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Review

Electrochemical and others techniques for the determination of malic acid and tartaric acid in must and wine

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This work is focused on a clear summary of the analytical techniques used for qualitative and quantitative analysis of malic acid and tartaric acid in wine and must. Particular emphasis is placed on electrochemical methods. The methods applied are divided into 5 basic groups — chromatographic, electrochemical, spectroscopic, enzymatic and titration. Some of these methods are already receding or are used only to a limited extent, mostly by small-scale winegrowers. The most widespread method in this field is high performance liquid chromatography combined with various types of detectors. The methods of capillary electrophoresis are on the rise. Expansion of spectroscopic and enzymatic techniques is not very significant and is mainly used in combination with other techniques.
With the constant development of instrumentation, the analysis of these basic acids has become very accurate and the analysis time has been minimized. Currently is experimentally tested a combination of these techniques to bring financial savings into sample analysis.

Keywords: malic acid, tartaric acid, chromatography technique, electrochemistry, spectroscopy, enzymatic and titration technique

1. INTRODUCTION

Many organic acids are represented in grapes and wine, the most important being malic acid and tartaric acid. The aim of the winemaker is to obtain the proportion of acids in wine corresponding to the given variety. The basis is to avoid low values below 5 g/l and at the same time high values above 12 g/l of acids. Especially for early varieties, the acid content is low, therefore it is necessary to carry out a regular analysis of titratable acid content, pH and possibly also of malic and vinous acids in grapes. The monitoring of the acid content of the grapes begins during their ripening. However, their proportional representation, which is predominantly influenced by the maturity of the grapes and their processing, is decisive.

The total amount of acids depends on the variety, the vineyard scale, the ripeness of the grapes and the vintage. During maturation, malic acid and later tartaric acid are first formed. These acids are the two major acids in the grapes [1, 2]. Their chemical structure allows them to take part in a series of enzymatic reactions and transport of energy in the plant.

The content of malic acid in grape juice is maximal just before maturation, where it can reach concentrations up to 20 g/l. During maturation, its content decreases and drops to 1 - 9 g/l in harvest. This decrease, caused by respiration, is more pronounced in warm climates [3, 4]. For most wines, the concentration is about 5 g/l [5, 6]. The taste of malic acid in the wine is acidic, its amount decreases in fruit with increasing maturity. Concentration also varies depending on the variety, with some varieties (such as Sylvanski, Barbera and Carignan) being planted due to the higher content of malic acid. Unlike tartaric acid, malic acid is lightly processed by microorganisms, which is often used in winemaking under the so-called malolactic fermentation to reduce the content of malic acid in wine. The fermentation is caused by lactic acid bacteria e.g. *Oenococcus oeni* (previously *Leuconostoc oenos*) [7, 8]. Chemically, it is decarboxylation of malic acid to form lactic acid and carbon dioxide [9]. The produced lactic acid has a finer flavour and gives the wine a more rounded and fuller flavour. Malolactic fermentation is mainly used in the production of red wines, to a small extent in white wines. Sensory is malic acid in the wine perceived as a sharp, pungent acid and the intention of the cellar masters is to produce a wine with a low content of malic acid. The content of malic and tartaric acids, the total content of titratable acids and pH can be seen in the table (Tab.1.) for different grape quality [10].

Table 1. Grape sorting by quality based on pH and organic acids [10].

Qualitative parameter	**Type of variety**	**Grape quality**		
		low	**average**	**high**
pH	White	2.8-3.0 3.4 or more	3.0-3.1 3.3-3.4	3.1-3.3
	Blue	2.8-3.0 3.5 or more	3.0-3.1	3.1-3.4
Titratable acids	Blue	3.0-5.5 11.0 or more	9.0-11.0 5.5-6.5	6.5-9.0
	Blue	3.0-5.0 10.0 or more	5.0-5.5 7.5-10.0	5.5-7.5
Tartaric acid	White	More than 9.0 Less than 4.0	7.0-9.0	4.0-7.0
	Blue	More than 5.0 Less than 9.0	8.0-9.0	5.0-8.0
Malic acid	White	More than 1.5 Less than 5.0	1.5-2.0 3.0-5.0	2.0-3.0
	Blue	Less than 1.0	1.0-1.5 3.0-5.0	1.5-3.0

2. CHARACTERISTIC OF MALIC ACID AND TARTARIC ACID

2.1. Malic acid

Malic acid is one of the carboxylic acids. According to the systematic nomenclature, it is a hydroxybutanedic acid with the molecular formula $C_4H_6O_5$. The chemical structure is shown in the figure (Fig. 1), where it generates two active forms, namely L-malic acid and D-malic acid[11]. The L-form of malic acid occurs in natural form (in fruit), while the D-form of the acid is synthetically produced [12]. In terms of sensory analysis, malic acid is included among bitters tasted in dicarboxylic acids. It naturally occurs in all kinds of fruits and vegetables but has been proven in meat and cheese. In many dishes, it produces an acidic perception and is created by so-called fruit metabolism [13]. Malic acid is abundantly present in apples and we perceive it as the sour taste of green apples. It can cause a bitter taste in wine, although its quantity in fruit falls with its maturity. During digestion, it supplies the body with 10 kJ/g of energy [15].

When added to foods, it is referred to as E 296; its dissociation constants are $pK_1 = 3.46$ and $pK_2 = 5.21$ [16]. The calcium and magnesium salts of the acid are well soluble, therefore there is no problem with using harder water (industrial beverage production) [17]. Its anion is called *malate* and is a cell in the citrate cycle [18]. It forms from *fumarate* by *fumarase* to the oxaloacetate is oxidised by the malate dehydrogenase enzyme. However, it can also be oxidised to pyruvate while reducing $NADP^+$ (Nicotinamide adenine dinucleotide phosphate) to NADPH.

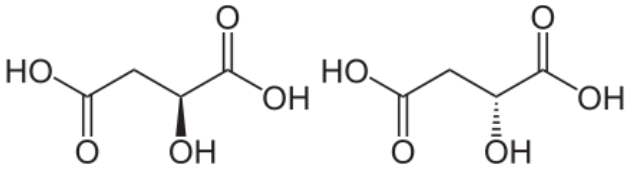

Figure 1. The structural formula of malic acid with two isomeric forms.

For food purposes, malic acid is made from apples where it naturally occurs. Malic acid was first isolated in apple must in 1785. It is industrially prepared enzymatically from fumaric acid using the enzyme *fumarase*. Microbial cells that produce this enzyme are used for this purpose [19]. A study by Takata et al. presents production using microorganisms *Brevibacterium flavum*, *Corynebacterium ammoniagenses* (previously *Brevibacterium ammoniagenses*), *Aspergilus oryzae* [21], *Penicillium viticola* [22], and *Saccharomyces cerevisiae* [8, 23] were studied for the production of malic acid.

2.2. Tartaric acid

Tartaric acid (2,3-dihydroxybutanedioic acid, sometimes dihydroxysuccinic acid) has the molecular formula HOOCCH(OH)CH(OH)COOH and is a colourless crystalline substance, well soluble in water with a characteristic acidic and fruity taste. Dissociation constants are $pK_1 = 2.96$ and $pK_2 = 4.16$. It occurs in three spatial isomers, has two asymmetric carbon atoms, so there is a

dextrorotatory D-form, a levorotatory L-form and an optically inactive meso-tartaric acid [16] (shown in Fig. 2). In nature, L-tartaric acid and racemic tartaric acid (mixture D and L form) are the most widespread, i.e. grapetic acid, which has been determined in grapes. In the international list of additives, it is designated with E-code 334 as L-tartaric acid. Seignette salt or sodium potassium tartrate KOOCCH (OH)CH(OH)COON is derived from tartaric acid, which is part of Fehling's reagent serving to proof reducing saccharides. It is used in the food industry to produce sparkling drinks and baking powders and in the dyeing industry [12].

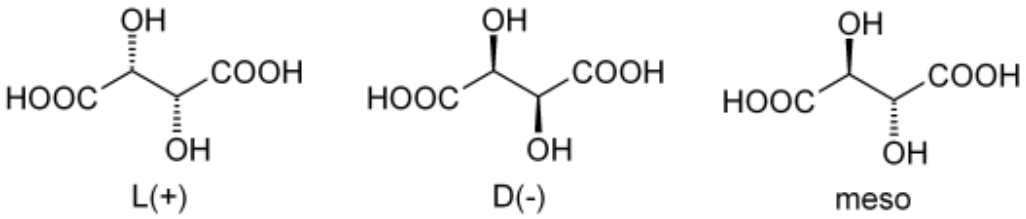

Figure 2. The structural formula of tartaric acid with three spatial isomers.

Tartaric acid is one of the most important acids in wine. Together with malic acid, they form the largest proportion of acids in must. An advantage is that tartaric acid does not consume yeast or microbes. During fermentation, only part of the tartaric acid precipitates in the form of tartar (0.5 - 1.5 g/l). Tartar solubility depends on the temperature, alcohol content, K^+ ions and tartaric acid content [24]. Tartaric acid is in a natural form part of many kinds of fruits especially grapes, some small berries (red currants, gooseberries, cranberries) or little-known tamarisk, sometimes referred to as sour or Indian dates [25, 26]. On the contrary, tartaric acid is not found in apples, blueberries or blackcurrants [16]. Tartaric acid is obtained industrially by extraction of tartar (potassium hydrogen tartrate). The racemic mixture of DL-tartaric acid can be obtained by chemical synthesis from maleic anhydride. Using various biotechnologies, it is possible to obtain L-tartaric acid where the conversion of calcium cis-epoxysuccinate with bacteria (e.g. *Acinetobacter tartarogenes*, *Agrobacterium aurem* [27], *Nocardica tartaricans* [28] and many others) are used. Another way to obtain D-tartaric acid is through microorganisms, that are only able to assimilate L-shaped acid from a substrate containing D, L-tartaric acid [29].

3. TECHNIQUES FOR DETERMINATION OF MALIC ACID AND TARTARIC ACID

The methods used are divided into 5 basic groups — chromatographic, electrochemical, spectroscopic, enzymatic and titration.

3.1. Chromatographic techniques

Liquid chromatography is the most commonly used chromatographic method. To a lesser extent, gas, ionic and thin-layer chromatography is used.

3.1.1. High-performance liquid chromatography (HPLC)

HPLC is one of the most appropriate and most commonly used techniques for determining organic acids in grape must and wine [30]. Before the analysis, it is advisable to pre-prepare the sample to prevent the interfering effects of sugars or dyes that could affect the development of the measurement. According to sample modification, the preparation can be simply divided into (i) sample modification by dilution, (ii) sample filtration, and (iii) more complex procedures. Normally, pre-treatment with solid-phase, ion-exchange or derivative extraction is used.

In their studies, Castellari *et al.* and Mato *et al*. compare direct injection with solid phase extraction using a SAX cartridge to separate organic acids [31, 32]. They assessed that direct injection with dilution or filtration pre-treatment was more accurate than solid phase extraction. In studies by Linget *et al.* and Vérette *et al.* [33, 34], a fully automated sample preparation system with on-line dialysis was developed before the HPLC analysis itself. Removal of the macromolecules and microparticles contained in the sample was performed. Several grape juice and wine tests were performed, and the results confirmed good repeatability and sensitivity. Scientists from the *Instituto Jean Piaget de Mirandela* [35] have developed a simple, fast, and accurate HPLC method for derivatisation and quantitative analysis of organic acids in port wines and grape must. The method was based on the use of O- (4-nitrobenzyl) -N, N'-diisopropylisourea (NBDI) as a derivatising reagent. Benzylmalonic acid was used as an internal standard.

Tartaric and malic acids in wine samples were determined as phenacyl esters[36].The sample was buffered at pH 6.8, mixed with a solution of phenacyl bromide and a crown ether in acetone, and heated at 100 °C for 40 minutes. Recoveries of acids were higher than 95%. The specificity of this assay was good as assessed by quantifying tartaric acid with other chromatographic techniques.

Methods with different separation mechanisms have also been developed, for example, HPLC with reversed phase, ion exchange HPLC or ion exclusion HPLC. The most commonly used methods are reverse phase separation techniques. Determination of organic acids by HPLC with reversed phase using UV detection and sample pre-dilution and filtration is presented in the study by Liorente *et al.* [37]. This is a simple method where the analysis time lasts 40 minutes.

For the determination of organic acids in grape juice and white wines, a special method of ion-exchange HPLC was developed with two spectrophotometric detectors (UV and IR) [31, 38] for direct determination. This direct analysis method provides acceptable resolution of chromatograms at lower cost and shorter analysis time. Ion-exclusion HPLC with an electrochemical detector and a refractive index detector allows mainly the determination of organic acids in grape juice and wine [32, 39, 40]. Preparing samples for HPLC analysis with ion exchanger and ion-exclusion HPLC is very simple, but chromatograms have a worse resolution than other methods.

Tusseau *et al.* [41] used reversible exchange columns and ion exchangers in their study. They found that the reverse exchange column is suitable for the determination of tartaric acid and malic acid. However, better ion exchange results have been obtained for citric acid and acetic acid. By using ion exchange, when organic acids occur in their ion form, pH control is required. Comparison of the three chromatographic systems: ion exchange, ion excluding and reversal phase is presented in the study [42]. Ding *et al.* concluded that narrow peaks were obtained by the ionic exclusion method, whereas

the reverse phase method has the fastest analysis. The advantage of the ion exchange method is its accuracy and easier processing of samples. Use of a phase inversion method in two-column HPLC coupled with a UV detector series [43]. The analysis of wine samples was carried out by direct injection without prior treatment. In their studies, Romero *et al.* and Tusseau *et al.* [41, 44] described an experiment by using two serial connected columns where solid phase extraction

was performed to remove organic acids. This connection of two columns in the series improved the resolution, but the analysis time was extended.

Jun described the HPLC method with reverse phase UV detection using the column with N-cetylpyridinium chloride [45]. Wine samples were pre-prepared by dilution and filtration. Several types of UV detectors have been used in this work. The most widely used detector is a UV spectrophotometer, a refractive index (RI) detector, a conductivity detector, an electrochemical detector or a photochemically induced chemiluminescent detector. The method of connection HLPC with Fourier Transform Infrared Spectroscopy (FTIR) method [46] provides the possibility of identifying substances that cannot be determined by UV-VIS techniques, but the low detection limit (0.2 g/l) is not very practical for use.

The Attenuated Total Reflectance (ATR) method is used to analyse solid and liquid samples and flow cell analysis directly with on-line detection in the mid-IR area [47]. Although there is a whole new combination of techniques, it has not proved to be better than other methods due to a long analysis time and detection of lower molecular weight acids. In addition, the sensitivity is not too high and the detection limit is about 0.2 g/l. Determination of organic acids in fruit wines by combining HPLC with electrochemical detection appears to be fast enough and simple without the necessary preparation of a derivative [48].

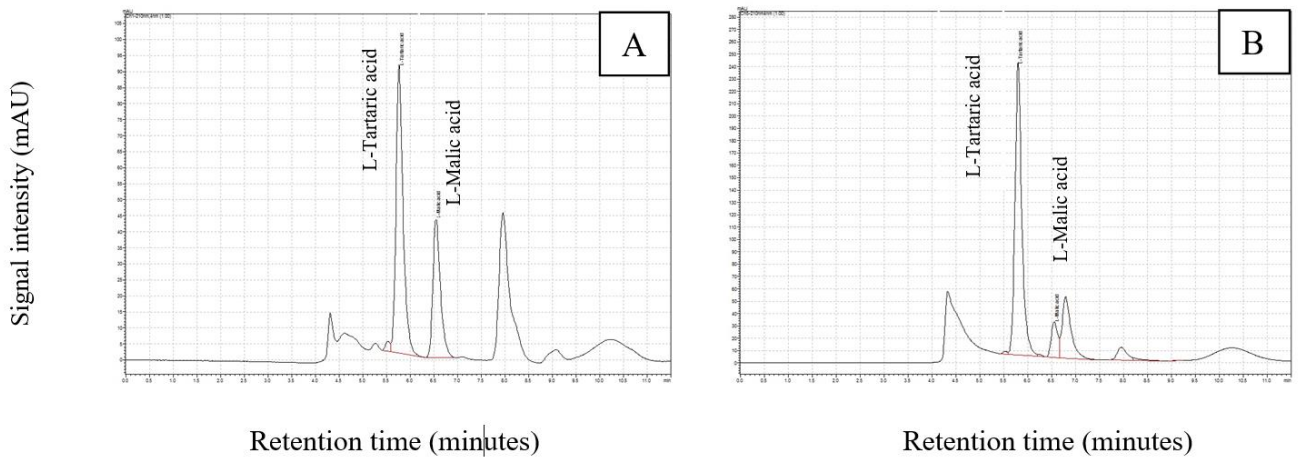

Figure 3. The chromatograms for determination of malic acid and tartaric acid in must (A) and wine (B)

The study of Tasev *et al.* [49] present a solid-phase extraction method followed by reverse phase high-performance liquid chromatog-raphy (RP-HPLC) was optimized and validated for the quantitative determination of tartaric, malic, shikimic, lactic, citric and succinic acids in wine *X*. Solid-

phase extraction was carried out with C18 car-tridges and extraction recoveries for all acids ranging from 98.3 to 103% were obtained. The wine pre-treatment involved a simple SPE method, which allowed for the successful elimination of the ma-trix components, resulting with good recoveries for all analytes. HPLC separation was performed with isocratic elution on a Supelco LiChrosorb RP-18 column protected with the appropriate guard column. The mobile phase was a 5 mM solution of H_3PO_4 with pH 2.1 at a flow rate of 1 ml/min. Detection of the organic acids was performed at 210 nm. Good linearity, sensitivity, precision and accuracy of the method confirmed its suitabil-ity for analysis of organic acids in red and white wines.

The chemical composition of grape berries during development exhibits large variations within a single bunch. To monitor the change in concentration of tartaric acid and malic acid between individual berries, a high-throughput method using UHPLC-MS/MS was developed in the study of Higginson *et al.* [50] to quantify these acids in berry extracts. The results from an analysis of single-vine datasets indicated that there was a large variation in the concentration of tartaric acid and malic acid between individual berries and also between bunches of berries across a vine. From this data, an optimum sampling size of 30 berries per vine was determined, which has an estimated standard error of less than 10% of the expected average berry acid concentration.

The aim of Monteiro Coelho *et al.* [51] study was to validate a method for the simultaneous determination of sugars and organic acids in wines and grape juices by HPLC with refractive index detection (RID) and diode array detection (DAD) and to characterise 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 $g \cdot L^{-1}$) and quantification (0.008–0.199 $g \cdot L^{-1}$) that are considered acceptable for application in the characterisation 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.

3.1.2. Ion chromatography

Ion chromatography with conductivity detection allows the separation and quantification of organic acids in both grape juice and wine. The technique has its advantages due to its specificity and sensitivity in the determination; it minimises the disturbing effect of sugars through the conductivity detector. In the method, it is not necessary to pre-treat the sample by extraction or derivative formation. This specific chromatographic method is reliable for routine quality control and ideal for the research of organic and inorganic anions as well as for analysing samples with very small amounts of organic acids.

In experiments by Kupin I and Saccani I [52, 53], a Dionex Omni PacPAX-500 column was used to separate and quantify significant organic acids and the separated anions were determined by a conductivity detector by using sodium hydroxide (NaOH) elution. More than 500 samples of fruit juices have been analysed. Masson [54] used a Dionex As11 column in his study. NaOH was used for

elution and the conductivity detector determined organic acids and inorganic anions in grape must. He studied the effects of three different solvents resp., namely methanol, ethanol and acetonitrile on the efficiency of the column. The best separation was in a mixture containing 13% methanol and 13% ethanol in water, which took place in just 20 minutes. Samples were prepared by 20-fold dilution and subsequent filtration.

3.1.3. Gas chromatography

Gas chromatography belongs to very sensitive and selective analytical techniques. The determination of organic acids with short chains is used in combination with the formation of derivatives. Acids may form derivatives with the following three compounds: trimethylsilyl [55-60] or methyl ester [61], derivatives with tert-butyldimethylsilyl [62] and ethyl ester derivatives [63]. Isolation of individual acids prior to the formation of derivatives is a necessity due to their complex representation in grape juice and wine. The isolation itself is most often carried out using lead salts and precipitation [56] with ion exchanger [64] and solid phase extraction [62]. All these steps are time-consuming and therefore the use of conventional gas chromatography is slowly receding.

Deng unified all steps of separation and derivation to reduce sample analysis time [63]. Linking the esterification with the ion exchanger resulted in the isolation of the individual organic acids within 60 minutes at 90 °C. Some organic acids such as acetic acid, lactic acid and malic acid can be determined directly by gas chromatography without the formation of a derivative [65]. Yang *et al.* [65] determined several organic short chain acids (up to 13 carbons) in 37 liquid food samples using a low detection detector. For higher detection limits, detectors such as flame ionisation detector (FID) [61, 65] and mass spectrometer (MS) can be used. Choosing these techniques for determining organic compounds is limited by the cost and complexity. Other alternatives such as liquid chromatography or capillary electrophoresis are more suitable for determining organic acids in grape juice and wine.

3.1.4. Thin-layer chromatography

The chromatographic process is based on the rise of the mobile phase with a thin layer of a fine-grained sorbent or a carrier of a fixed phase. The sorbent is either freely applied or more often fixed to a suitable substrate, which is either a glass plate, aluminium or plastic foil. The sample is applied to the start and after evaporation of the sample solvent; the plate is placed in a closed expansion chamber saturated with the vapours of the mobile phase. The mobile phase carries separated substances from the sample, which are delayed by interacting (by dissolving or adsorbing) with the stationary phase and thus dividing each other. As soon as the front of the rising mobile phase reaches the required distance, the chromatogram is removed and the detection is performed [66, 67].

Each substance is characterised by a position in the chromatogram, which is expressed by the retardation factor R_f. The retardation factor is characterised by the ratio of the distance of the centre of the stain from the start and the distance of the front from the start, depending on the particular

development system, the nature of the substance and other factors (e.g. temperature, amount of substance to be applied, etc.). A great advantage of TLC is the ease of execution, the speed of analysis, the availability of the relevant laboratory equipment and the relative economic ease. Other benefits include a broad choice of detection methods and the possibility of orientative or even very accurate quantitative evaluation [68].

Thin-layer chromatography has a wide use in terms of analytical methods for identifying tartaric, malic, lactic, succinic and citric acid [69]. For precise acid quantification, the content of each acid is determined separately using optical densitometry. This method is based on the measurement of optical density and the measurement procedure is similar to the photometric measurements, but it differs in the arrangement. The intensity of reflected light from the opaque pad is measured and the ratio of the intensity of incident and reflected light is evaluated.

Densitometry is the most common and most accurate technique, where scanning photo-densitometers convert staining intensity into a chromatogram with peaks whose surface is proportional to the amount of analyte. Another method used to identify and quantitate acids in wine and grape juice is gas chromatography, which was used in Ryan *et al.* study [56].

3.2. Electrochemical techniques

Electrochemical methods achieve considerable sensitivity and sensibility in terms of analysis. They are based on the principle of oxidation-reduction reactions associated with electron transfer. These methods are very widespread and represent a wide range of experimental techniques, the common feature of which is the transfer of electrical charge over the phase interconnection, at least one of which must be an ionic conductor of electrical current. This process is influenced by the existence of an electrical potential difference between the interconnecting phases induced either by external influences (external source of electrical voltage) or directly by the chemical composition of the whole system. Generally, electrical charge transfer is associated with a chemical change in the studied system [70]. Electrochemical methods are based on the measurement of voltage or current in the electrochemical cell. For electrochemical measurements, potentiometers, conductivity meters and polarographs and corresponding probes are used. Each type of probe has its own construction, matrices and areas of use.

From electrochemical methods, the most commonly used electrophoresis is used and to a lesser extent, isotachophoresis and bio-electrochemical methods and potentiometric titration with flow analysis.

The study of Nascimento Silva and Morgado Schmidt et al. [71] defines a rapid and simple method that requires minimum sample pre-treatment and no chromatographic separation. Based on direct infusion electrospray ionization mass spectrometry (ESI-MS), it was developed and validated to quantitate organic acids in wines and grapes. The main advantages are its speed (less than 5 min per sample of total analysis time), simplicity, and the analysis of the intact sample with no pre-separation or pre-derivatisation procedures. The linearity, precision, sensitivity and recuperation were as good as

those of the chromatographic methods using pre-separation via liquid chromatography or capillary electrophoresis.

3.2.1. Electrophoresis methods

In recent years, capillary electrophoresis has come to the forefront of analytical methods. It is used for the determination of organic acids in various types of food samples such as milk, cheese, beer, coffee or grape products [72-82]. This great development of electrophoresis has been due to its good parameters because it has high resolution, simplicity and automation. Other good features are short analysis time, low reagent consumption and minimal pre-sample preparation. The use of capillary electrophoresis to analyse organic acid content in grape juice and wine has erupted within a few years.

The method can separate small molecules in the analysed sample before the measurement begins. No complicated pre-preparation of thinning or filtration is required. Levi *et al.* [80] described a different sample processing consisting of purification by centrifugation and extraction of solids, compounds larger than C18 molecules and anthocyanins [83]. However, there are few differences between pre-treatment techniques. Two types of injectors were used to inject samples: hydrodynamic and electro-kinetic. Hydrodynamic injection of samples is the most widespread method, performed under reduced pressure (injection at the end of the capillary) or in a vacuum (capillary end detection) [84-88]. However, this type of injection does not depend on the sample parameters but only on its viscosity. The second injection method used is electrokinetic [83, 89, 90]. It is done by replacing the end of the injection container with a clamp and tension. Therefore, this method depends on the conductivity, the viscosity of the electrolyte, the sample properties and the mobility of the analyte. The use of electrokinetic injection increases the sensitivity of capillary electrophoresis, but accuracy is deteriorated and therefore is not suitable for quantification.

Electrophoresis methods often use electrolytes, which are important for good separation. Several types of electrolytes are used in the analysis of grape juice and wine. The most commonly used are bis (2-hydroxyethyl) imino-tris (hydroxymethyl) aminomethane, boric acid, 1,3,5-benzenetricarboxylic acid, 2-(N-morpholino) ethanesulfonic acid, chromate, 4-aminobenzoic acid, phosphate, phthalate, pyridinedicarboxylic acid, pyromellitic acid or tetraborate. In addition, the base electrolytes have been mixed with several modifiers (surfactants) to reduce electroosmotic flow, e.g. cetyltrimethylammonium bromide, ethylenediaminetetracarboxylic acid, myristyltrimethylammonium bromide, tetradecyltrimethylammonium bromide or tetradecyltrimethylammonium hydroxide.

In some methods, organic modifiers added to the electrolyte can affect migration of substances or even selectivity, including methanol [84, 85] or complex reagents such as Ca^{2+} and/or Mg^{2+} [87, 88, 91].

Most of the methods use capillaries to connect the entire system, which connected the receiving of the sample through the injector by passing it through the capillary to the detector. Previously, uncoated quartz capillaries with an added surfactant were used, but the adsorption of substances on the capillary walls caused reproducibility problems. Therefore, coated capillaries were used to prevent electroosmotic flow and the addition of surfactant was not necessary. A method of control with neutral

coated capillary (polyacrylamide) has been developed, where we achieve higher reproducibility and accuracy than with uncapped capillary methods [92].

Only two types of detectors are used, namely a conductivity and a UV spectrophotometer. The UV detector is a more widespread detection method due to its universal use [93]. Measurement can be done in two ways: directly and indirectly. Direct measurement means that the absorption of the electrolyte takes place in a lower UV area than the absorption of organic acids. Absorbance increases when the analyte passes through the detector. In contrast, the indirect measurement proceeds by absorbing the electrolyte in a higher UV range than the absorption of organic acids so that when the analyte passes through the detector, the absorbance decreases. The lower UV wavelength ranges from 185 to 254 nm and allows achieving high sensitivity in the determination of organic acids.

Klampfl *et al.* [82, 94] present a combination of two UV detectors together and prove that their combination allows for the quantification of most organic acids during one analysis. A short analysis time is another advantage of determining organic acids in grape juice and wine by this method. The analysis time is in the range of 3.5 to 20 minutes, although most analyses take less than 15 minutes. The work presents a determination of malic acid and lactic acid during malolactic fermentation by means of capillary electrophoresis and HPLC [83]. In this study, they concluded that both techniques, with a prerequisite for fast analysis, could be automated to process more samples. However, compared to HPLC, capillary electrophoresis has the additional advantage of low solvent consumption. They compare the quantitative data from the analysis of various organic acids for the interconnection of different methods with electrophoresis: the infrared spectrometry method for the determination of tartaric acid, malic acid and citric acid, acetic acid distillation, colorimetric method for the determination of tartaric acid and an enzymatic method for the determination of malic, lactic and citric acid [95].

Capillary electrophoresis can replace five current methods for organic acid analysis, namely HPLC, IC, enzymatic method, distillation and colorimetry. All results were almost in line with the results obtained from IC, colorimetry and distillation. The enzymatic method showed slight distortion due to the low ability for comparison with electrophoresis. Differences can be explained by the enzymatic method specific for D and L-isomers, electrophoresis linking both isomers to one peak [95].

Most electrophoresis methods allow for the determination of the main organic acids in grape juice (tartaric, malic and citric acid) and in wine (tartaric, malic, citric, succinic, acetic and lactic acid). The main disadvantage of capillary electrophoresis is its lower reproducibility compared to enzymatic and chromatographic methods, so some authors use standards or reference compounds to accelerate migration time. The used standards are butyric acid [96], formic acid [89] or glyoxylic acid [95, 97]. Oxalic acid was used as a reference compound for calculating the relative migration times of organic acids [88].

3.2.2. Isotachophoresis

This method is based on sample dosing at the interface of two electrolytes with different ion mobility. The mixture is divided by a constant current in a high voltage gradient. After dividing the mixture into individual zones, these zones are clamped between the lead and the terminating

electrolyte; they do not move away from each other and move at the same speed to the detection point. Masar *et al.* present [98] the determination of organic and inorganic acids in wine by isotachophoretic separation using a channel from poly (methyl methacrylate) with a CC (column-coupling) chip and detection on a conductivity column. Separation of the individual components of the sample using a 94 mm long channel with a chip, where separation takes 10-15 minutes at a low pH (2.9), gives better results. This method appears to be suitable for the determination of tartaric, lactic, malic and citric acid in wine.

3.2.3. Bio-electrochemical method

This method is based on biosensor technology and differential pH measurement for the determination of lactic and malic acid in wine [99] by using two procedures based on two lactate biosensors. Two electrodes, oxygen and peroxide, were used for the measurement [100]. Both electrodes were assembled using a polymeric membrane. Experimental parameters such as pH, temperature, concentration, and cofactor were optimised, and the determination took less than 1 minute. The detection limits are relatively high, so a dilution in the range of 1: 100 to 1: 200 is required, eliminating all potential electrochemical or enzymatic effects in the sample. A total of 14 samples were monitored. The determination of acids by this method using biosensors is fast, accurate and appropriate as an alternative to conventional methods [99].

3.2.4. Potentiometric titration and conjunction with flow analysis

For the determination of total acid content, pH, amount of magnesium and calcium can be used Flow Injection Analysis (FIA flow mode) with potentiometric detection. The pH was measured with a graphite/quinhydrone/silicone electrode, which was also used for determination of titration acidity [101]. The resulted amounts of acidity are consistent with the results obtained with the classical potentiometric titrations and pH measurement using a glass electrode. The method enables researchers to determine titration acidity about 40 times per hour and measure the pH about 30 times per hour. It is suitable for simple, quick and automatic determination of the total acid content, pH, calcium and magnesium content in wine in a small sample volume [102, 103].

3.3. Spectroscopic techniques

Spectrophotometric methods are based on the reaction of an organic acid with a light-sensitive or dye-sensitive substance that results in the formation of a compound or colour complex in which we then determine the wavelength. To avoid interfering effects, the organic acid is isolated by precipitation with a resin ion exchanger.

Rebelein presented one of the first spectrophotometric determination of malic, tartaric and lactic acid [104]. A resin ion exchanger separated the acids. The eluent passed through a sample several times to produce a colour difference of the individual compounds.

This colour difference was measured at several wavelengths; 490 nm tartaric acid, 420 nm malic acid and 530 or 570 nm lactic acid [104]. Other methods for determining tartaric acid are described in the publication. Essentially, these are methods that differ in the way samples are prepared to avoid disturbing effects of wine colour during process automation [30].

The use of infrared spectrometry for the analysis of grape must and wine can be divided into two areas of the electromagnetic spectrum: near infrared area = Near Infra-Red NIR [105, 106] and middle infrared area = Middle Infra-Red MIR [107-110]. IR-spectrometry methods represent a fast analysis of a large number of samples without expensive and time-consuming pre-treatment [111, 112].

The absorption in the near infrared area shows the spectrum in which C-H bonds are predominantly represented, while the middle infrared area shows spectrums rather for C-O, O-H and N-H bonds [113]. Strong absorption bands in the MIR area display many sharp peaks. On the other hand, this area highly absorbs the samples containing high amounts of water and organic compounds such as wine or juices. Therefore, it is not appropriate to use a long-wavelength MIR area for wine analysis. The need for a long-wave way up to several microns leads to constructional problems, especially regarding highly viscous and abrasive samples. Harrick and Fahrenfort in their studies for the first time present a possible alternative in this field by applying a technique that uses attenuation of the total reflection of ATR FTIR spectrometry [114, 115].

The principle is passing the infrared beam through a sample, which has a higher refractive index than the ambient air. It comes to full reflection and the beam proceeds inside the prism similarly to the inside of the optical fibre. Part of the beam is lost when it touches the sample and the reflected beam is attenuated, allowing us to obtain the infrared spectrum of the sample. Typically, this method is used to analyse liquid and solid samples [116]. In ATR spectrometry, the rate of IR beam absorption into a sample is dependent on wavelength and sample fracture index (up to 3 μm depending on setting). Much lower is for transmission measuring spectrometers (10-50 μm). Due to these parameters, ATR spectrometry can be partially used in the analysis of water samples which absorb more in greater rate of absorption spectrum.

Infrared spectrometry (IR) has been successfully used for monitoring fermentation. In this work, an IR calibration was used to analyse individual stages of fermenting must [117]. During the fermentation, the following substances were examined: glucose, fructose, glycerol, ethanol and organic acids; malic, tartaric, succinic, lactic, acetic and citric acids [107, 108]. Further advantages of the method are a high degree of automation, high sample throughput, simple sample preparation and low cost of analysis. The disadvantage is the high cost of the device and the need for precise calibration of the specified substances. In general, these methods are sufficiently accurate, but the previous calibration with a large number of samples is important. Good results from FTIR analysis were obtained in the total characterisation of samples, monitoring of ethanol, total acid content, total sugar and sulphate content [106, 118]. Ethanol, organic acids and other compounds present at higher concentrations can produce interfering substances for absorption bands of infrared analysis.

Visible (VIS) and near infrared spectrometry (NIRS) were used to measure the concentration of elements in Australian wines, 32 white and 94 red varieties. This study demonstrated the relationship between spectra from the NIR area and some of the elements contained in the wine. In the case of

quantitative analysis of the studied substances, calibration is necessary [119, 120]. The study was complemented by examining the temperature effect on the VIS-NIRS spectrum, which was confirmed in both red and white wines. Major changes observed in NIR spectra of wine samples were around 970 nm and 1400 nm in OH bonds. For samples measured at 30 °C and 35 °C, no change was found this temperature is suitable for further analysis.

Fourier transform with infrared spectroscopy can be used for the overall characterisation of grape juice and wine. This method enables complex use for the analysis of ethanol, sugar and acids. Due to the possibility of variable track length adjustment, this setting is suitable for acid determination. On the other hand, the fixed path length is more suitable for ethanol and sugars [121]. The usefulness of infrared spectroscopy can be said to be unlimited in view of its ability to determine all components of grape juice and wine [122].

3.4. Enzymatic techniques

Enzymatic methods are used predominantly for the quantitative determination of malic, lactic and lemon acid in grape juice and wine. They can also be used to determine other acids such as tartaric, acetic, ascorbic, formic, gluconic, citric, oxalic and succinic acid [123, 124].

The principle of enzymatic methods is to measure the increase or decrease in absorption of NADH (nicotine amide-adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate) coenzymes that absorb in the wavelength region [124-126]. A spectrophotometer and a determination at a wavelength of 340 nm are usually used to measure absorption. The main advantage of this method is high specificity. It can be used to determine the L and D isomers of certain acids. However, only one of the organic acids can be analysed for such determination, which makes this method time-consuming.

One of the ways to reduce the time of analysis is using Flow Injection Analysis = FIA. Puchades *et al.* [127] determined both malic and lactic acid in a sample of wine using FIA in an open reactor with enzymatic immobilisation.

A study by Lima *et al.* also presents the determination of two acids in wine using FIA and spectrophotometric detection [128]. The injection system is interconnected with the injection unit in one plane for monitoring the exact composition of the sample for analysis. Following an enzymatic reaction with NADH or NADPH, we can observe two peaks corresponding to the monitored acids.

Mataix *et al.* present a shift in this method using FIA with photometric detection along with fluorimetric detection at wavelengths 340 and 460 nm [129, 130]. Comparison of these two detection techniques shows that fluorimetry is cheaper, allows for repeatability, and requires no such amount of enzyme but shows a lower detection limit in comparison with photometry. Silva brought the optimisation of the measurement process in this technique to meet the following characteristics of the method: fast, accurate, does not require any sample preparation and linear response [131, 132]. A major development in this technique has been the modification of existing measurement and the creation of a new multifunction device. The existing flow system of the device was extended by a three-way valve to multi-analysis by changing the sample flow [133]. A multifunctional flow system

linked with the dialysis unit for sample dilution allows for the spectrophotometric determination of tartaric acid and potassium in port wine [134]. The proposed method was used to analyse 30 samples.

Biosensors were used for the analysis of malic and lactic acids in five white and five red wine samples [132, 135]. This method is characterised by high repeatability, short response time and low cost of analysis.

Enzymatic methods are often used as reference methods to verify the correctness of chromatographic methods, namely HPLC and ion chromatography [52, 136] as well as for verification of capillary electrophoresis [95]. Their great advantage is the ability to monitor the process of malolactic fermentation and, if necessary, to regulate it.

3.5. Titration techniques

Titration is one of the basic analytical techniques commonly used in quantitative analysis laboratories [5, 137]. It is primarily intended to determine the unknown concentration of the known sample volume by slowly adding a certain volume of titration standard (of known concentration). We use the amount of titrating agent so that the test substance reaches the so-called equivalence point without any residue. In order to clearly and accurately determine when the equivalence point occurred, a indicator is added to the titrated solution, which changes its colour in the equivalence point. Other analytical methods used to determine the equivalence point are so-called instrumental, e.g. potentiometric and acconductometric titration.

In their studies, Norton *et al.* and Ryan *et al.* [56, 138] present the determination of acids in grape juice and wine using the titration method. By titration, we determine the total acid content or tartaric acid content in grape juice or wine. This method cannot be used to determine individual acids.

Potentiometric titration belongs to techniques that provide reliable results on the acid content of the sample. Rajkovic [139, 140] monitored the influence of other organic substances contained in grape juice or wine on the acid content of white and blue varieties. In determining the total acid content of various types of wine by potentiometric titration, the effect of other substances on their composition was not recorded. Potentiometric titration or rather classic voltammetric titration supplemented with electrochemical potential measurement shows very good results for the analysis of white and red wines in 37 samples [141]. Both methods can be used to detect total acid content in different samples. Their main advantage is time efficiency, objectivity and accuracy as well as the minimisation of possible contamination of the sample because the sample is closed during the determination [141].

Classical titration is typically performed four times because the first time is only indicative. It is working with a 1-10 ml sample, which is diluted to 150 ml using distilled water and titrated with 0.1 M sodium hydroxide by using a glass electrode with pH 8.8 [142]. The endpoint or also point of equivalence is considered to be the value at which the pH reaches the maximum change with the addition of an alkaline base [10]. This value is different for each type of fruit, ranging from 7.2 to 8.4 [3, 143].

During titrating red grape juice or wine, the sudden change in colour can be hard to see. For a more accurate determination of acids, a pH meter or indicator paper can be used. It is also possible to apply the determination of acids using sodium hydroxide titration with activated carbon, which discolours the sample at the point of equivalence [144].

A sensitive, fast, and inexpensive square wave voltammetric method using a cobalt phthalocyanine modified carbon paste electrode was developed for simultaneous determination of citric, lactic, malic and tartaric acids in fruit juices. To overcome the strong overlap of voltammetric signals caused by calibrated and uncalibrated constituents, a multivariate curve resolution with alternating least squares (MCR-ALS) was used. The data were previous treated for correction of baseline and potential shift. The MCR-ALS calibration models were constructed and evaluated using a validation set obtained from a Taguchi design. As far as the authors Silva *et al.* [145] are aware, a voltammetric method that simultaneously determines four organic acids in complex samples such as fruit juices without any previous pre-treatment has not yet been reported in the literature.

4. CONCLUSION

The finding of malic acid and tartaric acid content in grape must and wine plays an important role in the wine production process. Their representation refers to the quality and maturity of the grapes, affects the final taste or may be an indicator of material adjustment.

Classical titration methods are rather retreating and replaced by instruments using special and professional analytical equipment. With titrations, we can meet with small winemakers who still use these methods for an indicative determination of the acid content. For more accurate analysis, however, they turn to accredited laboratories, which use special analytical instruments.

The most commonly used method in laboratories is HPLC. Its interconnection with spectroscopic or enzymatic techniques leads to a significant acceleration of the analysis with a possible identification of other substances contained in the wine. According to the prevalence of electrochemical methods, capillary electrophoresis is the most widespread. In some cases, this method is displaced by HPLC as it achieves considerable sensitivity and sensibility. Spectroscopic and enzymatic methods are not widespread and are used in combination with other techniques. Their association with chromatographic techniques leads to better results.

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