripe style olives	+	+	+	+	+
Lye-cured fermented					
Spanish style green olives	+	+			+

2.2 Factors Affecting Quality of Table Olives

a) Pre-harvest factors

- Environmental factors,

-Variety,

- Pruning, irrigation and fertilization,

- Maturity status

- The effects of diseases and pests

b) Factors in harvest

- Time of harvest,
- Harvest method,
- Transport and storage of olives conditions.

c) After harvest and in production

-Sorting process,
-Olive processing operations
-Packing material,
-Improper hygienic conditions,
-Varietal diversity,
-Use of uncontrolled additives or pesticides,
-Long waiting period before processing,
-Acidity, pH, salt concentration, yeast growth and circulation to be done at periodic operations during processing in olives (I.Ulusal Zeytin Öğrenci Kongresi, 2008).

2.3 Effect of Salt Varieties on Table Olive Fermentation

Lactic acid bacteria (LAB) are used for a variety of dairy, vegetable, and meat fermentations . Among them, *Lactobacillus spp.* are present in cucumber, sauerkraut, and olives. *Saccharomyces cerevisiae* is present in many food fermentations and it has been identified as the most abundant yeast species in table olives, and can be related to practically any processing style.

Fermented vegetables are traditionally prepared using common salt as a main ingredient, with the aim of flavoring and preserving the final products. Common salt, initially consisting of sodium chloride, lowers the water activity, increases the ionic strength of the solution, reduces the solubility of oxygen in water, and renders the product less prone to spoilage . However, producers must also consider new concerns of the population with respect to the effect of common salt on cardiovascular diseases, and the fact that overall sodium intake has declined since the early 1980s. To improve consumers' opinion of high-salt, fermented vegetables, sodium chloride (NaCl) could be replaced, at least partially, with other chloride salts with more favorable effects on health such as potassium chloride (KCl), calcium chloride ($CaCl_2$), or magnesium chloride (MgCl), whose cations are

macroelements, and whose contents must be declared in nutritional labeling, according to the legislation of most countries .

Marsilio et al. (2002) studied the sensory analysis of green table olives fermented in different saline solutions (NaCl, KCl, and their mixtures) and obtained acceptable products, although slightly bitter. Tassou et al. followed the microbiological and physicochemical changes of naturally black olives at different temperatures and NaCl levels in brine and obtained the best conditions at 25C and 6% NaCl. Tsapatsaris and Kotzekidou studied the effects of a substitution of NaCl with 50% KCl *on L. plantarum* and *Debaryomyces hansenii* growth in olive juice obtained from the natural black Greek variety Kalamon. However, information about the individual effects of the different chloride salts (with nutritional interest for consumers) on *L. pentosus* growth, a bacteriocin- producing LAB , and *S. cerevisiae*, a ubiquitous microorganism in table olive fermentations, is still scarce ($CaCl_2$ and KCl) or nonexistent ($MgCl_2$).

A quantitative investigation on the individual effects of sodium (NaCl), potassium (KCl), calcium ($CaCl_2$), and magnesium ($MgCl_2$) chloride salts against *Lactobacillus pentosus* and *Saccharomyces cerevisiae*, two representative microorganisms of table olives and other fermented vegetables, was carried out. In order to assess their potential activities, both the kinetic growth parameters and dose-response profiles in synthetic media (deMan Rogosa Shrpe broth medium and yeast-malt-peptone-glucose broth medium, respectively) were cultured and analyzed. Microbial growth was monitored via optical density measurements as a function of contact time in the presence of progressive chloride salt concentrations. Relative maximum specific growth rate and lag-phase period were modeled as a function of the chloride salt concentrations. Moreover, for each salt and microorganism tested, the noninhibitory concentrations and the MICs were estimated and compared. All chloride salts exerted a significant antimicrobial effect on the growth cycle; particularly, $CaCl_2$ showed a similar effect to NaCl, while KCl and $MgCl_2$ were progressively less inhibitory. Microbial susceptibility and resistance were found to be nonlinearly dose related (J. Bautista-Gallego et al., 2008).

2.4 Methods to Determine the Salt Content

Sodium occurs naturally in virtually all foods, albeit in relatively small amounts. Table salt, in the form of sodium chloride (NaCl), is a common additive to food products and is used as a preservative and a flavor enhancer. Traditionally, salt was added to food as a form of preservation. Since the advent of refrigeration, salt is more commonly used to enhance flavor but its ability to reduce microbial growth, improve texture, and increase shelf life are still utilized. Sodium may be added in forms other than table salt, such as sodium nitrate, sodium bicarbonate (baking soda), and monosodium glutamate. Sodium can also be added during food production from more complex sources, such as in soy sauce, garlic salt, or other condiments.

Mainly there are three methods for determining the salt content of a substance.

2.4.1 Refractometry

This method determines the salt content of a substance based on its refractive index. Refractive index is determined by passing a light through a prism into a sample and measuring how the light bends and establishing the critical angle. The critical angle is the angle at which no light is refracted and all light is internally reflected.

Refractometry can be used to determine a wide variety of parameters including sugar, propylene glycol, gelatin, and salt. Based on the types of dissolved solids in a sample, a refractive index is generated and converted to a measurement unit such as % Brix (sucrose) or % salt. It is important to note that refractometers are not specific, and only measure total light refraction. This makes them ideal for quantitative use in binary solutions, such as a salt brine solution, or for qualitative measurements in a finished product as a measure of consistency from batch to batch.

In mechanical refractometers, the sample is placed on a prism, and the user looks through an eyepiece to determine the “shadow line” to determine this critical angle. Since temperature greatly affects refractive index, temperature compensation is achieved using bimetal strips that move the lens or scale as they expand or contract due to changing temperature. Manual refractometers are a low cost investment, but have limited accuracy

due to subjectivity of determining the “shadow line,” variations in ambient light wavelengths, and limited temperature compensation.

Digital refractometers utilize an internal light source at a fixed wavelength. This internal light passes through a prism and into the sample and an internal light detector identifies the critical angle and therefore, the refractive index. Digital refractometers eliminate the subjectivity of determining the shadow line manually and have improved temperature compensation due to the use of programmed algorithms. As a result, digital refractometers can perform measurements in wider temperature ranges at a low-moderate price investment.

Refractometers are beneficial due to their low startup cost and lack of chemical reagents required to perform tests. However, this method is not specific to salt, and therefore prone to interferences from substances present in the sample that alter refractive index. These substances include fats, sugars, and salts other than sodium chloride. If salt is the only variable present in a complex sample, refractometers can be useful for qualitative measurements.

2.4.2 Use of Ion-Selective Electrode

Another method used for determining salt content in food is through the use of an ion-selective electrode, more commonly referred to as an ISE. An ISE is a chemical sensor with a sensing tip used to determine the concentration of a specific ion in a solution. In sodium ISEs, the sensing tip is a specially formulated sodium-specific glass bulb. ISEs obey the Nernst Equation, which allows us to correlate a millivolt (mV) reading to a proportional concentration value. However, much like refractometry, changes in temperature can also affect measurement accuracy. This is mitigated one of two ways: by monitoring temperature and applying a temperature correction using the electrode’s isopotential point or by maintaining a constant temperature between standards and samples during calibration and measurement.

Like a pH meter, ISEs require care to ensure accurate measurements. The glass bulb of the sodium ISE must be hydrated at all times in an electrolyte solution. In addition, the electrode bulb needs periodic etching to ensure that a fresh layer of sensing glass is

exposed prior to measurement. Proper function of the electrode can be validated by performing a slope check using sodium standards. The slope check ensures that the electrode conforms to Nernstian behavior and is operating correctly.

The ISE must be calibrated daily in order to ensure accurate measurements. Calibration standards should be bracketed the expected concentration of the sodium content of the food measured. For example, one calibration standard should have a higher concentration than the expected concentration, and another standard should have a lower concentration than your expected value. The standards should also be a decade apart from one another (i.e. 100 parts per million, or ppm, and 1,000 ppm).

Ionic strength adjuster (ISA) must also be added in a fixed ratio to both calibration standards and samples for accurate readings. Electrode response is affected both by ion concentration, as well as ion activity. The ISA standardizes ion activity between calibration standards and samples, therefore ensuring changes in the electrode response are based on changes in ion concentration, rather than ion activity. Once calibration is complete, measurements on liquid or solid samples can be performed. Solid samples can be extracted with water. The amount of water used to extract the solid samples must be accounted for so that a dilution factor may be applied.

Sodium ISEs are very specific to sodium measurement, and are prone to little interference. The startup cost of measurement with an ISE is moderate. However, the care involved with ISE tends to require a trained technical staff and a longer startup time before measurements may be taken.

2.4.3 Titration

This is the most common method of analysis in in-house laboratories for determining salt in foods. Titrimetric methods have been adopted as the reference method by organizations such as the Association of the Official Analytical Chemists (AOAC) for a variety of food matrices, which include cheeses, meats, and vegetables. A titration is a procedure where a solution of a known concentration (titrant) is used to determine the concentration of an unknown solution (analyte). Results are calculated based on the amount of titrant used to

reach the endpoint. Endpoint can correspond to a color change of an indicator, or detected with a potentiometric sensor.

Mohr Titration

The Mohr method is a manual titration method using silver nitrate. In this titration, a burette is used to manually add silver nitrate to a sample, allowing for a reaction to occur between silver ions in the titrant and chloride in the sample between each dose. The pH of the sample must be buffered to around 7.0 for the reaction to occur. This reaction between silver and chloride produces an insoluble silver chloride (AgCl) precipitate.

Silver nitrate is added until chloride is no longer present in the sample solution. When silver nitrate is added to the sample in excess, it binds with a chromate ion indicator to produce a red color in solution, signifying the endpoint. Chloride concentration is calculated, which can then be used to sodium or sodium chloride content. This method has the benefit of high accuracy when performed by skilled operators, although determining when the color indicator has sufficiently changed makes this method prone to overestimation of salt content. The investment for manual titration is very low for silver nitrate titrant, color indicator, a manual burette, and other necessary volumetric glassware.

Titration with silver nitrate may be automated with a potentiometric titration system. The titration system can be equipped with an ISE sensitive to the concentration of chloride or silver ions. However, this electrode would not be used to directly determine concentration during a titration. Instead, the electrode would monitor the solution for a change in the mV potential as a result of silver ions being in excess, or depletion of chloride ions in solution. As a result, calibration of ISEs is not necessary for titration, making the startup time for analysis immediate.

These titration systems automatically control titrant dosing and endpoint detection. Automatic endpoint detection increases titration precision by eliminating human subjectivity associated with manual titration. Instead of a visual color change indicator, the titrator will determine the endpoint by measuring changes in mV potential. Also, the automated dosing system dispenses smaller, more precise doses than a technician using a

manual burette. Dynamic dosing is available on many titration units, which permits the unit to control how much titrant is dosed based on the progress of the titration. Dynamic dosing allows for larger doses to be dispensed in the beginning of the titration, with progressively smaller doses being dispensed as the endpoint is approached. This saves time and reduces the likelihood of overshooting the endpoint. Automatic titrators require a moderate to large investment (Masulli, 2015).

Titration method was applied in this work and validated.

2.5 Method Validation

Method validation is necessary for all kind of laboratory analysis. Validation has three important parts, these are:

1.the specific intended use or application, is the analytical requirement which derives from the problem that the analysis is intended to solve; this is clarified during the discussions between the laboratory and the customer as part of contract review.

2.the objective evidence is usually generated data from planned experiments, from which the appropriate method performance parameters are calculated;

3.the confirmation is taken as a satisfactory comparison of the performance data with what is required, i.e. demonstrating that the method is fit for purpose. Advice on how to do method validation is laid out in a number of guides - the actual procedures may vary from sector to sector. It is always worth following any guidance available for the particular sector, so that validation procedure is compatible with that in peer laboratories. Where particular conventions have been followed these should be stated. There are a number of different definitions of validation but they are broadly in line with the definition of ISO Guide 99:2007 (International vocabulary of metrology – Basic and general concepts and associated terms (VIM)) which defines validation as, verification, where the specified requirements are adequate for an intended use and verification as, provision of objective evidence that a given item fulfils specified requirements (Method Validation Course 1125, 2015).

2.5.1 Method Performance Acceptability Criteria

In method validation studies, several performance characteristics may be investigated, depending on the type of method and its intended use. These are summarized below:

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components. In drug assays, specificity takes into account the degree of interference from other active ingredients, excipients, impurities, degradation products, or matrices, and ensures that a chromatographic peak corresponds to a single component. Specificity can be demonstrated by the resolution between peaks of interest. Modern chromatographic methods typically include a peak-purity test based upon photodiode-array detection or mass spectrometry.

Accuracy is the closeness of test results to the true value. For drug substances, accuracy measurements are obtained by comparing test results to the analysis of a standard reference material or to a second, well-characterized method. For drug products, accuracy is evaluated by analyzing synthetic mixtures (containing all excipient materials in the correct proportions) spiked with known quantities of analyte. Guidelines recommend that data be collected from a minimum of six determinations over at least three concentration levels covering the specified range. The data should be reported as the percent recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals (such as ±1 SD). Accuracy determination ranging 70-130% of expected content will satisfy requirements. Statistical analysis can be applied using a one sample t-test.

Precision measures the degree of agreement among test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. Precision is commonly described in terms of repeatability, intermediate precision, and reproducibility. Repeatability is investigated by analyzing a minimum of six determinations using the same equipment and sample, covering the specified range of the procedure, or a minimum of six determinations at 100% of the test concentration and reported as percent relative standard deviation (RSD). Intermediate precision refers to the agreement among the results from a single laboratory, despite potential variations in sample preparation, analysts, or

equipment. Reproducibility refers to the agreement among the results from different laboratories. Results are reported as % RSD, and the percent difference in the mean values between the analysts must be within specifications. Less than 2% RSD is often recommended, but less than 5% RSD can be acceptable for minor components.

The limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected. The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

The limit of quantitation (LOQ) is the lowest concentration of an analyte in a sample that can be quantified with acceptable precision and accuracy under the stated operational conditions of the method. In a chromatography laboratory, the most common way to determine both the LOD and the LOQ is using signal-to-noise ratios (S/N), commonly 3:1 for LOD and 10:1 for LOQ. An appropriate number of samples must be analyzed to fully validate the method performance at the limit.

Linearity is the ability of a method to provide results that are directly proportional to analyte concentration within a given range. Range is the interval between the upper and lower concentrations of an analyte that have been demonstrated to be determined with acceptable precision, accuracy, and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method (for example, nanograms per milliliter). Guidelines specify that a minimum of five concentration levels be used to determine the range and linearity, along with certain minimum specified ranges depending on the type of method. Data to be reported generally include the equation for the calibration curve line, the coefficient of correlation (R^2), standard deviation of relative residuals and the curve itself.

Ruggedness is a measure of a method's capacity to obtain comparable and acceptable results when perturbed by small but deliberate variations in procedural parameters; it provides an indication of the method's suitability and reliability during normal use. During a ruggedness study, method parameters (such as eluent composition, gradient, and detector

settings) are intentionally varied to study the effects on analytical results. Common chromatography parameters used to measure and document robustness include critical peak pair resolution (R s), plate number (N) or peak width in gradient elution, retention time (t R), tailing factor (T F), peak area (and height) and concentration.

Dynamic range is the range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity ("Analytical Procedures and Method Validation.", 2000).

2.5.2 How should methods be validated?

The laboratory using a method is responsible for ensuring that it is adequately validated, and if necessary for carrying out further work to supplement existing data. Usually national or international organizations, such as AOAC International, ISO, have undertaken the interlaboratory validation of the method in a method performance (collaborative) trial. The extent of laboratory internal validation and verification depends on the context in which the method is to be used. If a method is being developed which will have wide-ranging use, then collaborative studies involving a group of laboratories is probably the preferred way of carrying out the validation. However, it is not always a suitable option for industrial laboratories, since those that might be interested could be competitors. Whether or not methods validated in a single laboratory will be acceptable for regulatory purposes depends on any guidelines covering the area of measurement concerned. The type of method and its intended use indicates which validation parameters need to be investigated. The laboratory has to decide which performance parameters need to be characterised in order to validate the method. Characterisation of method performance is an expensive process and inevitably it may be constrained by time and cost considerations. Some of the parameters may have been determined approximately during the method development stage. Often a particular set of experiments will yield information on several parameters, so with careful planning the effort required to get the necessary information can be minimised. Validation requirements may be specified in guidelines within a particular sector of measurement relevant to the method and it is recommended that where these are available they are followed.

2.5.3 Method Validation Strategy

The necessity for laboratories to use a 'fully validated' method of analysis is now universally accepted or required within many sectors of analysis. Most method validation guides start with discussions on how criteria such as specificity, accuracy and precision of the method shall be established. The analytical problem, requirements of the customers and choices of analytical principles are seldom mentioned in this context. The first step in a 'full validation procedure' therefore should be to identify and document 'customer requirements' and the analytical problem, what is analytically and economically possible and other specific requirements on sampling, laboratory environment, external environment, etc.

This proposed procedure assumes that the instrument has been selected and the method has been developed. It meets criteria such as ease of use; ability to be automated and to be controlled by computer systems; costs per analysis; sample throughput; turnaround time; and environmental, health and safety requirements. Faced with a particular analytical problem, ideally, the laboratory should firstly agree with the customer an analytical requirement, which defines the performance requirements that a method must achieve to solve the analytical problem. In response to this requirement, the laboratory can evaluate existing methods for suitability and if necessary develop a new method. This iterative process of development and evaluation continues until the method is deemed capable of meeting the requirement; further development is unnecessary and the analytical work can proceed. This process of evaluation of performance criteria and confirming that the method is suitable.

Here are some recommendations for the use of a singlelaboratory method validation:

• Wherever possible and practical, a laboratory should use an analysis method whose performance characteristics have been evaluated through a collaborative trial that conforms to an international protocol.

• When such methods are not available, an in-house method must be validated before being used to generate analytical data.

• Single-laboratory validation requires the laboratory to select appropriate characteristics for evaluation (e.g., selectivity, calibration,accuracy, etc.).

• Evidence that these characteristics have been assessed must be made available.

During method validation, the parameters, acceptance limits and frequency of ongoing system suitability tests or quality control checks should be defined. Criteria should be defined to indicate when the method and system are beyond statistical control. The aim is to optimize these experiments so that, with a minimum number of control analyses, the method and the complete analytical system will provide long-term results to meet the objectives defined in the scope of the method.

2.5.4 Revalidation

Most likely some method parameters have to be changed or adjusted during the life of the method if the method performance criteria fall outside their acceptance criteria. The question is whether such change requires revalidation. In order to clarify this question upfront, operating ranges should be defined for each method, either based on experience with similar methods or else investigated during method development. These ranges should be verified during method validation in robustness studies and should be part of the method characteristics. A revalidation is necessary whenever a method is changed, and the new parameter lies outside the operating range. Possible changes may include: new samples with new compounds or new matrices; new analysts with different skills; new instruments with different characteristics; new location with different environmental conditions; new chemicals and/or reference standards; and modification of analytical parameters.

2.5.5 Transferring Validated Routine Methods

When validated methods are transferred between laboratories the receiving laboratory should demonstrate that it can successfully perform the method and their validated state should be maintained to ensure the same reliable results in the receiving laboratory. This means the competence of the receiving laboratory to use the method should be demonstrated through tests, for example, repeat critical method validation experiments and run samples in parallel in the transferring and receiving laboratories. Typical instances when method transfer occurs are from the Research and Development (R&D) laboratory to the Quality Control (QC) laboratory. Currently, there is no official document available that can be used as a guide for performance demonstration of the receiving laboratory.

However, the USP has published an article where the most common practices of method transfer are described : comparative testing, co-validation between two laboratories or sites, complete or partial method validation or revalidation, and the omission of formal transfer, sometimes called the transfer waiver. The transfer should be controlled by a procedure. The recommended steps are: (1) designate a project owner; (2) develop a transfer plan; (3) define transfer tests and acceptance criteria (validation experiments, sample analysis: sample type, replicates); (4) describe rational for tests; (5) train receiving laboratory operators in transferring laboratory on equipment, method, critical parameters and troubleshooting; (6) repeat 2 critical method validation tests in routine laboratory; (7) analyze at least three samples in transferring and receiving laboratory; and (8) document transfer results.

CHAPTER 3

RELATED RESEARCH

Method validation has received considerable attention in the literature and there are several guidelines available for analytical and bio-analytical aspect and they are as follows:

a. The United States FDA established two industrial guidelines. First one for the validation of analytical methods (this guidance provides recommendations to applicants on submitting analytical procedures, validation, data and samples to support the documentation of the identity, strength, quality, purity and potency of drug substances and drug products) and second one for the validation of bioanalytical methods (this guidance applies to bioanalytical methods used for human or non-human clinical, pharmacological, toxicological studies and preclinical studies-based on bioanalytical procedures such as chromatography, immunology and microbiology).

b. ICH developed two guidelines for method validation that were later merged in one: Q2-R1. It discusses the considered characteristics (terminology and definitions) and methodology to be used during the validation of the analytical procedures.

c. International Union of Pure and Applied Chemistry published “Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis”. This guideline provides minimum recommendations on procedures that should be employed to ensure adequate validation of analytical methods.

The above-described guidelines are mainly focused on seven common parameters that should be considered during bio-analytical method validation in order to establish the method “fit-for-purpose”. The definition for these seven parameters has been (ShamaNaz et al., 2014).

There are various researches about method validation in literature. Some of researches are summarised below:

H. Soo Lim et al.'s studied HPLC method was developed and validated to determine the presence of ferrocyanide ions (FeCNs) in food grade salts. An analytical column coupled with a guard column and mobile phase comprised of sodium perchlorate and sodium hydroxide (NaOH) were employed at a detection wavelength of 221 nm. Samples were dissolved in 0.02 M NaOH solution and filtered. For processed salts including herbs and spices, a C18 cartridge was applied to minimize interference from salt matrices. The method validation was based on linearity, accuracy (recovery), precision, LOD, LOQ, and measurement uncertainty. This method exhibits good linearity from 0.110 mg/L (r2 = 0.9999). The LOD and LOQ values were determined to be 0.02 and 0.07 mg/kg, respectively. The FeCN recoveries in six salt matrices ranged from 80.3102.2% (RSD = 0.3–4.4%). These results indicate that the proposed method is suitable for FeCN ion determination in various food grade salts (H. Soo Lim et al., 2018).

Organic acids and sugars are related to the chemical balance of wines and grape juices, besides exerting a strong influence on the taste balance and sensorial acceptance by consumers. The aim of this study was to validate a method for the simultaneous determination of sugars and organic acids in wines and grape juices by high-performance liquid chromatography (HPLC) with refractive index detection (RID) and diode array detection (DAD) and to characterize commercial products from northeast Brazil. The method provided values for linearity ($R > 0.9982$), precision ($CV\% < 1.4$), recovery (76–106%) and limits of detection (0.003–0.044 g L^{-1}) and quantification (0.008–0.199 g L^{-1}) which are considered acceptable for application in the characterization of these types of matrices. Principal components analysis (PCA) was used to verify the applicability of the method in the quality control of the products and resulted in the correct separation of the samples according to their type of processing. The results obtained in the characterization of the samples studied showed high levels of glucose and fructose in grape juice and the organic acids content was similar to those found in products originating from other regions around the world (Emanuela et al., 2017).

A reliable and sensitive method was developed for simultaneous determination of glyphosate and glufosinate in various food products by liquid chromatography-tandem mass spectrometry. Based on extraction, derivatization with9fluorenylmethylchloroformate and purification on solid phase extraction column, quantification was done by using isotopic-labeled analytes as internal standard and calibration in matrix. Good selectivity and sensitivity were achieved with a limit of quantification of 5 μg/kg. The recoveries of these two pesticides ranged from 91% to 114% with inter-day and relative standard deviation of 3.8–6.1% in five matrices of cereal group spiked at 5, 10, and 20 μg/kg. An accuracy profile was performed for method validation, demonstrating the accuracy and precision of the method for the studied food groups. The verification results in expanded food groups indicated extensive applicability for the analysis of glyphosate and glufosinate. Finally, the developed method was applied to analyze 136 food samples including milk-based baby foods from the French Agency for Food, Environmental and Occupational Health & Safety. Glyphosate residues were detected in two breakfast cereal samples (6.0 and 34 μg/kg). Glufosinate residues were found in a sample of boiled potatoes (9.8 μg/kg). No residues were detected in the other samples, including milk-based baby foods with limits of detection ranging from 1 to 2 μg/kg. The method has been applied for routine national monitoring of glyphosate and glufosinate in various foods (Yang Liao et al., 2017).

Shrikant H. Patil et al. (2011) searched for a novel and simple titrimetric method for determination of commonly used angiotensin-II-receptor antagonists (ARA-IIs) is developed and validated. The direct acid base titration of four ARA-IIs, namely eprosartan mesylate, irbesartan, telmisartan and valsartan, was carried out in the mixture of ethanol:water (1:1) as solvent using standardized sodium hydroxide aqueous solution as titrant, either visually using phenolphthalein as an indicator or potentiometrically using combined pH electrode. The method was found to be accurate and precise, having relative standard deviation of less than 2% for all ARA-IIs studied. Also, it was shown that the method could be successfully applied to the assay of commercial pharmaceuticals containing the above-mentioned ARA-IIs. The validity of the method was tested by the recovery studies of standard addition to pharmaceuticals and the results were found to be

satisfactory. Results obtained by this method were found to be in good agreement with those obtained by UV spectrophotometric method. For UV spectrophotometric analysis ethanol was used as a solvent and wavelength of 233 nm, 246 nm, 296 nm, and 250 nm was selected for determination of eprosartan mesylate, irbesartan, telmisartan, and valsartan respectively. The proposed titrimetric method is simple, rapid, convenient and sufficiently precise for quality control purposes.

R. N. Haddadin and A. Y. Issa studied a simple and inexpensive titrimetric method for the determination of magnesium ion in esomeprazole magnesium raw material was developed and validated according to International Conference on Harmonization guidelines and the United States Pharmacopoeia. The method depends on complex formation between EDTA and magnesium ion. The method was proven to be valid, equivalent and useful as an alternative method to the current pharmacopeial methods that are based on atomic absorption spectrometry (R. N. Haddadin and A. Y. Issa, 2011).

K. Basavaiah (2009) applied two simple titrimetric methods have been developed for the determination of hydroxyzine dihydrochloride (HDH) in pure form and in tablets. The principle of the methods are simple acid–base reactions in which the hydrochloride content of the drug was determined by titrating with an aqueous standardized NaOH solution either visually using phenolphthalein as indicator (method A) or potentiometrically using glass-calomel electrode system (method B). The methods were applicable over the range of 2-20 mg HDH. The procedures were also applied for the determination of HDH in its dosage forms and the results were found to be in good agreement with those obtained by the reference method. The precision, expressed by intra-day and inter-day relative standard deviation values, was satisfactory (RSD $\leq$ 2.76%). The accuracy was satisfactory as well (RE $\leq$ 2.67%). Excipients used as additives in pharmaceutical formulations did not interfere in the proposed procedures as shown by the recovery study via a standard addition technique with recovery percentage in the range 97.48–106.3% with a standard deviation of 1.76–3.42 %.

Baldut et al. (2015) searched for an easy, sensitive and inexpensive volumetric method for the determination of rosuvastatin calcium in raw material has been developed.

The titrimetric method is based on the reaction of calcium with a solution of Disodium ethylene diaminotetraacetate (EDTA) - Magnesium 0.01 M. Hydroxynaphtol blue was used as indicator. It changes from pink to blue at pH = 10 at the end point of the titration. The method was validated for linearity, precision and accuracy, following the suggestions of the International Conference on Harmonization (ICH).

The linearity of the volumetric method was determined by analysis of six replicates at 80%,100% and 120% and three replicates at 90% and 110% of analyte concentration. The calibration curve was linear, with r = 0.9998. Assay method precision was evaluated by carrying out six independent assays of bulk drug and the intermediate precision was also verified using different analyst and different day in the same laboratory. Accuracy (mean recovery 99.0%) and precision were found to be satisfactory.

The proposed method can be used for quality control assay of rosuvastatin calcium in bulk drug.

Silva B. et. al. (2004) analysed the sulfate ion content found by the two validated methods was compared by the statistical t-student test, indicating that there was no statistically significant difference between the methods.The response factor, defined as the relation between consumed volume and sulfate ion quantity, was applied for the determination of repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by the relative standard deviation (RSD) of the response factors calculated from the standard calibration curve points (n = 15). Intermediate precision was determined within two days by comparing the response factors calculated from the standard calibration curve points obtained on the first and second day (n = 30). RSD below 2.0% is recommended for repeatability and intermediate precision.

The accuracy was studied by recovery of known amounts of standard sulfate ion added to indinavir sulfate raw material within two consecutive days of analysis. Volumes of 1.0, 2.0 and 4.0 mL of standard sulfate ion stock solution, corresponding to 16.87, 33.74 and 67.48 mg of sulfate ion were added to conical flasks containing 250 mg of indinavir sulfate (33.74 mg of sulfate ion) and 80 ml of methanol/water (1:1, v/v). A blank solution containing 250 mg of indinavir sulfate (33.74 mg of sulfate ion) and 80 ml of

methanol/water (1:1, v/v) was also prepared. For each recovery point the titration was performed in triplicate within two consecutive days (n = 6). The percent recovery of added sulfate ion was calculated from the volumes consumed in the titrations.

Rele R. (2016) searched for a simple precise, rapid accurate and sensitive non-aqueous potentiometric titration method was developed for quantitative determination of candesartan cilexetil from pharmaceutical dosage form. The titration was carried out using standardized 0.1 N perchloric acid. The proposed method was found to be precise with % RSD < 1 (n = 6). The method showed strict linearity (r2 > 0.9999) between 20% to 100% of 0.100 mg of drug substance weight. The percentage recovery of candesartan cilexetil in the optimized method was between 99.49 to 99.91%. The method is also found to be rugged when checked by different analysts and using different lots of reagents and different makes of titrators.

CHAPTER 4
MATERIALS AND METHODS

4.1 Materials

Water, olive brine and olive samples fortified with NaCl at three different level i.e. 3, 5 and 7% were used as material for validation experiments.

4.1.1 Fortified Water Samples with Salt

Pure water was fortified with pure sodium chloride (NaCl) at 3 different levels i.e, 3, 5 and 7% and 6 replicates at each level. Preparation of fortified water samples:

1. 1.5g of NaCl was dissolved in 50 ml pure water for 3% NaCl solution in water

2. 2.5g of NaCl was dissolved in 50 ml pure water for 5% NaCl solution in water

3. 3.5g of NaCl was dissolved in 50 ml pure water for 7% NaCl solution in water

4.1.2 Fortified olive brine

Olives were picked from tree with hands (Figure 4.1). They were at green colour maturity period. Samples were replaced in plastic box and transfered to factory (Figure 4.1).

Figure 4.1: Picking of olives and olives boxes

Firstly, olives were washed in washing machine and seperated accoring to size in sorting machine(Figure 4.2). Then they were cracked with crushing machine (Figure 4.3) and put into pure water as same as in practice. Olives were waited in the water for 3 days. After that olives were seperated from water and water was fortified with pure sodium chloride (NaCl) at 3 different levels i.e, 3, 5 and 7 % and 6 replicates at each level. (Figure 4.4).

1. 3g of NaCl was dissolved in 50 ml water for 3% NaCl solution in water

2. 5g of NaCl was dissolved in 50 ml water for 5% NaCl solution in water

3. 7g of NaCl was dissolved in 50 ml water for 7% NaCl solution in water

Fiqure 4.2: Seperating olives from sorting machine

Fiqure 4.3: Craking olives in crushing machine

Figure 4.4: Different concentration of brine

4.1.3 Sampling of olive

2 kg of olives were picked from tree. They were transfered to laboratory. Olives were washed and crushed, then comminuted and homogenized. Homogenized olive samples were fortified with pure sodium chloride (NaCl) at 3 different level i.e, 3, 5 and 7% :

1. 15g of NaCl was dissolved in 500g olive extract for 3% NaCl solution

2. 25g of NaCl was dissolved in 500g olive extract for 5% NaCl solution

3. 35g of NaCl was dissolved in 500g olive extract for 7% NaCl solution

Fortified samples be waited for one day, so that NaCl can incorporate with olive matrix. Next day, salty olive samples were filtered through filter cloth.

Figure 4.5: Filtering of fortified olive for salt analysis

4.1.4 Duplicate Sampling of Olive's Brine During the Fermentation Period

In çakıstes production, washed and crushed olives put into polyester buxees and then filled with brine and allow fermentation. During fermentation period, brine samples are taken to check salt concentration. For the internal quality check, we also took brine samples and analyzed.

Picked olives were washed in washing machine and sorted. Then they were cracked with crushing machine and put in polyester boxes. When box was full with olive, brine was added. Brine was containing 7% salt and 1% citric acid.

A laboratory that has to analyze small batches and has to perform a wide variety of tests, as a general approach all of the test materials or a random selection from them are analyzed in duplicate (Harmonised Guidelines for Internal Quality Control in Analytical Chemistry Laboratories, 1995).

During fermentation period, olives, brine samples were taken from selected boxes in duplicates and analyzed in different days. Total 18 analyses were done and following formula (4.1) was used to calculate laboratory uncertainty from differences of duplicate measurments of the some samples.

$$s = \frac{1}{\sqrt{2}}\sqrt{\frac{\sum d^2}{n}} = \sqrt{\frac{\sum d^2}{2n}} \qquad (4.1)$$

4.2 Reagents and other Materials/ Equipments

0.1N silver nitrate ($AgNO_3$) solution

%5 potassium chromate(K_2CrO_4) solution (Indicator)

High Purity Silver Nitrate

Potassium chromate

Volumetric Flask

Analytical Balance

De-Ionized -or- Distilled Water

Buret

Pipet

Erlenmeyer

Filter

Beaker

Balloon jojoe

4.2.1 Preparation Of 0.1N Silver Nitrate ($AgNO_3$) Solution

-16.987 gr of High Purity Silver Nitrate is carefully weighted.

-Add the Silver Nitrate powder to the empty 1 l volumetric flask.

- Add about half of the water, swirl the flask to dissolve all of the silver nitrate.

- Carefully add the rest of the water and make up to the volume.

- Solution will be stored into another bottle at this point.

4.2.2 Preparation Of %0.5 Potassium Chromate (K_2CrO_4) Solution (Indicator)

- 5 gr of potassium chromate is carefully weighed.

-Add the potassium chromate powder to the empty 100ml volumetric flask.

- Add about half of the water, swirl the flask to dissolve all of the potassium chromate.

- Carefully add the rest of the water and make up to the volume. .

- Solution will be stored into another bottle at this point.

4.3 Method

4.3.1 Sample Processing For Salt Analysis

All fortified samples and olive brine samples taken during the olive fermentation period were analyzed for the salt content by Mohr titration by 6 replicates at the level of 3,5 and 7% salt and at three different times.

Procedure is described below :

a) One ml sample was taken from brine and put into erlenmayer

b) 0.5 ml %5 potassium chromate (indicator) was added into same erlenmayer

c) Burette was filled with $AgNO_3$ and zero was set

d) Titrate with standardized $AgNO_3$ solution until first perceptable pale red-brown appears

e) The colour should remain constant for 30 seconds

f) Titration volume is recorded

g) The following formula (4.2) was used to calculate the amount of salt.

$$\text{Percent salt} = (SxFxNxMx100)/(Ax1000) \qquad (4.2)$$

S: Consumption of 0.1 N AgNO3 in titration (ml)

F: Factor of 0.1 N AgNO3

N: Normalite of AgNO3

A: Amount of sample

4.3.2 Testing Matrix Effect

The potential for matrix effects to occur should be assessed at method validation. They are notoriously variable in occurrence and intensity but some techniques are particularly prone to them.

In this study, salt analyses were made by fortifying pure water, olive brine and olive to test accuracy. And mean recoveries were compared with t-student test.

Significance tests are used to compare two or more sets of results in a variety of ways. The t-test is very frequently used to compare the means of two small samples. If these samples have means $\bar{x}_1$ and $\bar{x}_2$, standard deviations s_1 and s_2, and sizes n_1 and n_2, then the difference between the two means can be studied by comparing $|\ \bar{x}_1 - \bar{x}_2\ |$ with zero. First, the pooled variance, s^2, is obtained from the weighted average of the two sample variances, the weights being the number of degrees of freedom corresponding to each sample. The value of s (pooled standard deviation) derived from below equation:

$$s_p^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \qquad (4.3)$$

The value of s derived from equation (4.3) can be used in equation (4.4) to calculate the t value. The t statistic takes values which depend on the sample size and the probability level, P, of interest.

$$t = \frac{\left|\bar{x}_1 - \bar{x}_2\right|}{s_p\sqrt{\frac{1}{n_{1_1}} + \frac{1}{n_2}}} \qquad (4.4)$$

The experimental value of t is compared with tabulated critical values at $n_1 + n_2 - 2$ degrees of freedom and the desired P-level, or the P value is obtained directly. If the experimental t value exceeds the critical value, this is a sign that the difference between the means, $\left|\overline{x_1} - \overline{x_2}\right|$ is larger than might be expected, i.e. that the two means differ significantly at the P level in question.

The difficulty that arises is the number of degrees of freedom to be used when comparing such t values with critical values. If $n_1 = n_2 = n$, then it may be permissible to use $n - 1$ degrees of freedom.

4.3.3 Testing Matrix Effect and Limit of Detection (LOD)

Additionally, the matrix effect (ME) was assessed by employing matrix-matched standards in this study. Matrix-matched calibration standards were prepared by using blank brine sample at seven different concentrations 1, 2, 3, 4, 5, 6 and 7 %.

The linearity of calibration curve was determined by computing correlation coefficient (R) and standard deviation of relative residuals (Srr) by using below formula 4.5:

$$s_{(y/x)w} = \left\{ \frac{\sum_i w_i (y_i - \hat{y}_i)^2}{n-2} \right\}^{1/2} \qquad (4.5)$$

Where:

y_i is the response obtained from injecting x_i analytical standard.

$\hat{y}_i$ is the point corresponding with x_i on the regression line

n is the total number of standard injections (e.g. when the calibration is made at three level with duplicate injections, then **n** is replaced with n*k= 3+2 equal to 6).

m is the number of replicate injections made to determine the analyte concentration

b is the slope obtained from the weighted linear regression.

y_o is the mean response (**m** replicate injections) used to calculate the concentration valueX_0

w_0 is the weighing appropriate to value of y_0

w_i is the weighing appropriate to value of $y_i : x_i$ pairs

$\bar{y}_w$ is the arithmetic mean of the weighted y_{iw} responses from all standard injections ($y_I w_i/n$).

$\bar{x}_w$ is the arithmetic mean of the weighted x_{iw} concentrations of standards ($w_I x_i/n$).

Limit of detection is the minimum concentration or mass of the analyte that can be detected with acceptable certainty, though not quantifiable with acceptable precision. LOD is also estimated through calibration curve (Tiryaki, 2016).

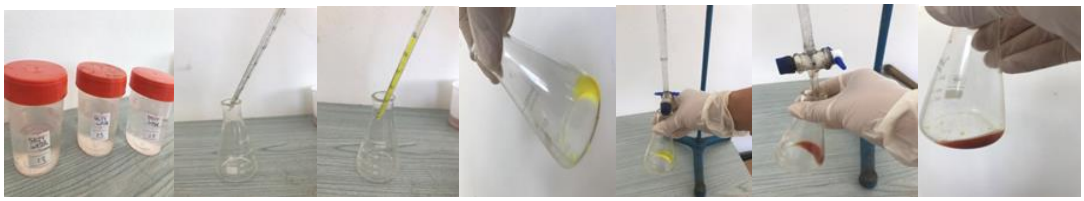

Figure 4.6: Salt analysis of salty water

Figure 4.7: Salt analysis of cracked olive's brine

Figure 4.8: Salt analysis of olive

Figure 4.9: Salt analysis of olive's brine during the fermentation period

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Results

5.1.1 Validation Results of Fortified Water Solutions

The results obtained from fortified water solutions for 3 different levels at 3 different time were summarized in Table 5.1.

When we look at recoveries and the repeatability values of the daily salt analysis of pure water solutions, recoveries were found as 109, 110 and 113 % for day 1, day 2 and day 3 of salt concentrations and relative standard deviations 4, 4 and 5 % respectively . Repeatability values are satisfactory since they are lower than 5%.

Salt recoveries from pure water were changing form 101 % to 121 %. Avarage recoveries of three different days were found as 116, 105 and 111 % for 3, 5 and 7 % salt concentrations, with 3, 2 and 2% RSD respectively.

5.1.2 Validation Results of Olive Brine

Cracked olive's brine at three different levels were analysed in 6 replications and at three different time. Table 5.2 represents the recoveries from cracked olive's brine at three different levels and three different time.

Salt recoveries from brine are changing form 94 % to 117 %. Avarage recoveries of three different days were found as 107, 105 and 103% for 3, 5 and 7 % salt concentrations, with 6, 3 and 1% RSD respectively.

When we look at recoveries and the repeatability values of the daily salt analysis, recoveries were found as 103, 105 and 107 % for day 1, day 2 and day 3 and relative standard deviations 3, 3 and 5 % respectively . Repeatability values are satisfactory since they are lower than 5%.

5.1.3 Validation Results of Fortified Olive

Fortfied olive with salt at three different levels were analysed in 6 replications and at three different time. Table 5.3 represents the recoveries from fortified olive solutions at three different levels and three different time.

Salt recoveries from olive solutions are changing form 100 % to 119 %. Recoveries of three different days were found as 110, 105 and 103 % for 3, 5 and 7 % salt concentrations, respectively.

When we look at recoveries and the repeatability values of the daily salt analysis, recoveries were found as 107, 106 and 104 % for day 1, day 2 and day 3 and relative standard deviations 6, 4 and 3 % respectively. Repeatability values are satisfactory since they are lower than 7%.

Table 5.1: Recoveries from fortified salt solutions at three different levels and three different time.

	3%						5%						7%						Ravg %	SD	RSD
Time 1	115	114	114	113	113	111	101	104	104	105	105	105	108	109	109	110	111	111	109	4	4
Time 2	117	115	116	117	114	115	102	105	103	105	105	106	110	111	108	111	110	110	110	5	4
Time 3	117	120	121	119	121	119	106	107	108	108	105	108	114	114	113	114	113	114	113	5	5
Ravg %	116						105						111								
SD	3						2						2								
RSD	3						2						2								

Table 5.2: Recoveries from cracked olive's brine at three different levels and three different time.

	3%						5%						7%						Ravg %	SD	RSD
Time 1	107	104	103	94	105	103	104	104	104	103	104	104	106	101	101	101	102	102	103	3	3
Time 2	109	103	103	105	104	105	102	104	106	117	107	105	102	104	104	104	103	103	105	3	3
Time 3	111	126	102	114	107	110	105	109	105	108	104	104	101	104	102	104	104	104	107	6	5
Ravg %	107						106						103								
SD	6						3						1								
RSD	6						3						1								

Table 5.3: Recoveries from fortified olive at three different levels and three different time.

	3%						5%						7%						Ravg %	SD	RSD
Time 1	117	114	115	119	111	110	105	103	108	101	104	105	103	100	103	104	101	100	107	6	6
Time 2	115	111	110	105	117	104	111	108	102	104	105	105	104	102	105	104	101	101	106	5	4
Time 3	113	109	103	104	101	102	103	105	105	105	106	103	104	103	104	105	103	102	105	3	3
Ravg %	110						105						103								
SD	6						2						2								
RSD	5						2						2								

5.1.4 Overall Recovery and Reproducibility of the Method

Recovery studies were carried out to determine the accuracy of the method.

Mean recovery for olive brine and olives were found as 105% and 106% respectively, where as 111 % for direct water solutions (Fiqure 5.1).

Reproducibility values were calculated as relative standard deviation of mean recoveries obtained from three different time analysis of the fortified samples.

Reproducibility values were 4.19% and 4.44% for fortified brine and olive samples.

Finally overal recovery, in other words, accuracy of the Mohr titration method is 105% with 4.55% of RSD (n=108) for olive and olive brine.

Mohr titration method for the determinatin of salt amount in olive and/or olive brine was succesfully validated, since the accuracy and precision of the method were with in the acceptable ranges.

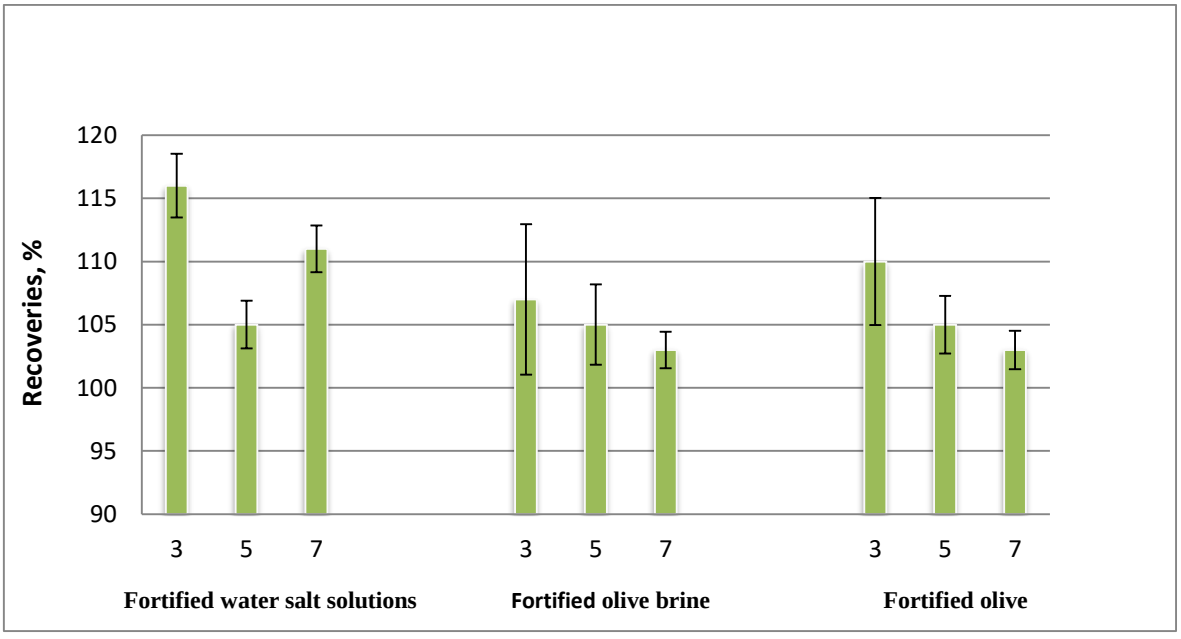

Figure 5.1: Mean recovery and RSD for fortified water solutions, fortified olive brine and fortified olive

5.1.5 Internal Quality Check Results Obtained from Duplicate Analysis of Olive Brine

Olive brine samples were taken during the fermentation period for the estimation of laboratory uncertainty, 3 sampling party were selected during one month period. 3 samples were taken from each barrel with onr week interval.

Sample 1 was analyzed on the day of sampling, the 2nd sample was analyzed the next day and sample 3 was stored as a control sample. Total 18 samples were analysed and laboratory uncertainty was calculated as 3% (Table 5.4).

Table 5.4: Laboratory uncertainty

Sample	Duplicate1	Duplicate2	Difference between duplicates	Square of Differences	Sum of squares	Cvlab
1	4,50	4,79	-0,062	0,003897845	0,01213	0,0259
2	5,82	5,55	0,047	0,002255623		
3	6,40	6,40	0	0		
4	4,38	4,38	0	0		
5	6,05	6,14	-0,014	0,000218041		
6	6,61	6,55	0,009	8,35E-05		
7	5,50	5,90	-0,070	0,004924592		
8	6,31	6,14	0,027	0,000745794		
9	6,23	6,25	-0,003	1,02728E-05		

Laboratory uncertainty value is in accordance with reproducibility value of direct water analysis.

5.1.6 Matrix Effect

Statistical significance of differences, by using Student t test, between water and olive brine, water and olive, brine and olive is summarized in Table 5.5. It was considered that the differences between all the fortified samples were statistically important from each other, since calculated t-statistics are greater than the t-critical value of 2.01 (df=53 and P=0.05, two sided).

Table 5.5: Comparison of matrix effect on accuracy by t-student test

Matrix	Mean Recovery	SD	Tested matrices	t_{calc}
Pure water	111	4.63	Water-Brine	6.61
Brine	105	4.40	Water-Olive	5.56
Olive	106	4.71	Brine-Olive	2.19

5.1.7 Linearity and Limit of Detection (LOD)

Linearity and LOD are important parameters to be determined in method validation experiments. One simple way to estimate linearity and LOD is use of the calibration curves in matrix. The standard deviations of relative residuals (Srr), which is a decisive parameter in internal quality control of linearity, should be ≤ 0.1 (Gozek, K. et al., 1995), (Miller, J.N. & Amburs, A., 2000) . This was the case in the study that correlation coefficient R was 0.998 and Srr was 0.03.

Typical LOD value for salt calculated from the calibration curve was found 0.01%.

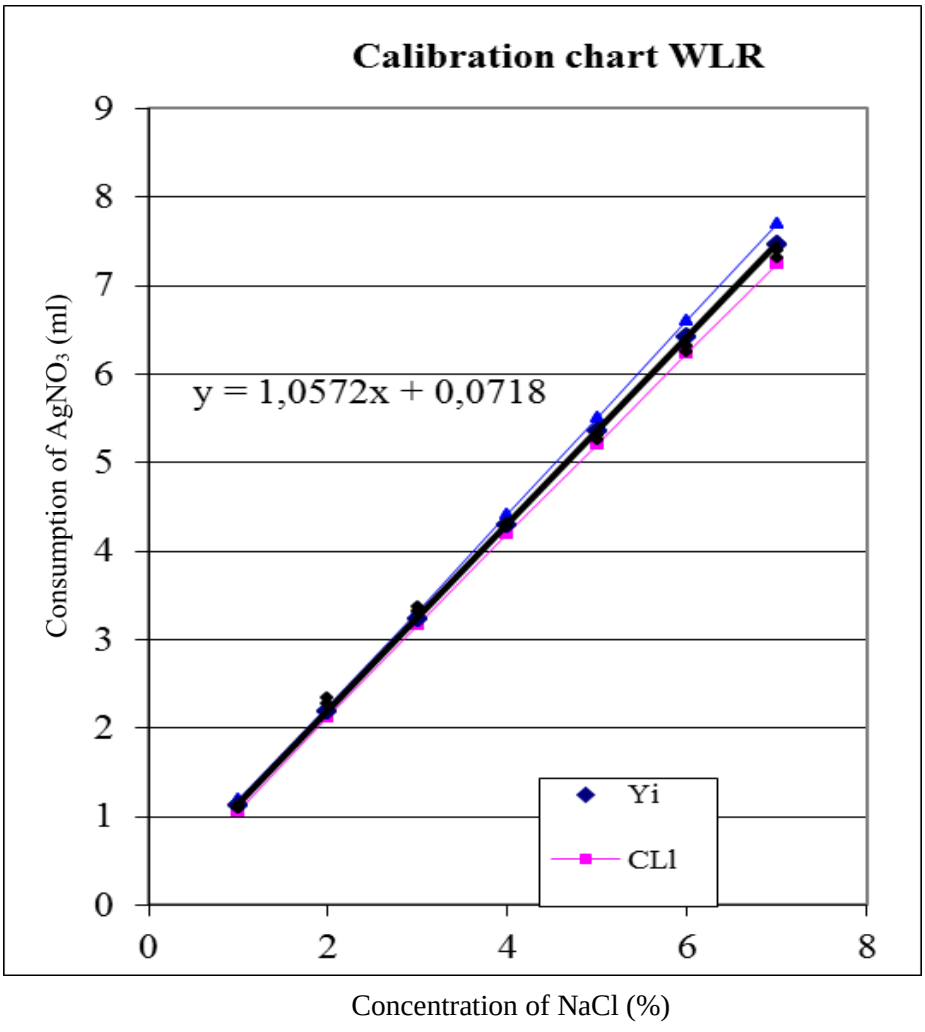

Figure 5.2: Calibration curve

5.2 Discussion

For two of the matrices, olive and olive brine, recoveries averaged as 105 % over the validation range with a relative standard deviation of 4 % (n=108). All these findings comply with the values recommended for mean recovery range (70–130%) and reproducibility (RSD 5%). Our findings are also in accordance with the similar titrimetric method's validation criteria.

Basavaiah (2009) applied two simple titrimetric methods for the determination of hydroxyzine dihydrochloride (HDH) in pure form and in tablets. The methods were applicable over the range of 2-20 mg HDH. The procedures were also applied for the determination of HDH in its dosage forms and the results were found to be in good agreement with those obtained by the reference method. The precision was satisfactory (RSD $\leq$ 2.76%). The accuracy was satisfactory as well. Excipients used as additives in pharmaceutical formulations did not interfere in the proposed procedures as shown by the recovery study via a standard addition technique with recovery percentage in the range 97.48–106.3% with a standard deviation of 1.76–3.42 %.

Rele and Terse (2011) studied simple precise, rapid accurate and sensitive non-aqueous potentiometric titration method for quantitative determination of azelnidipine from pharmaceutical dosage form. The proposed method was found to be precise with % RSD <1 (n = 6). The percentage recovery of azelnidipine in the optimized method was between 100.03 % to 101.85 %. The method is also found to be robust when checked by different analysts and using different lots of reagents and different makes of titrators.

Baldut et al. (2015) used a titrimetric method for the determination of rosuvastatin calcium in raw material. The method was validated for linearity, precision and accuracy according to the International Conference on Harmonization (ICH). Accuracy (mean recovery 99.0%) and precision were found to be satisfactory.

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

All the testing laboratories wish to demonstrate that they operate a management system, are technically competent and are able to generate technically valid results. They have to also comply with international standards such as ISO 17025 and ISO 9001 and operate in accordance with them. The use of international standards will facilitate cooperation between laboratories and other bodies and also in the harmonization of standards and procedures.

Many factors determine the correctness and reliability of the test results performed by a laboratory, such as accommodation and environmental conditions, test and calibration methods and method validation, equipment, sampling and handling of test and calibration items. Laboratory should validate the methods to confirm that the methods are fit for the intended use.

In this study, Mohr titration method was successfully validated for salt analysis in all two matrices, olive and olive brine, and all three fortification level and can be used for routine analysis by implementing internal quality control measures during its use.

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