HPLC-IMER
as a Fast and Sensitive Method for the Determination of Sulfur Dioxide in Foodstuffs

Dissertation

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Bild auf dem Cover: Sulfitoxidase aus *Arabidopsis thaliana*.
(Schrader et al., *Structure* (2003))

Contents

Abstract

Sulfur dioxide is one of the oldest food additives and probably the most versatile one. For centuries, it has been used for the preservation of foodstuffs, to prevent browning reactions and to preserve natural food colors.

Due to possible adverse health effects and pseudoallergic potential, the application of sulfur dioxide and sulfites in foodstuffs is limited by the European Union.

Until today, the numerous official methods for sulfite analysis are not satisfying, as they are not only laborious but, most important, insufficiently sensitive and selective. Especially for the analysis of food not allowed to contain sulfites, or claiming not to contain any, a reliable and very sensitive method for the analysis of sulfites near the current legal limit of 10 mg/L is still owing.

This research project was supported by the FEI (Forschungskreis der Ernährungsindustrie e. V., Bonn), the AiF and the Ministry of Economics and Labour. It was accomplished in cooperation with the Forschungsanstalt Geisenheim.

HPLC-IMER (HPLC with immobilized enzyme reactor) is a combination of an isocratic HPLC system with an integrated immobilized enzyme reactor and electrochemical detection for the analysis of sulfites.

The goal of this work was to optimize the method for different food matrices, to compare different sulfite oxidases, and a comparison of the HPLC-IMER with the official method for sulfite analysis.

Several HPLC-IMER parameters were optimized, with a focus on an effective sample preparation for different foodstuffs. With a programmable autosampler, the effects of different parameters (reaction times of water, carbonate buffer, and sodium hydroxide) on the release of bound sulfites were studied. It was found that treatment with sodium hydroxide is essential in the release of bound sulfites, with longer reaction times required for samples rich in anthocyanins.

For the first time, an enzyme reactor with plant sulfite oxidase from *Arabidopsis thaliana* was employed in biosensor analysis, and its performance was compared with the animal sulfite oxidase from chicken liver (EC 1.8.3.1). The novel plant sulfite oxidase has a much broader linear range (0.04 up to more than 20 mg/L) than the animal enzyme (0.04–0.8 mg/L). Furthermore, the immobilized enzyme from *Arabidopsis thaliana* was shown to be a lot more stable in the course of

many analyses. Thus, regarding the application in HPLC-IMER, the plant sulfite oxidase is superior to the animal sulfite oxidase.

The general applicability of the HPLC-IMER for a number of different foodstuffs was shown. Many samples like fruit juices, fruit nectars, sugar sirups, jelly, wine and smoothies were successfully analyzed. In a storage study over seven months, the alteration of sulfite contents in a commercial red grape juice was examined with the HPLC-IMER.

Two modifications of the HPLC-IMER were developed and tested:

1. Replacement of the amperometric detector by a coulometric electrode array detector (CEAD) did not lead to the expected improvement in sensitivity.

2. For the analysis of low sulfite contents in very complex matrices, a distillation was performed prior to the HPLC-IMER (DE-HPLC-IMER). This modification, even though time consuming and laborious, allows for a reliable detection of sulfites in complex sample matrices, e. g. in an onion powder. As opposed to the official distillation method, the danger of detecting false positive results is minimized.

The optimized HPLC-IMER method was compared to the official distillatory method of sulfite analysis (IFU 7a) and the newly developed DE-HPLC-IMER in the diploma thesis of Kothe [55].

In almost all cases, the HPLC-IMER leads by far to the highest sulfite findings. The results for sulfite contents are up to 100% above those of the IFU 7a and the DE-HPLC-IMER. Additionally, the HPLC-IMER method generally shows a significantly lower detection limit, as well as a lower standard deviation.

After all, this method has many advantages compared to the official methods, as it is not only more sensitive and precise, but also simple to perform, automatable and fast.

Zusammenfassung

Schwefeldioxid ist vielleicht der älteste und vielseitigste bekannte Lebensmittelzusatzstoff. Seit Jahrhunderten wird SO_2 unter anderem zur Konservierung, zum Bleichen oder zur Farberhaltung von Lebensmitteln eingesetzt.

Aufgrund möglicher unerwünschter gesundheitlicher Nebenwirkungen, wie zum Beispiel pseudoallergischer Reaktionen, sind Schwefeldioxid und seine Salze, die Sulfite, in der EU nur begrenzt als Zusatzstoffe zugelassen.

Die analytischen Möglichkeiten zur Quantifizierung der Sulfitgehalte in Lebensmitteln sind bis heute so zahlreich wie unbefriedigend: die offiziellen Methoden nach §64 LFGB[1] sind methodisch aufwändig und nicht immer ausreichend spezifisch. Insbesondere für die Untersuchung (angeblich) ungeschwefelter Lebensmittel fehlt bislang eine zuverlässige und empfindliche Messmethode, die SO_2-Gehalte um den rechtlichen Grenzwert von 10 mg/L sicher bestimmen kann.

Die vorgelegte Arbeit wurde im Rahmen eines Forschungsprojektes aus Haushaltsmitteln des Bundesministeriums für Wirtschaft und Technologie (BMWi) über die Arbeitsgemeinschaft industrieller Forschungsvereinigungen „Otto von Guericke“ e.V. (AiF) gefördert und in Kooperation mit der Forschungsanstalt Geisenheim durchgeführt.

Die HPLC-IMER (HPLC mit immobilisiertem Enzymreaktor) ist eine Kombination aus einem isokratischen HPLC-System mit einem integrierbaren Enzymreaktor zur empfindlichen Analyse von Sulfiten in Lebensmitteln.

Ziel dieser Arbeit war eine Optimierung dieser Methode für verschiedene Probenmatrizes, der Vergleich unterschiedlicher Sulfitoxidasen und der Vergleich der HPLC-IMER mit der offiziellen Methode zur Sulfitanalytik.

Ein Schwerpunkt lag auf der Probenaufarbeitung für unterschiedliche Lebensmittel. Ein programmierbarer Autosampler ermöglichte die reproduzierbare Untersuchung der Auswirkungen verschiedener Parameter auf die Wiederfindung (z. B. Behandlung mit Wasser, Carbonatpuffer oder Natronlauge mit unterschiedlich langen Einwirkzeiten). Für die meisten Proben ist eine Vorbehandlung mit Natronlauge essenziell zur Freisetzung der gebundenen Sulfite. Dabei zeigte sich, dass die nötige Einwirkzeit für Lebensmittel mit hohen Anthocyangehalten länger ist als für andere Proben.

[1] Lebensmittel- und Futtermittelgesetzbuch; Methoden siehe Anhang B.4.

Zum ersten Mal wurde in der Analytik von SO_2 ein Enzymreaktor mit pflanzlicher SOx eingesetzt und mit tierischer Sulfitoxidase verglichen.

Der lineare Bereich der bereits zuvor in der Biosensor-Analytik eingesetzten tierischen SOx (0.04 bis 0.8 mg/L SO_2) aus Hühnerleber (EC 1.8.3.1) wird von dem pflanzlichen Enzym weit übetroffen (0.04 bis über 20 mg/L SO_2). Darüber hinaus zeigt sich die immobilisierte pflanzliche SOx auch durch ihre bessere Stabilität der tierischen überlegen.

Die Praxistauglichkeit der optimierten HPLC-IMER mit pflanzlichem Enzymreaktor wird für eine große Anzahl verschiedener Lebensmittelproben demonstriert: diverse Fruchtsäfte, Nektare, Zuckersirupe, Gelee, Wein und Smoothies wurden erfolgreich untersucht. Ein Lagerversuch mit rotem Traubensaft zeigte die Veränderung des Sulfitgehaltes über einen Zeitraum von über sieben Monaten.

Zwei Modifikationen der HPLC-IMER wurden entwickelt und getestet:

1. Der Ersatz des amperometrischen Detektors durch einen coulometrischen Elektrodenarray Detektor (CEAD); dies führt jedoch nicht zu der erwarteten Empfindlichkeitssteigerung.

2. Die Kopplung der HPLC-IMER mit einer vorgeschalteten Destillation (DE-HPLC-IMER) ermöglicht den sicheren Nachweis geringer Sulfitgehalte auch in chemisch sehr komplexen Probenmatrizes, wie zum Beispiel Zwiebelpulver.

Die optimierte HPLC-IMER wird in der Diplomarbeit von Kothe mit der offiziellen Methode für die Bestimmung von Schwefeldioxid in Fruchtsäften (IFU 7a) und mit der im Rahmen dieser Arbeit neu entwickelten DE-HPLC-IMER verglichen [55].

Dabei führt die HPLC-IMER in fast allen Fällen zu den höchsten Wiederfindungsraten für Sulfit. Die gefundenen Werte liegen bis zu 100% über denen der anderen beiden Methoden. Des Weiteren sind sowohl die Nachweisgrenze als auch die Standardabweichung der HPLC-IMER deutlich niedriger als die der destillativen Methoden.

Insgesamt zeigt die HPLC-IMER zahlreiche Vorteile gegenüber den üblichen Methoden. Sie ist nicht nur empfindlicher und genauer, sondern auch automatisierbar, schnell und einfach durchführbar.

Publications

Parts of this thesis have been published before:

Posters

Tschoepe M., Herzig B., Sprenger C., Theisen S., Galensa R.
Bestimmung von Schwefeldioxid in Aprikosen und daraus hergestellten Fruchtprodukten mittels HPLC/Biosensorkopplung (Enzymreaktorkopplung)
138th Regionaltagung NRW der Lebensmittelchemischen Gesellschaft, Bonn, 8th March 2006.

Theisen S., Hänsch R., Mendel R., Galensa R.
Sulfit-Bestimmung mittels HPLC-Biosensorkopplung - Vergleich von tierischer und pflanzlicher Sulfitoxidase
36. Deutscher Lebensmittelchemikertag, Nürnberg, 10–12th September 2007
Abstract: Lebensmittelchemie *62*, p. 115 (2008).

Theisen S., Bonn/D, Kothe L., Bonn/D, Galensa R.
HPLC-IMER as a sensitive method for analysis of SO_2 in fruit juices
27th International Symposium on Chromatography, Münster, 21–25th September 2009.

Oral Presentations

Theisen S.
Bestimmung von Schwefeldioxid in Früchten und Fruchtprodukten durch HPLC-Biosensorkopplung
1. Sitzung des projektbegleitenden Ausschusses des Forschungsvorhabens 14583, Bonn, 27th September 2006.

Theisen S.
Bestimmung von Schwefeldioxid in Früchten und Fruchtprodukten durch HPLC-Biosensorkopplung
2. Sitzung des projektbegleitenden Ausschusses des Forschungsvorhabens 14583, Geisenheim, 5th December 2007.

Theisen S., Dietrich H., Galensa R., Giehl A., Herzig B., Patz C., Tschoepe M.
DE-HPLC-IMER – eine Möglichkeit zur spezifischen und empfindlichen SO_2-Bestimmung in festen und flüssigen Lebensmitteln
Regionaltagung NRW der Lebensmittelchemischen Gesellschaft, Bonn, 5th March 2008.
Abstract: Lebensmittelchemie *62*, p. 137 (2008)

Theisen S., Kothe L., Tschöpe M., Galensa R.
HPLC-IMER als empfindliche Methode zur Bestimmung von SO_2 in Fruchtsäften
BGS-Professoren-Treff (Baumann-Gonser-Stiftung), Bonn, 6th November 2008.

1. Introduction

1.1. Sulfites as Food Additives

1.1.1. Use of Sulfur Dioxide in History

Sulfur (S_8) has been known and utilized by mankind since prehistorical times. In the ancient world, sulfur was, besides carbon, the only known nonmetal element. Its use as a bleaching agent for cotton, as part of matches or gunpowder and application in early pharmacy has been described [45].

Sulfur dioxide ($SO_{2(g)}$) is probably the oldest known food preservative. In ancient cultures in China, Greece or Assyria, sulfur was burned in order to get rid of evil ghosts and for disinfection purposes [62]. Egyptians and Romans are said to have used sulfuric vapors in the wine making more than a thousand years ago [10]. In medieval times, using sulfur dioxide had already become a common practice in the making of wine and must. Back then, the form of use was rather empirical, based on family customs and traditional recipes.

Some of the dangerous effects of sulfur dioxide have already been known hundreds of years ago, according to Beythien [9]. The sulfurization of wine became forbidden throughout Europe in the early 15th century due to its toxic effects on some people. It was a milestone, when Kaiser Maximilian (1459–1519) officially permitted the use of burned sulfur as an additive in 1487. He enacted the first legal limit on the sulfurization of wine, allowing to burn 1 loth of sulfur (about 16 g) for one funder (1200–1500 litres) of wine.

Very early in the 19th century, the use of sulfur dioxide as a common food additive – not only for wine – has already been established in Europe and the United States of America [82]. In 1810, Proust introduced calcium sulfite ($CaSO_3$) as a bleaching agent for the sugar beet industry in Europe, which was later replaced by sulfur dioxide gas. Especially in the United States, the application of sulfur dioxide was already common practice at that time. It was used for preservation purposes in many different foodstuffs, including meat and fish [10]. In 1902, the extensive use of sulfur dioxide on dried fruits that had been imported from the United States gave reason for the first health related publications on the use of sulfur dioxide in foodstuffs other than wine in Germany [8]. At that time, the scientific research on the effects of sulfur dioxide in food began to develop. France was one of the first countries to investigate the harmful effects of sulfites. As a consequence, in 1902 legal limits for the use of sulfites in winemaking were given by the French government [82].

Since then, a lot of research has been done on the helpful and harmful effects of sulfites in foodstuffs.

1.1.2. Sulfur Dioxide, Sulfite and Bisulfite

The terms "sulfur dioxide" or "sulfite" are commonly used synonymously to describe the oxo-species of sulfur in the oxidation state IV.

Sulfur dioxide is produced industrially by burning elementary sulfur (see equation 1), hydrogen sulfur (H_2S, shown in equation 2) or sulfite-containing ore like Pyrit (FeS_2, shown in equation 3) in an oxygen stream.

$$S_8 + 8\,O_2 \longrightarrow 8\,SO_2 \tag{1}$$

$$2\,H_2S_{(g)} + 3\,O_{2\,(g)} \longrightarrow 2\,H_2O_{(g)} + 2\,SO_{2\,(g)} \tag{2}$$

$$4\,FeS_{2\,(s)} + 11\,O_{2\,(g)} \longrightarrow 2\,Fe_2O_{3\,(s)} + 8\,SO_{2\,(g)} \tag{3}$$

Sulfur dioxide is a colorless gas that easily dissolves in water. 1 vol of water dissolves 80 vol of SO_2 at 0 °C, and 40 vol of SO_2 at 20 °C [45]. The gas has a pungent, irritating odour and is toxic to plants, animals and humans. The MAK-value is 5 mg/m^3, which is equivalent to about 2 ppm. It is nonflammable, corrosive to metals, and reacts violently with ammonia, acetylene, chlorine and ethylene. The structure of SO_2 is angulate, with a C_{2V}-symmetry. The angle between the sp^2-hybrided S-atom and the O-atoms is 119.5°.

In aqueous systems, SO_2 reacts with water to form sulfurous acid:

$$SO_2 + H_2O \rightleftharpoons H_2SO_3; \qquad K \ll 10^{-9}$$

Predominantly, the equilibrium is more on the left side of the equation, so there is a lot more free, dissolved SO_2 than actual sulfurous acid.

Sulfurous acid dissociates as shown in the following reaction:

$$H_2O + SO_2 \overset{pK_a=1{,}86}{\rightleftharpoons} HSO_3^- + H^+ \overset{pK_a=7{,}2}{\rightleftharpoons} SO_3^{2-} + 2\,H^+$$

The different states of dissociation depending on the pH range are displayed in Fig. 1.1.

In the normal pH range of food (pH 3–6), sulfites occur predominantly in the bisulfite form. HSO_3^- is usually in equilibrium with small amounts of sulfite and aqueous sulfur dioxide, their amounts depending not only on the pH value, but also on other factors like salt concentration (ionic strength) or the presence of certain non-electrolytes like ethanol [91].

At high concentrations, e. g. in the process of food drying, bisulfite (hydrogen sulfite) may dimerize to disulfite, which cristallyzes at very high concentrations:

$$2\,HSO_3^- \rightleftharpoons S_2O_5^{2-} + H_2O \text{ (disulfite/metabisulfite)}$$

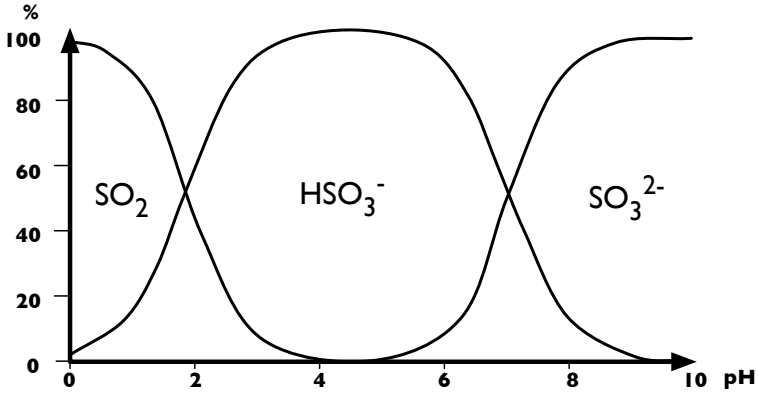


Figure 1.1.: pH curve of sulfur (IV) as described by Lück [62].

The sulfite ion has a trigonal pyramidal structure with a $C_{3\,V}$-symmetry and an O–S–O angle of 107.4°. It can be described with three equivalent resonance structures. Each consists of the central sulfur atom, which is singly bonded to two oxygen atoms (each with a negative charge) and double bonded to one oxygen atom with a formal charge of zero.

The expression "free sulfites" usually refers to the above mentioned forms of sulfite ions in aqueous medium, as there are $SO_{2\,(aq)}$, H_2SO_3, HSO_3^-, SO_3^{2-} and $S_2O_5^{2-}$.

In this work, the word “sulfites” will be used as the general term for all of these forms. When referring only to one specific form, this will be indicated by using the correct chemical term (e. g. sulfur dioxide or bisulfite) or, in the case of sulfite, by displaying the chemical formula (SO_3^{2-}).

In food, there are even more forms of sulfites than just the different dissociation forms. One has to differentiate between free and bound sulfites. Bound sulfites may be either reversibly or irreversibly bound to reactive molecules.

The free forms of sulfites can undergo several reactions with the different components in food. In the presence of transition metal ions (like iron, copper or manganese) and oxygen, sulfite is easily oxidized to sulfate. Thereby, the reducing effects are stronger in alkaline than they are in acidic solution [45].

This autoxidation of S(IV) has been described thoroughly by Danilewicz [24]. In acidic conditions, the reaction starts with the formation of a sulfite radical ($SO_3^{\cdot -}$) (oxidation state V) via metal sulfito complexes:

$$[Fe^{+III}(H_2O)_6]^{3+} + SO_3^{2-} \longrightarrow [Fe^{+III}(SO_3^{2-})(H_2O)_4]^{+} + 2\,H_2O$$
$$\longrightarrow [Fe^{+II}(H_2O)_6]^{2+} + SO_3^{\cdot -}$$

In the radical chain propagation, the sulfite radical reacts rapidly with an oxygen molecule to form the peroxomonosulfate radical ($SO_5^{\cdot -}$). This, in turn, may react with a free bisulfite ion to form the disulfate ion ($S_2O_7^{2-}$), which then rapidly hydrolyzes into two sulfate ions. This reaction can be inhibited by chelating agents such as EDTA or citrate, as well as alcohols (e. g. mannitol, ethanol), polyols and organic acids [109].

1.1.3. Reversibly Bound Sulfites

In foodstuffs, the predominating forms of bound sulfites are hydroxysulfonates, that are formed by reaction of HSO_3^- with reactive carbonyl groups. HSO_3^- is the most neutrophilic one of the S(IV) species, it is able to take part in addition reactions via either the sulfur atom or one of the oxygen atoms. The adduct resulting from bisulfite addition reaction through the oxygen atom leads to a sulfinate, whereas the adduct bound through the sulfur atom is called a sulfonate. It was demonstrated by Berké, that the formation of sulfonates predominates [7]. The basic mechanism of this reaction is shown in figure 1.2.

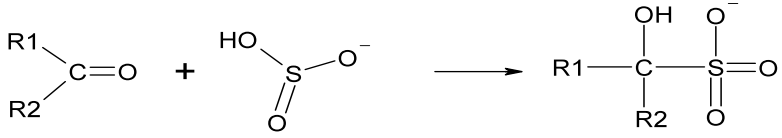


Figure 1.2.: Reaction of a carbonyl compound with bisulfite ion, leading to a sulfonate.

Typical carbonyl components in fermented beverages reacting with sulfites are acetaldehyde, arabinose, 2,5-diketogluconic acid, galacturonic acid, D-threo-2,5-hexodiulose, 2-ketoglutaric acid, pyruvic acid and L-xylosone [13, 15, 16, 14]. In unfermented beverages such as apple juice, the main S(IV) binding components are the sugars glucose, xylose and L-xylosone [39].

These adducts are decomposed only slowly upon acidification, but more rapidly when heated to boiling temperature. In alkaline media, the decomposition is significantly faster. In the analytical quantification of sulfites, this behaviour is used to determine the free, as well as the bound sulfites.

Besides the addition to aldehydes and ketones, an ionic addition to C–C double bonds may also lead to reversibly bound sulfites in foods. Examples are the addition to pyridine and flavin nucleotides, the addition to menadione (Vitamin K_3), as well as the addition reactions with uracil and cytosine [41]. Another important example is the addition reaction to anthocyanins, which leads to their decolorization as described in chapter 1.4.

1.1.4. Irreversibly Bound Sulfites

S(IV) species that are not recovered when boiling with acid for up to two hours and distilling the evolved SO_2 are considered "irreversibly bound". The majority of those are intermediates in non-enzymic browning reactions and products of the addition to disulfide bonds of proteins.

1.1.5. Preservative Effects on Food

Sulfur dioxide is probably the most versatile food additive. Due to its chemical reactivity, there are many different possible effects on food ingredients that make the sulfur species so interesting for food industries. Sulfites are most famous for their preservative character, as they have antimicrobial effects against bacteria, yeast and mold fungus. But sulfites are also able to inhibit enzymic and non-enzymic browning reactions. Furthermore, they are alleged to prevent oxidative spoiling, and sulfites are also helpful in industrial processes like extracting pectins from citrus, or anthocyanins from grapes [109, 82, 84, 115, 44].

1.1.5.1. Antimicrobial Effects

Undissociated sulfonic acid and dissolved sulfur dioxide, respectively, are the most effective forms of S(IV) against microbial organisms, whereas sulfites (SO_3^{2-}) have only little or no effects. Hydrogen sulfite, the sulfite form most prevalent at normal food pH values, still has remarkable effects against microbial spoilage, but the effects are less strong than those of the undissociated acid [82].

Furthermore, the intensity of the preservative effects is different for bacteria, yeast and mold fungus, and even within these groups, there are also some significant variations [62]. Rehm and Wittmann [82] described, that sulfur dioxide and hydrogen sulfites are most effective against bacteria and less effective against mould and yeast. The minimum concentration for an inhibitory effect on bacteria at a pH value of 6 varies between 500 mg/L and 2000 mg/L of sodium sulfite. For an inhibitory effect on yeast and mold, generally lower pH values or higher concentrations of sodium sulfite are required [82]. Some of that data is shown in table

1.1. Gram-negative bacteria are typically more sensitive to sulfur dioxide than the gram-positive ones [114].

Table 1.1.: Inhibitory effects of sulfite on microorganism, as reported by Rehm et al. [82]. MIC: minimum inhibitory concentration of Na_2SO_3.

Species	**pH**	**°C**	**MIC**
Bacteria			
Pseudomonas fluorescens	6	30	50 mg/100 mL
Bacillus subtilis	6	30	50 mg/100 mL
Escherichia coli	6	37	200 mg/100 mL
Aerobacter aerogenes	6	30	200 mg/100 mL
Yeast			
Saccharomyces cerevisiae	6	20	1750 mg/100 mL
Saccharomyces ellipsoideus	3.5	-	80 mg/100 mL
Pichia membranaefaciens	6	20	2500 mg/100 mL
Willia anomala	6	20	2500 mg/100 mL
Mold fungus			
Penicillium glaucum	6	20	1250 mg/100 mL
Aspergillus niger	5	30	350 mg/100 mL
Aspergillus niger	6	20	1250 mg/100 mL

1.1.5.2. Effects on Bacteria

Bacterial growth, in general, is supported by alkaline pH values. The addition of sulfur dioxide and a decline in pH value lead to an inspecific inhibition of bacterial growth. Besides that, there are three different mechanisms that may lead to the inactivation of bacteria. There is a) the inhibition of genetic sequences/processes, b) possible damage via the cell membrane and c) the interference with enzymes or enzyme reaction intermediates [82]. Enzymes have disulfide bonds to stabilize their tertiary structure. These bonds, if accessible, may be attacked by sulfite ions. A cleavage leads to an irreversible change in structure, and usually inactivates the enzyme or changes its functionality [108]. The reaction mechanism of the sulfite ion with disulfide bonds is described by Wever as follows [114]:

$$R_1{-}S{-}S{-}R_2 + HSO_3^- \rightleftharpoons R_1{-}SH + R_2{-}S{-}SO_3^-$$

1.1.5.3. Inhibition of Yeast

Of the different forms of sulfite, only the non-ionic form, the SO_2, is able to enter the yeast cell. SO_2 passes the cell wall through free diffusion across the lipid domains of the membrane. As mentioned before, the amount of SO_2 in aqueous solution is strongly dependant on the pH value, and its percentage increases in acidic media (see figure 1.1). This explains the strong inhibitory effects of sulfur species at low pH values. Once inside the cell, sulfur dioxide can execute its destructive power as a preservative. The mechanism of yeast inhibition is based on three main effects: firstly, the pH value inside the cell is less acidic, usually it is slightly below 7. Therefore, most of the acidic SO_2 is converted into hydrogen sulfite (see figure 1.1). The concentration of SO_2 decreasing inside the cell, allows for even more SO_2 to pass the yeast cell membrane. Secondly, the conversion of SO_2 into HSO_3^- generates acid inside the cell, which is challenging the internal buffering capacity of the yeast cell. Thirdly, as mentioned before, the hydrogen sulfite ion is a very reactive molecule, leading to various chemical additions to and modifications of vital cell constituents. All of this will eventually lead to the death of the cell [84].

Not all yeast stems are equally sensitive to the exposure to sulfur dioxide. Some yeasts are even known to produce and release sulfites themselves. These yeast stems may account for up to 20 mg/L SO_2 in unsulfurized alcoholic products, some are even capable of producing up to 100 mg/L SO_2 [12, 27, 26].

1.1.5.4. Inhibition of Enzymic Browning

The most relevant enzyme involved in enzymic browning is the polyphenol oxidase. Enzymic browning occurs on cut surfaces e. g. of apples and potatoes. Through the cutting, the polyphenol oxidase is released from cell vacuoles and gets into contact with plant polyphenols, especially monophenols and o-diphenols. In the presence of oxygen, a browning reaction is visible.

Sulfite is able to prevent this browning reaction by adding to the quinone intermediate to give a substituted o-diphenol which does not continue its reaction to brown high molecular weight products [48, 30]. Another proposed mechanism is the reduction of the quinone, leading to oxidation of the sulfite ion to sulfate ion as presented in figure 1.3.

Furthermore, Schroeter stated, that a sulfite-induced inactivation of phenolases may also contribute to the inhibition of browning reactions through sulfites [90].

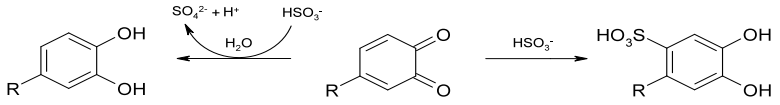


Figure 1.3.: Reactions of bisulfite ions with quinones, figure according to Danilewicz et al. [24].

1.1.5.5. Inhibition of Non-enzymic Browning

The Maillard reaction is probably the best-known example for non-enzymic browning. Reducing sugars and amines start this reaction, forming a glycosylamine and then a Schiff base. A cascade of reactions follows, leading to manifold aromatic and brown products, i. e. the melanoidins. The very reactive intermediates of this reaction have α, β-unsaturated dicarbonyl groups that may react readily with sulfites to form hydroxysulfonates. Wedzicha [111] gave detailed information about the mechanism of the Maillard reaction with glucose, glycine and sulfite. The hydroxysulfonates then prevent any further steps in the browning reaction. Sulfites that are bound to glucose were reported by Ingles et al. to be irreversibly bound, as these sulfonic acid derivatives do not break down to yield sulfur dioxide in the Monier-Williams distillation [47].

The non-enzymic browning reaction involving ascorbic acid is interrupted by a similar reaction with sulfites, also leading to 3,4-dideoxy-4-sulfo(-pent-/-hex-)osuloses [110, 68]. Not only does sulfur dioxide prevent a non-enzymic browning reaction, it is also capable of bleaching the melanoidins of an already browned product. This mechanism is considered to involve the reaction of a polarized C=C bond of the melanoidin with sulfite, leading to irreversibly bound sulfur dioxides [109, 68].

1.1.6. Undesired and Toxicological Effects of Sulfites

Sulfites used to be considered safe food additives for a long time, but there are several adverse health effects that have caused new considerations.

A reaction, rather undesired in foodstuffs, is the cleavage of thiamin (vitamin B_1) by the sulfite ion [114, 108, 62], shown in figure 1.4. This reaction is irreversible. Since meat is generally regarded a valuable source of thiamin, the use of sulfites in meat products is restricted in many countries [9].

The capacity of the human body to metabolize sulfites is extremely high. Humans produce about 1680 mg of endogenous sulfite every day (mostly out of cystine),

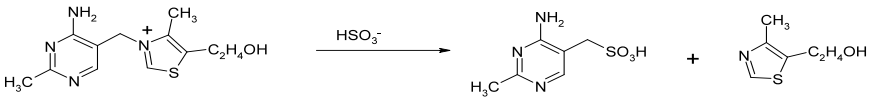


Figure 1.4.: Irreversible thiamin cleavage by the sulfite ion, as described by Wever [114].

which are quickly metabolized and excreted as sulfates [41, 100, 32]. It is reported, that the human body is able to metabolize up to 40 times more sulfites than the typical amount of intake through foods [32].

Theoretical calculations based on *in vitro* assays of sulfite oxidase suggest, that the enzyme is theoretically able to oxidize up to 48 g/kg(bodyweight)/day of SO_2 [21], this showing the high capacity of the sulfite oxidase enzyme. Considering these calculations, it is not amazing, that sulfites were formerly categorized GRAS (generally recognized as safe) [77].

Nevertheless, the external intake of sulfites may cause severe health effects in some people, as will be described below. It has even lead to some cases of death [94], especially with the very sensitive group of people like those with asthmatic diseases.

1.1.6.1. Metabolism

The oxidation of sulfite to sulfate by sulfite oxidase is the final step in the catabolism of sulfur-containing amino acids, particularly cysteine and methionine. The same pathway is relevant for the oxidation of exogenous sulfites, where sulfites are ingested with the food [41].

The acidic medium of the stomach releases bound sulfites and leads to the formation of sulfur dioxide. Sulfur dioxide can be resorbed by the mucosa cells of the gastrointestinal tract. From there, it is transported to the liver via the portal vein blood stream, where the metabolization to sulfate takes place [114].

The human liver has sufficient oxidation capacity for large amounts of ingested sulfites, the half-life of sulfites in a human organism is described to be about 15 minutes [114]. The enzyme responsible for the oxidation of sulfites is the sulfite:cytochrome *c* oxidoreductase, the sulfite oxidase (E.C. 1.8.3.1). This enzyme is found to be present in all mammalian tissues, but mainly in the liver, followed by kidney and heart. It is located in the mitochondrial intermembranous space.

In the in-vivo oxidation of sulfites, a pair of electrons of the sulfite ion is transferred to the Mo(VI) and then to the heme group of the enzyme. From there, the electron

pair is passed on to cytochrome c of the respiratory chain, where $\frac{1}{2}O_2$ is reduced to H_2O, producing one molecule of ATP in the process. The resulting sulfate can be rapidly excreted in the urine [100].

For humans, there is no general intake limit for sulfites, that can be regarded to have no adverse effects. For some people, the intake of as much as 4 g of sulfites shows no health effects at all, whereas others suffer after the intake of very small amounts.

In animals, subchronical toxicity symptoms are mainly a deficiency of vitamin B_1, accompanied by diarrhea, depression of growth and a reduction of feed intake and conversion [62]. Symptoms of chronic toxicity in animals are impairment of the nervous system, as well as damage to the reproductive organs, the bone tissue, the kidneys and other organs [62].

1.1.6.2. Intolerance Reactions to Sulfites

The danger of sulfite-intake lies in the wide variety of possible intolerance reactions of sulfite sensitive people.

By the U.S. Food and Drug Administration (FDA), sulfites were considered to be generally recognized as safe (GRAS) when the Federal Food, Drug, and Cosmetic Act was amended in 1958. This status was later abolished after reevaluation, due to several reported cases of adverse health reactions to sulfited foods [77].

Typical acute symptoms after excessive SO_2-intake are asthma, headaches, nausea, and gastritic reactions like abdominal pain, vomiting and diarrhea [59, 62, 32]. An ingestion of sulfite salts may cause allergic reactions, but far more often it leads to pseudoallergic reactions. The most common adverse effects in sensitive or allergic persons are acute bronchospasm, bradycardia, hypotension and skin lesions like urticaria and severe flushing [94, 114, 33]. Tingling and pruritus have been described as well. Even a few cases of death after consumption of sulfited foods have been reported [100]. Lately, also a relationship of sulfite intake with tinnitus has been discussed, hypothesizing that the avoidance of sulfites may bring relief of the tinnitus for individuals with sulfite intolerance [56].

It is known that asthmatics who show severe symptoms and are dependant on corticosteroids are especially prone to sulfite sensitivity [77]. There are different data in the literature on the prevalence of sulfite sensitivity in asthmatic population, ranging from 1–11% of asthmatics suffering also from sulfite intolerance [114, 94, 97]. In a study with sulfite-treated lettuce, all five out of five patients with asthma showed adverse reactions after the intake of about 64–108 mg of sodium bisulfite (equivalent to 32–64 mg of sulfur dioxide). The intensity of reactions varied, including flushing and itching in the mouth, throat and skin, up to a severe reaction with progressive dyspnea [46, 17].

Drugs for asthmatic people, which contain sulfites, may be especially harmful and dangerous [83], since asthmatic persons are most at risk of suffering from an intake of sulfur dioxide.

The rapid onset of symptoms after the ingestion of sulfited food is typical for the pseudoallergic asthmatic reaction [42]. This reaction is usually triggered as soon as a few seconds up to two minutes after the exposure. The pathway here is different from the typical pathway of sulfite oxidation in the human body. In the pseudoallergic reaction, ingested sulfites are transported from the stomach to bronchial mucosa, where, at the surface, SO_2 is formed and released into the air containing bronchi and alveoli, thus causing the asthmatic reactions [97].

Until today, sulfite threshold levels have not been systematically assessed in asthmatic or non-asthmatic persons. Amounts as low as 1 to 5 mg of ingested potassium metabisulfite (equal to 0.3 to 1.4 mg of SO_2) have been reported to have caused reactions in some sulfite sensitive individuals [96], and most sulfite sensitive people show adverse reactions to ingested metabisulfites in the ranges of 20 to 50 mg [33].

Very few challenge studies with sulfited foods have been conducted. Most of the studies used sulfite containing capsules or liquids, or the patients inhaled sulfite containing sprays. The described adverse reactions were varying depending on the form and the amount of application. The situation of sulfites in food may even be different, as e. g. some of the combined forms may not contribute to the allergic potential. Food challenges will help to define the risks associated with sulfited foods and thus eventually lead to an adjusted threshold for sulfites in food.

The European Food Safety Authority (EFSA) states, that the threshold for sensitivity reactions may be lower than the legal limit of 10 mg/kg SO_2, "however, threshold levels have not been systematically assessed and the smallest concentration of sulfites able to provoke a reaction in a sensitive person is unknown" [33].

Sulfur dioxide amounts of less than 10 mg/kg in food are considered not to be existent, not because they are of no danger for sulfite sensitive individuals, but because "the assay used to detect the level of sulfites in foods is not sensitive enough to detect amounts less than 10 mg/kg" [33].

1.1.7. Sulfites in food law

1.1.7.1. Former judicial legislations

Sulfites have been used in winemaking for hundreds of years. Not surprisingly, the first German regulations about the use of sulfur dioxide were concerning wine (Weingesetz (1892)) and fruit (Fruchtbehandlungsverordnung (1961-1967)) [119]. The first general regulation about SO_2 in foodstuffs in Germany was enacted in 1969 (Bundesgesetzblatt I S. 1326), the Verordnung über die Verwendung von Schwefeldioxid. This regulation covered the maximum addition limits of sulfur dioxide and sulfites to foods, as well as their declaration [89].

1.1.7.2. Actual legislations

In German food regulations, sulfur dioxide and sulfites, when added to foods for technological purposes, are considered food additives.

General regulations concerning food additives are found in the Lebensmittel- und Futtermittelgesetzbuch (LFGB)[1]. §2 (3) LFGB gives the definition of food additives, whereas §§ 6 and 7 cover the prohibition of unallowed use and the legislative authorization. Based on this authorization, the German Zusatzstoff-zulassungsverordnung (ZZulV) lists sulfur dioxide and seven sulfite salts as permitted additives (see table 1.2).

Table 1.2.: Sulfur dioxide and sulfites allowed for use in food (according to ZZulV).

E-Number	Additive
E 220	Sulfur dioxide
E 221	Sodium sulfite
E 222	Sodium hydrogen sulfite
E 223	Sodium disulfite
E 224	Potassium disulfite
E 226	Calcium sulfite
E 227	Calcium bisulfite
E 228	Potassium bisulfite

[1] See appendix A.2 for detailed information on all legislations mentioned in the text.

Their use is permitted for specified foods only, and the given limits must not be exceeded. The allowed concentrations vary in the range of 10–2000 mg/kg for solid samples (or mg/L for liquid samples, respectively) of sulfites, calculated as SO_2. For example, an amount of 10 mg/kg is the legal limit for SO_2 in grapes, fresh litchi and sugars (except glucose sirup), 20 mg/L for beer, whereas dried fruits may contain sulfur dioxide up to 1000 mg/kg (bananas) or even 2000 mg/kg (e. g. apricots, grapes, figues). The complete list is presented in appendix B.2 on page 134.

In all cases, added sulfites and sulfur dioxide exceeding 10 mg/kg or 10 mg/L in the food, have to be declared. There are different ways to properly inform the consumer. If there is no list of ingredients with the food, the addition of sulfites has to be declared as "geschwefelt" (engl. “sulfurized”), according to §9 (1) no. 5 ZZulV. In §9 (6) ZZulV it is specified how this declaration has to be accomplished: the information has to be marked in a conspicuous place in such a way as to be easily visible, clearly legible and indelible. The information may either be posted on a sign close to the foodstuff, on the package of the foodstuff or, e. g. in a restaurant, on the menu. Alternatively, the additive can be listed as a regular ingredient in the list of ingredients (§9 (8) no. 2 ZZulV in conjunction with §3 (1) 3 LMKV).

In addition to the ZZulV, there are also regulations concerning the declaration of sulfites and sulfur dioxide as potential allergens in foodstuffs.

Sulfites are known to have pseudoallergenic potential, as described in chapter 1.1.6.2. Since 2003, the European Union requires the explicit declaration of twelve, since the year 2006 of fourteen, ingredients that are known to have an allergenic potential, among them sulfur dioxide and sulfites.

The DIRECTIVE 2000/13/EC in its actual version (last changed by the DIRECTIVE 2007/68/EC) was transposed into german national law by the latest version of the Verordnung über die Kennzeichnung von Lebensmitteln (LMKV).

According to the LMKV, sulfites in foodstuffs have to be declared whenever the concentration of SO_2 exceeds 10 mg/kg or 10 mg/L, respectively. Lower amounts are considered to be non-existent. The declaration has to be either part of the list of ingredients or, if there is no list of ingredients, the information has to be given elsewhere on the package or near the foodstuff, comprising the word "contains", followed by the name of ingredient(s) concerned (Art. 6 paragraph 3a of DIRECTIVE 2000/13/EC).

As mentioned before, the information is to be "easy to understand and marked in a conspicuous place in such a way as to be easily visible, clearly legible and indelible. They shall not in any way be hidden, obscured or interrupted by other written or pictorial matter" (Art. 14, no. 2 of DIRECTIVE 2000/13/EG).

These regulations are supposed to ensure "better information and to protect the health of certain consumers", as mentioned in the recital no. 11 of the DIRECTIVE 2003/89/EC.

1.2. Quantitative Analysis of Sulfites

1.2.1. Methods for Sulfite Analysis

The analysis of sulfur dioxide and sulfite salts is not trivial. As described earlier, sulfites are very reactive substances, that may be difficult to capture. Not only are they easily oxidized to sulfate, they may also escape from the analysis as gaseous sulfur dioxide or react readily with other compounds before being detected. A reliable analysis becomes even more difficult, as chemically similar molecules are sometimes erroneously mistaken for sulfites.

Until today, there are very many different methods for the analysis of sulfur dioxide in food. This great amount of analytical methods shows the great interest in a reliable, reasonable and straightforward sulfite analysis, and it is an indication for the difficulty to find one method that serves well for all cases. Most of the analytical approaches have drawbacks that limit their application to certain prospects.

Maybe the first, and the most simple method for sulfite analysis is the direct titration of a sulfite containing sample with iodine solution. Sulfite is easily oxidized to sulfate by reducing iodine to iodate. As sulfites are not the only potential reactants with iodine, this method works well only in solutions without interferences.

Probably the most common and widespread method until now, is the one developed by Monier-Williams in 1927 [73]. This method is composed of a destillation with a titrimetric quantification of the distilled sulfur dioxide. It can be applied successfully to almost all kinds of foods for the determination of free, as well as of total SO_2. For many years, this method was considered to be the most reliable method for the determination of SO_2 in food [49].

There have been numerous refinements over the years, adjusting the method to particular applications, but the basic approach is always the same. Because of its importance for sulfite analysis, the basic setup will be described in the following paragraphs:

Acidic solution (phosphoric acid or hydrochloric acid) is added to the food sample, turning the free sulfites into gaseous SO_2. When the mixture is heated to boiling temperature, not only the free sulfites are turned into SO_2, but additionally most of the bound sulfites are liberated as SO_2 as well. The mixture is refluxed, typically under nitrogen flow as carrier gas, and the gas is then bubbled through a solution of hydrogen peroxide. Oxidized by the peroxide, the sulfur dioxide turns into sulfuric acid and can then be quantified by end-point titration with standardized hydroxide solution. The amount of sodium hydroxide necessary to neutralize the

acidic solution allows for the calculation of the initial sulfite concentration of the sample.

This procedure requires only little more than basic glassware and standard commercial reagents, making it a rather low-cost and easy-to-apply method, even in small, low-tech laboratories. However, for the analysis of a large number of samples, the immense time effort for one analysis becomes a disadvantage. Probably the main drawback of the Monier-Williams method is the difficult handling of the apparatus. To achieve the required selectivity and sensitivity for a precise result in a complex matrix requires a lot of skill and experience [48]. Also, the method is rather laborious, as there are no possibilities for automatic sample preparation or analysis.

The Monier-Williams method serves well for the determination of rather large amounts of SO_2. Yet in the critical concentration range of 10 ppm and lower, the results are not reproducible and reliable enough to ensure a correct analytical result. The destillation is prone to false-positives, as volatile acids may accompany the refluxed sulfur dioxide, lowering the pH in the receiving flask and so leading to an overestimation of the amount of sulfites. On the other hand, there is also a danger of too low findings of sulfur dioxide, especially with unexperienced personnel. If the gas flow is set too fast, or the apparatus is not perfectly leakproof, some of the liberated SO_2 may escape before being oxidized to sulfate.

Trying to overcome the disadvantages of the Monier-Williams method, there have been innumerable attempts to find a better method for determination of sulfites in food matrices. These methods include ion-chromatography [4, 54, 61, 85], flow-injection analysis [18, 19, 65, 40, 63, 72, 86, 104, 99], and numerous electroanalytical methods, like the cyclic voltammography [80, 87] to mention only a few.

Also several less common methods have been described, like the isotachophoretic determination [58], differential pulse polarography [118], diffuse reflectance fourier transform infrared spectroscopy (DRS-FTIR) [105] and capillary electrophoresis [11, 103]. To give an impression of the variety of different methods for the quantification of sulfites in foods one has to mention also the gasdiffusion separation [34, 92], the chronopotentiometry [93] or the combination of liquid core waveguide and light intensity difference technique [102].

Most of the methods mentioned above require either some very expensive laboratory equipment, a laborious calibration or sample preparation procedure, or their detection limits are above the legal limit of 10 mg SO_2 per litre or kilogram. Most of the methods are found to be inaccurate or have limited uses, especially with more complex matrices.

The HPLC-IMER presented in this work is able to overcome these drawbacks, as it is applicable for almost all kinds of food samples with little sample preparation

and short times for analyses. The HPLC-IMER coupling combines the benefits of chromatographic separation with the specificity of the enzyme sulfite oxidase and the sensitivity of an electrochemical detector to easily yield accurate results with an extremely low detection limit.

1.3. HPLC Coupled with an Immobilized Enzyme Reactor (HPLC-IMER)

The coupling of an HPLC system with an integrated sulfite oxidase reactor between the column and the detector was first described in the dissertation thesis of Pabel in 1993 [75]. At that time, the method was still at a very early stage, but a general applicability for sulfite standard solutions was shown. This combination of an enzyme coupled with an electrochemical detector is a special form of a biosensor, as will be described in chapter 1.3.1.1. The goal of this new setup for the analysis of sulfites in foodstuffs was to profit from a separation of the analyte from other sample compounds prior to the very sensitive detection of hydrogen peroxide, selectively produced by the enzyme sulfite oxidase.

In the book "Food biosensor analysis", Galensa described the principle of postcolumn detection and its advantages in food analysis thoroughly [107].

In the first experiments with HPLC-IMER, Pabel found that the linear range for sulfite determination was between 0.002 and 0.05 mmol/L Na_2SO_3 (or 0.125–3.15 mg/L SO_2). The detection voltage was set at 700 mV, the eluent was a phosphate buffer (0.1 mol/L, pH 7.3) and the best suited column was an Aluspher 60, RP-select (Merck, 125x4 mm i. d.). The flow rate was 0.5 mL/min and the retention time was 2 minutes. However, with these parameters it was not possible to prevent a tailing of the sulfite peak. Analyses of real food samples were not presented in that work.

Several modifications and advancements of the method were later presented by Weßels [113, 112]. In his thesis, Weßels investigated and optimized most of the method parameters. The phosphate buffer was replaced by a carbonate buffer, making it possible to lower the detection potential to 0.1 V. This is advantageous, as a higher cell potential typically forces a reaction of more substances, making the detection less specific. Also, a faster electrode fouling was observed with higher potentials. The lower the potential on the detector cell, the less interferences occur, and the longer an electrode is not affected by electrode fouling. Part of this improvement was also the change of the detector model from a biometra with thin-layer electrode to a Trace® system with a thick layer electrode.

Weßels exchanged the Aluspher column with an anion exchange column (Carbopak PA-100, Dionex) for better long-term stability.

The former steel-cartridges for the immobilized enzyme were replaced by polycarbonate cartridges with a larger volume. In contrast to the steel cartridges, the polycarbonate cartridges can be filled by hand without applying pressure. The different packing method did not affect the dead volume of the HPLC as suspected, the peak half-width did not change significantly.

An optimization of the stability of the sulfite standard solution was achieved by addition of EDTA and fructose to the stem solution as well as to the diluted standard solution. A stem solution with EDTA and fructose was shown to be stable during the period of one week when properly stored in a refrigerator at 4°C.

The HPLC-IMER was successfully applied to different food matrices like beer, wine, grape juice, apple juice and even pepper. The sample preparation included the dilution of the sample with carbonate buffer (pH 10.6) for the release of combined sulfites. The dilution factor was between 10 and 20 for beer samples, whereas grape juices sometimes needed more (or more concentrated) buffer for a successful release of the bound sulfites.

The linear range was between 0.02 and 4.0 mg/L SO_2 with a correlation coefficient of 0.9997, the injection volume was 10 μL.

The results of the HPLC-IMER for beer and red wine samples were compared to the results of other well established, distillative methods. For the higher SO_2-contents of the wine samples there was a fairly good correlation to the method of Rebelein [81], the correlation coefficient was 0.984. For the beer samples with SO_2-contents of 3.8–7.6 mg/L, the correlation coefficient was only 0.9137.

A decrease of sulfite peak areas was observed at long running times of the system. Therefore, a one point calibration was recommended after every three to four sample injections. The decrease in performance has two main potential causes: a weakening of enzyme activity, or a fouling of the electrode surface. In his experiments, Weßels showed that it was the electrode fouling that was responsible for the observed slow decrease in sulfite peak areas over longer periods of working times. In contrast, the enzyme reactors were very stable over long periods of time.

Further optimization of the method was in 1997 presented by Patz, Galensa and Dietrich [78]. An interlaboratory comparison based on the method presented in the work of Weßels [112] had shown difficulties in finding the true total amount of sulfites, especially in samples that were rich in polyphenols as i. e. wines and grape juices. For those samples, the authors suggested a more intense alkaline pre-treatment in order to release larger amounts of the bound sulfites.

Taking into account all of the research that has been previously conducted, the improved HPLC-IMER conditions were chosen as a starting point for our research. Knowing that the intensity and the reaction time of the alkalization procedure prior to injection had strong effects on the release of bound sulfites, theses parameters were to be optimized for a large amount of different food samples.

1.3.1. Elements of the HPLC-IMER

The HPLC-IMER as it is presented in this work consists of an isocratic HPLC system with an integrated sulfite oxidase reactor. A schematic view of the setup is presented in figure 1.5.

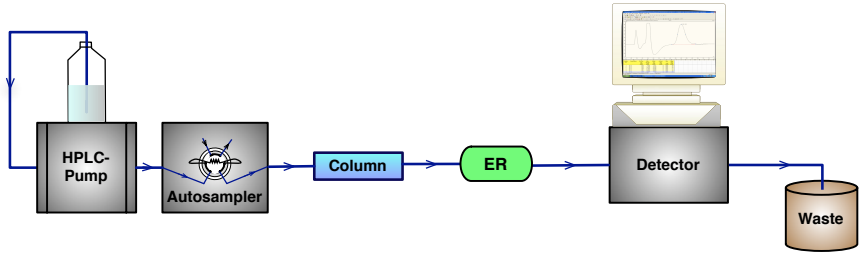


Figure 1.5.: Basic setup of the HPLC-IMER. The enzyme reactor (ER) is located between the column and the electrochemical detector.

Prior to injection into the HPLC, the sample is treated with alkaline solution in order to release bound sulfites and to turn all forms of sulfur(IV)-oxo species into the desired ionic sulfite (SO_3^{2-}) form. Figure 1.6 gives a rough overview of the pH dependency of the sulfur-oxo (IV) species. More detailed information is given in chapter 1.1.

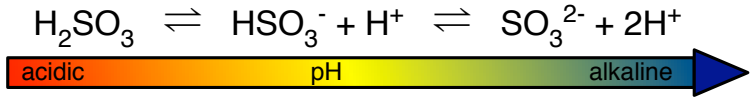


Figure 1.6.: pH dependant forms of sulfites in aqueous solution.

When passing through the column, the analyte (SO_3^{2-}) is separated from other compounds of the sample. The enzyme sulfite oxidase, which is immobilized on the carrier material within the enzyme reactor, specifically oxidizes sulfite ions to sulfate, producing equivalent amounts of hydrogen peroxide (see figure 1.7).

The hydrogen peroxide is then electrochemically detected by an amperometric detector.

Not only hydrogen peroxide, but also the sulfite ion is electrochemically active. It is possible to detect sulfites without an enzyme reactor, but higher voltages are

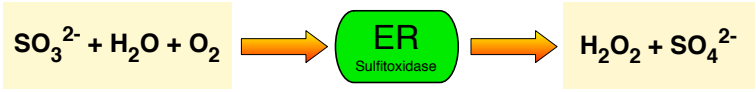


Figure 1.7.: Oxidation of sulfite to sulfate by the sulfite oxidase enzyme reactor. Equivalent amounts of hydrogen peroxide are generated.

necessary for a sensitive result. At the applied voltage of 0.2 V, the signal caused by hydrogen peroxide is about 40 times higher than a signal induced by sulfites, as will be discussed in chapter 3.2.5.

1.3.1.1. Biosensors

Biosensors are a subgroup of chemical sensors. By the IUPAC[2], a biosensor is defined as "a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" [67]. Typical elements in a biosensor are a biological recognition element, a physical transductor, an electrical amplifier and a data processing system [95]. The basic setup of a biosensor is presented in figure 1.8.

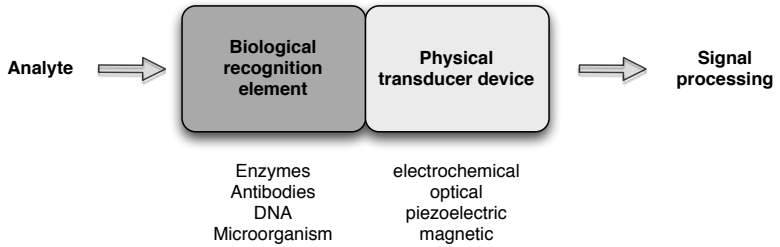


Figure 1.8.: Typical components and setup of a biosensor. Figure according to Schmid et al. [88].

The biological sensing element of the biosensor can either selectively change (i. e. with an enzyme or microorganism) or detect (i. e. with the aid of an antibody)

[2]International Union of Pure and Applied Chemistry

the specfic analyte in a complex, diluted sample matrix [88]. Until today, several enzymes, immuno-compounds, receptors, nucleic acids, microorganisms or even plant or animal tissues have been described as the biological compound of a biosensor [88, 95, 37].

Physical transductors for biosensors may be optical, electrochemical, thermoelectric, piezoelectric, or magnetic devices [107].

In 1962, the first biosensor system was described by Clark et al., who immobilized the enzyme glucose oxidase (EC 1.3.3.1) on an oxygen electrode in order to measure the blood sugar content [20]. Since then, hundreds of different biosensors have been developed, and many of them have become standard applications in the analysis of complex samples.

The amperometric biosensor with an oxidase as biological element and a platinum electrode for the detection of hydrogen peroxide, similar to the HPLC-IMER method described in this work, is a combination commonly used for numerous analytical tasks.

The combination of an enzyme reactor with an electronic device for detection is called a "biosensor", in the strict sense of the term, only when there is a direct spatial combination of both. In the method presented in this work, the biologically active substance is not directly connected to the detector, as they are divided by a capillary. Therefore, it is more precise to speak of HPLC with an immobilized enzyme reactor (HPLC-IMER) than of a biosensor, even though both concepts are very similar, and the term "biosensor" is better-known.

1.3.1.2. The Enzyme Sulfite Oxidase

Enzymes are ubiquitous in all living organisms. Most of all enzymes are globular proteins. They are biomolecules that catalyze chemical reactions by lowering the activation energy, thus dramatically increasing the rates of reactions by factors of at least a million. The reactions catalyzed by enzymes are very specifc, as enzymes are extremely selective for their substrates.

There are six enzyme subgroups named after the sorts of reactions that are catalyzed:

1. oxidoreductases
2. transferases
3. hydrolases
4. lyases
5. isomerases

6. ligases

All enzymes are categorized by numbers by the NC-IUBMB[3] [74]. Sulfite oxidase from plant or animal, for example, is categorized with the Enzyme Commission (EC) number 1.8.3.1. The groups and subgroups are: 1 (Oxidoreductase), 8 (Acting on a sulfur group of donors), 3 (With oxygen as acceptor), 1 (number of enzyme within the subgroup).

Three different kinds of sulfite oxidizing enzymes are presently known [35]:

1. sulfite oxidase in animals (E.C. 1.8.3.1)
2. sulfite oxidase in plants (E.C. 1.8.3.1)
3. sulfite dehydrogenase in bacteria (E.C. 1.8.2.1)

The chemical reaction catalyzed by all sulfite oxidases is the oxidation of sulfite to sulfate, leading to equivalent amounts of hydrogen peroxide in the presence of oxygen and water (see chapter 1.7 on page 22).

Sulfite oxidizing enzymes protect cells against damage caused by exposure to internal and external sources of sulfite. As explained earlier (in chapter 1.1.5 on page 6), sulfites can cause fatal damage to DNA and proteins within a cell.

All sulfite oxidases are mononuclear molybdenum proteins, containing a molybdopterin cofactor (Moco). They are found to have nearly identical square pyramidal coordination of five ligands around the Mo atom in the fully oxidized Mo(VI) state. Those ligands are two oxo-ligands (one of which is axial and one equatorial) and three equatorial sulfur ligands [31]. The proposed catalytic cycle of sulfite oxidation to sulfate by a sulfite oxidizing enzyme is shown in figure 1.9. In step 1 (**A** → **B**), the equatorial oxo-ligand forms a complex with the sulfite ion, leading to a sulfate ion coordinated to the Mo(IV). In a second step (**B** → **C**), the sulfate ion is replaced by water or hydroxide. Via the oxidation state (V) (**D**) of the molybdenum centre, the enzyme is brought back into the fully oxidized resting state Mo(VI)(**A**).

Even though the basic underlying mechanism is the same for all sulfite oxidizing enzymes, there are relevant differences in the structure and the complete oxidation mechanism between all three sulfite oxidizing enzymes.

Animal Sulfite Oxidase

The animal sulfite oxidase is a homodimeric enzyme, located in the intermembrane space of mitochondria of higher animals and birds [35]. Animal (and human) sulfite oxidase is found in almost all parts of the body, with especially large quantities

[3] Nomenclature Committee of the International Union of Biochemistry and Molecular Biology

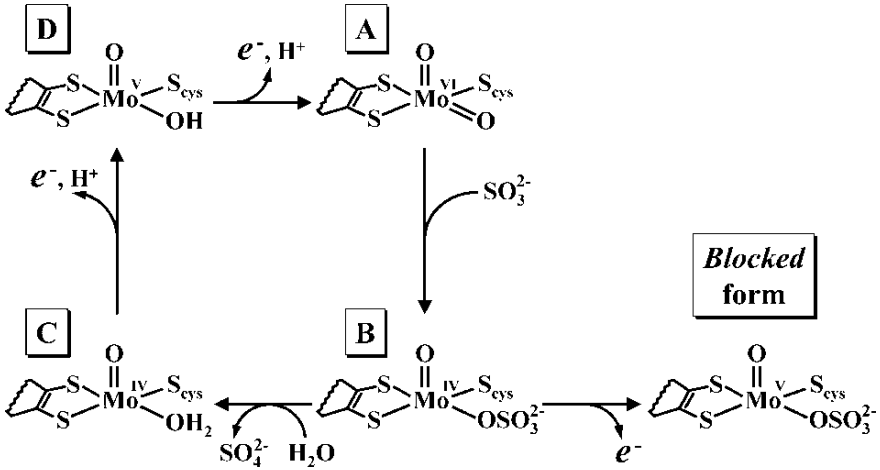


Figure 1.9.: Catalytic cycle of sulfite oxidizing enzymes. Illustration by Enemark et al. [31].

located in the liver, kidney and heart, whereas only small amounts are found in the spleen, brain, skeletal muscle and blood.

The enzyme has already been successfully isolated from the livers of e. g. rats, chicken, mice, guinea pigs, hamsters, goat, rabbit, cattle, frog and eel, as well as other fish [57, 3, 101].

The oxidation of sulfite to sulfate with the physiological electron acceptor cytochrome *c* is catalyzed by the sulfite oxidase. This oxidation is the terminal step in the physiological degradation of of the sulfur-containing amino acids cysteine and methionine [36] and of other, sulfur-containing cell membrane components such as the sulfatides [28].

The very important role of sulfite oxidase in humans and animals is revealed when it is absent: Humans with sulfite oxidase deficiency suffer from major neurological abnormalities and early death [53].

Animal sulfite oxidase, as well as bacterial sulfite dehydrogenase, contains an additional heme domain as prosthetic group, whereas plant sulfite oxidase only has the molybdenum domain [35]. The heme consists of an organic part, the protoporphyrin – a ring out of four pyrrole rings linked by methene bridges – and an inorganic part, the iron atom (see figure 1.10).

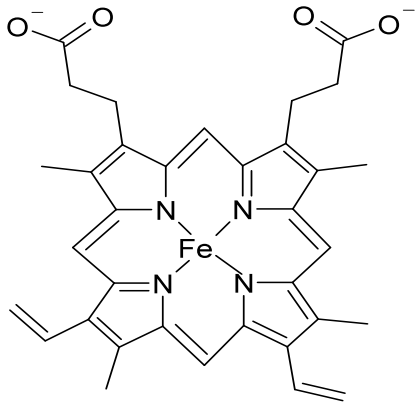


Figure 1.10.: Heme domain as present in animal and bacterial sulfite oxidase, figure by Stryer [98].

The iron atom binds to the four surrounding nitrogen atoms. Iron binds oxygen only in the +2 oxidation state [98].

The molecular weight of the animal sulfite oxidase dimer is 115 kDa, with heme as prosthetic group [22]. It is a homodimer, consisting of two subunits with each a molybdenum cofactor and bound to it, a heme-containing domain of the cytochrome b_5 type [36]. The heme group accounts for the slightly brown colour of the enzyme.

Animal sulfite oxidase, isolated from chicken liver, is commercially available. The enzyme has been applied successfully to biosensor analysis, as described by many authors [117, 36, 2, 1].

Hepatic sulfite oxidase isolated from chicken liver used to be the only enzyme available. It was shown to be well suited for immobilization and application purposes in the HPLC-IMER method [78, 113, 75, 76, 107].

Plant sulfite oxidase

Plant sulfite oxidase is a homodimeric, molybdenum-containing enzyme without a heme group, located in the peroxisomal fraction of plant cells [29].

It has been shown to play a major role in protecting plants from damages caused by SO_2. Plants that are exposed to high levels of sulfur dioxide in the air show

a severe reduction in plant growth and even cell death due to the inactivation of proteins like thioredoxins through sulfitolysis [60].

The lack of a heme domain of the plant sulfite oxidase is evident both from the amino acid sequence and from its enzymological and spectral properties [29]. It is therefore the smallest eukaryotic molybdenum enzyme presently known, with a weight of 43 kDa for the monomer, or 90 kDa for the dimer [69, 70].

In order to achieve biological activity, the molybdenum ion in plant sulfite oxidase has to be complexed by a pterin compound, forming the molybdenum cofactor [69].

The plant sulfite oxidase from *Arabidopsis Thaliana* has been identified, isolated and biochemically characterized for the first time by Eilers et al. [29]. Sulfite oxidase derived from the cloned sulfite oxidase gene of *Arabidopsis Thaliana* was provided by Hänsch et al. for use in our experiments with HPLC-IMER.

As it is not commercially available yet, this plant enzyme has not before been employed in enzyme supported analysis. In this work, this enzyme was tested for analytical purpose for the first time, showing very good activity and stability features.

Bacterial sulfite dehydrogenase

Bacterial sulfite dehydrogenase is located in the periplasm of bacteria [35]. The enzyme was isolated from the soil bacterium *Starkeya novella* and examined by Kappler et al. [52].

Sulfite dehydrogenase oxidizes sulfite during the chemolithotrophic growth of *Starkeya novella*, using thiosulfate as an energy source.

It is a heterodimeric, heme *c* and molybdenum-containing bacterial enzyme that cannot transfer electrons to molecular oxygen and is therefore classified as a sulfite dehydrogenase instead of an oxidase [52].

Each subunit contains one redox center, the larger 40.2 kDa SorA subunit with molybdopterin cofactor, the smaller 8.8 kDa SorB subunit with the c-type heme.

In the dissertation thesis of Arndt, a marine bacterium, *sulfitobacter pontiacus*, with large amounts of a sulfite oxidizing enzyme was examined [5]. Compared to other thiothrophic organisms, *sulfitobacter pontiacus* showed very high activities towards the oxidation of sulfites even in an unpurified state. These findings led Arndt to the hypothesis, that the purified sulfite oxidase derived from *sulfitobacter pontiacus* would potentially show great performance in the bioanalytical determination of sulfites in foodstuffs.

1.3.1.3. The Amperometric Detector

An amperometric detection method in electrochemical analysis is "a detection method in which the current is proportional to the concentration of the species generating the current" (definition by IUPAC[4] [67]). Amperometric detection is a subgroup of the electrochemical detection. In the case of the HPLC-IMER, the concentration measured is the amount of hydrogen peroxide produced by the enzymic transformation of sulfite to sulfate. Hydrogen peroxide is oxidized at the electrode, the released electrons leading to the detector signal.

$$H_2O_2 \longrightarrow O_2 + 2\,H^+ + 2\,e^-$$

For the detection of hydrogen peroxide, an amperometric detector with a platinum electrode was used. The advantages of this detection method are its sensitivity and a good selectivity with low applied voltages.

The detector cell consists of three electrodes: the working electrode, the auxiliary electrode and the reference electrode. The flow cell is of the wall-jet type with the eluent flowing vertically onto the electrode surface before exiting the conically shaped flow chamber to the side. Due to a constant flow past the electrode, only about 1–10% of the analytes react at the electrode surface, where 90–99% pass the electrodes without being oxidized. Despite the low reaction rates, an electrochemical detector shows very good sensitivity.

The reduction or oxidation of the analyte takes place at the working electrode, depending on the voltage applied between the working and the reference electrode. The electrical output results from the electron flow caused by the chemical reaction that takes place at the surface of the electrodes. The current flow is proportional to the concentration of the analyte, and its value depends on the applied electrode potential and the flow rate of the eluent [107].

The relationship between current and analyte concentration is described with the following equation shown in figure 1.11:

The auxiliary electrode compensates for the changes in current flow and provides for a constant cell potential level between the working and the reference electrode [71].

A platinum electrode as working electrode and a Ag/AgCl-reference electrode are common in the detection of hydrogen peroxide [88]. The applied potential for the oxidation of hydrogen peroxide on the electrode is 0.2 V. With this low potential, the noise level is moderate, and the detection of hydrogen peroxide is very specific as only few substances are oxidized at that potential.

[4] International Union of Pure and Applied Chemistry

$$I \sim \frac{d * A * C}{l} \tag{1.1}$$

Figure 1.11.: I=limit current; d=diffusion coefficient, A=electrode surface, C= concentration of the electrochemically active analyte, l=thickness of the electric double layer [38].

1.4. Anthocyanins in HPLC-IMER Sulfite Analysis

The HPLC-IMER method shows good results in the analysis of sulfites for foodstuffs like beer and champagne [76]. Other products, like grape juices or red wine, tend to produce too low results. Up to 30% of the expected sulfites disappear, and can not be detected with the HPLC-IMER. Unlike beer and champagne, grape juices and red wine contain considerable amounts of polyphenols, especially anthocyanins, which are known to interact and even covalently bind with sulfites. This effect was assumed by Patz et al. to be the reason for the low recovery of sulfites in grape products [78].

1.4.1. Anthocyanins

Anthocyanins are natural colorants that are located in the cell vacuoles of plants. They are responsible for many of the colors found in flowers and fruit. Their color nuances may vary from red over purple and blue, covering most colors naturally found in plants. The many different color variations are derived from only a few main anthocyanidins that change their color due to pH variations and depending on accompanying metal ions.

Anthocyanins are flavonoids with a flavylium ion structure as presented in figure 1.12.

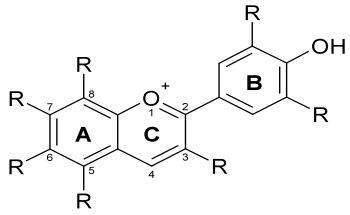


Figure 1.12.: Basic structure of anthocyanins: The flavylium cation.

If all R are either -OH, -H or -OCH_3, this structure represents an anthocyanidin. Six anthocyanidin forms are the most common in fruits: Pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (all strucutres shown in figure 1.13).

The anthocyanidin form is not very stable and it is insoluble in water. In plants, anthocyanidins are commonly linked to one or more mono- or diglycosides, most commonly glucose and rhamnose. But other aliphatic and aromatic substituents,

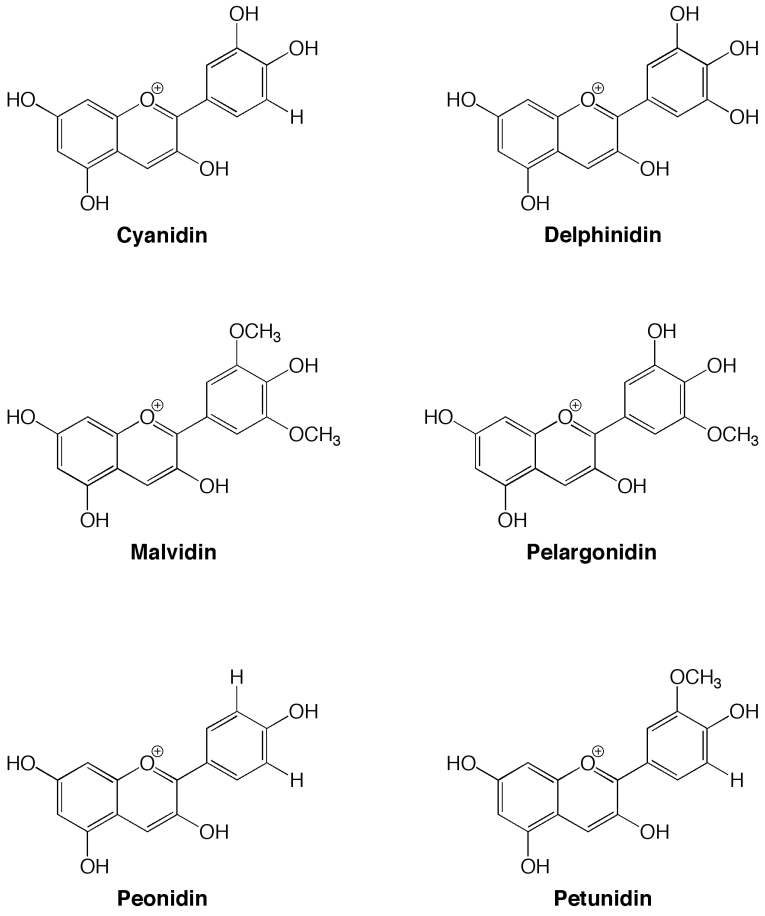


Figure 1.13.: Chemical structures of the six main anthocyanidins.

like malonic acid or caffeic acid, have also been reported [43]. The anthocyanidins linked to sugars or other substances are called anthocyanins.

1.4.2. Anthocyanins and Sulfites

Addition reactions of sulfites with anthocyanins have been well-known for many years [50]. The decrease in colour intensity of a red grape juice or a red wine after addition of sulfur dioxide gas or sulfite salts has often been described. Even a complete decolorization of a fresh juice can be achieved with large amounts of sulfites. Since only monomeric anthocyanins are considered to react readily with SO_2, this reaction is also used to determine the amount of coloured polymeric anthocyanins in grape juices and wines [79, 106].

This addition is a reversible reaction. At very low or very high pH values, the bond is split, and with the release of the sulfite ion, the anthocyanin regains its natural colour.

The addition product of an anthocyanin with sulfite is a colourless sulfonate as described by Berké [7]. Berké was the first to discover and describe the correct structure of Malvidin-3-sulfonate by using ^{1}H, ^{13}C and ^{33}S NMR analysis. The proposed addition reaction is shown in figure 1.14.

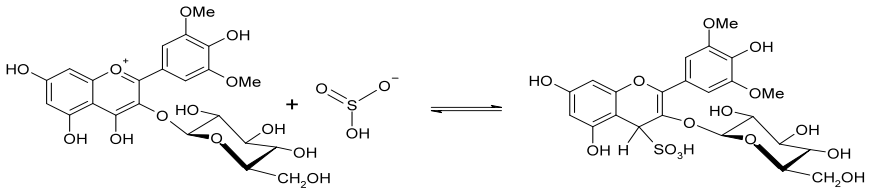


Figure 1.14.: Addition reaction of Malvidin 3-glucoside with hydrogensulfite, as proposed by Berké et al. [7].

In the production of red grape juices, sulfites are commonly used to achieve a better extraction performance of anthocyanins from the grape peel. In the sulfonate form, which is stable at the natural pH value of grape juices, anthocyanins are more stable and less susceptible to degradation and polymerization processes.

The alkaline pH value applied in HPLC-IMER should theoretically be sufficient for the cleavage of the sulfur-anthocyanin bond. If anthocyanin-sulfite adducts are responsible for the low recoveries in anthocyanin containing products, the identification of the responsible, very stable molecules may help to understand

the mechanism, and potentially lead to a better suited sample preparation for these matrices.

For the identification of these stable products, a chromatographic separation and detection with HPLC-UV-MS analysis was employed, as described in chapter 3.1.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Sodium carbonate	p. a., Merck (Darmstadt, Germany)
Bicarbonate of soda	p. a., Merck (Darmstadt, Germany)
Monopotassium phosphate	p. a., Merck (Darmstadt, Germany)
Sodium phosphate dibasic dihydrate	Fluka (Buchs, Schweiz)
Sodium hydroxide	pellets GR, p. a., Merck (Darmstadt, Germany)
Sodium hydroxide solution 0.01 mol/L	p. a., Merck (Darmstadt, Germany)
Sodium hydroxide solution 1 mol/L	prepared from pellets
Phosphoric acid	p. a., Merck (Darmstadt, Germany)
Hydrogen peroxide solution	p. a., Merck (Darmstadt, Germany)
Methyl Red, Methylene Blue	Indicator, Merck (Darmstadt, Germany)
Sodium sulfite	anhydrous, p.a., 1.06657.0500, Merck (Darmstadt, Germany)
D (−)-Fructose	p. a., Merck (Darmstadt, Germany)
Edetate disodium dihydrate disodium salt dihydrate (EDTA)	p. a., Merck (Darmstadt, Germany)

2.1.2. Solvents

Acetonitrile	HPLC Gradient grade, Fisher Scientific (Leicestershire, UK)
Methanol	HPLC for gradient analysis, Acros Organics (New Jersey, USA)
Purified water	Millipore Direct-Q® 3UV with Pump (Molsheim, Germany)
Ethanol	denaturated
Trifluoroacetic acid	extra pure, 99%, Acros Organics (New Jersey, USA)

Carbonate buffer pH 9.1; 0.04 mol/L
1 L contains 0.43 g sodium carbonate and 3.04 g bicarbonate of soda

Carbonate buffer pH 10.6; 0.06 mol/L
1 L contains 4.7 g sodium carbonate and 1.2 g bicarbonate of soda

Phosphate buffer pH 8.0 for enzyme immobilization; 1 mol/L
0.12 g KH_2PO_4 and 3.00 g Na_2HPO_4 x 2 H_2O, filled to 20 mL with purified water

Phosphate buffer pH 7.5 for enzyme immobilization; 0.1 mol/L
0.22 g KH_2PO_4 and 1.45 g Na_2HPO_4 x 2 H_2O, filled to 100 mL with purified water

2.1.3. Standard Compounds

Malvidin 3-O-glucoside chloride	>95%, CAS number: 7228-78-6, Extrasynthèse (Genay, France)
Cyanidin 3-O-glucoside chloride	from Blackberry concentrate, > 90%

2.1.4. Further Supplies

Disposable syringe filter	Chromafil® RC-45/25, regenerated cellulose, Macherey-Nagel (Düren, Germany)
Frit	porous plate HDPE (high density polyethylene), Reichelt (Heidelberg, Germany)
Shaker	Gerhardt (Königswinter, Germany)
Vacuum-box	BAKER spe-12G Column Processor® (PTFE Design), Mallinckrodt Baker (Phillipsburg, NJ, USA)

2.2. Methods

2.2.1. HPLC-IMER

All devices by Dionex (Idstein, Germany) if not otherwise indicated.

HPLC-Pump	ICS-3000: SP, PEEK, isocratic pump with degasser
Injection valve	25 μL, Rheodyne
Autosampler	AutoSelect AS 50
i-Valve	Motor valve, 10 port, 2 way, PEEK, RS 232
i-Valve	Solvent Selector, 6 port, Teflon, RS 232
Detector	TED 4020, Trace (Braunschweig, Germany)
Cell	Platinum, potential: 0.2 V; Range 20 nA, Trace (Braunschweig, Germany)
Analytical column	CarboPac PA-100 Guard, 4 x 50 mm, resin composition: 8.5 μm diameter, ethylvinylbenzene/divinylbenzene substrate (55% cross-linking) agglomerated with 275 nm MicroBead^{TM} quaternary ammonium functionalized latex (6% cross-linked); column construction: PEEK with 10-32 threaded ferrule-style end fittings, all components are nonmetallic. Mobile phase compatibility: pH 0–14; 100% compatible with common organic solvents
Software	Chromeleon Version 6.70 SP1 Build 1842 and Version 6.80 SP1 Build 2238
Eluent	Carbonate buffer, 0.04 mol/L, pH = 9.1
Flow rate	0.6 mL/min, isocratic
Detection	0.2 V, Range 200 nA

2.2.2. Sample Preparation

The high reactivity of the analyte sulfite, especially in the presence of oxygen, requires a fast performance of the sample preparation.
Immediately after opening the package, 1 mL of the liquid sample (or 1 g for solid samples, respectively) is added to 2 mL of sodium hydroxide solution (1 mol/L). This mixture is blended by carefully swaying the flask, and is then allowed to stand for one hour at room temperature. Carbonate buffer (pH 10.6; 0.06 mol/L) is then added up to a volume of 25 mL. Further dilution may be necessary depending on the concentration of sulfur dioxide in the sample. After mixing the liquids again the sample is ready for injection.

2.2.2.1. Preparation of the Sulfite Standard Solution

The standard solution is prepared by diluting the parent solution.

Parent solution:
About 0.2 g of sodium sulfite, 1.4 g of fructose and 0.09 g of EDTA are weighed into a 50 ml volumetric flask. All chemicals are dissolved in purified water, and filled up to 50 ml. This solution can be used for one week, if stored in a refrigerator at about 4 °C.

Standard solution:
The parent solution is diluted in two steps.
Step one: 1 ml of the parent solution is diluted 1:100 with carbonate buffer (pH 10.6; 0.06 mol/L).
Step two: this solution is diluted again, 1:50, by adding 0.011 g of fructose and 0.009 g of EDTA as stabilizers. The sulfite standard solution can be used only for one day, and thus has to be prepared freshly out of the parent solution every day.

2.2.2.2. Quantification

Data is collected by the electrochemical detector, which is set at 0.2 V at a range of 200 nA. The amount of sulfite in a sample is quantified by calculating the area under the sulfite peak of a sample in relation to the area under the sulfite peak of the standard solution. It is important to only compare the areas of two consecutive chromatograms, or of two that are preferably temporally very close together, as the enzyme activity may vary over time and can not be considered stable throughout a day or longer.

The peak area depends on different parameters and can never be regarded to be absolute. The amount of sulfite oxidase successfully immobilized on the carrier beads of the enzyme reactor limits the capacity of the reactor. However, the performance of the enzyme reactor also depends on the age of the reactor as well as the time it has actually been in use in the HPLC. For more details on enzyme stability and performance see chapter 3.3.

The quantification of sulfites in the experiments for proper sample preparation was adjusted, as it was not possible to inject a standard sulfite solution after each sample injection (see chapter 3.2.5). For those experiments, a standard solution was injected twice, once at the beginning, and once at the end of one experiment. The decrease in the peak areas for both standard injections was calculated and the sample peak areas were adjusted mathematically to that decrease. Thus, the decrease in the method performance over time was considered.

2.2.3. Enzyme Reactor

Cartridge	polycarbonate cartridge, Trace, Braunschweig, Germany
Immobilizing agent	Eupergit® C 250 L, oxirane acrylic beads, Röhm, Darmstadt, Germany

2.2.3.1. Enzymes

Plant sulfite oxidase (At-SO)
A homodimeric molybdenum enzyme, cloned and expressed from *Arabidopsis thaliana* [28]. Provided by Prof. Mendel, Botanical Institute of the Technical University of Braunschweig.
Structure: At-SO is a molybdenum enzyme with molybdopterin as an organic component of the molybdenum cofactor. It has no heme domain. For one reactor, ca. 500 μg protein is immobilized on ca. 40 mg Eupergit®.

Animal sulfite oxidase (hepatic)
From chicken liver, Sigma-Aldrich (Steinheim, Germany), Enzyme Commission (EC) 1.8.3.1.
Suspension in 3.2 mol/L ammonium sulfate containing 1.6 mM molybdic acid, pH 7.5.
Activity: One unit oxidizes 1.0 μmole of sulfite per minute at pH 8.5 at 25 °C; KM = 1.4 x 10^{-4} mol/L (Cytochrome as electrone acceptor).
Molar mass: 115000 Da
Structure: the sulfite oxidase is a homodimeric molybdenum enzyme, consisting of an N-terminal heme domain and a C-terminal molybdenum domain
pH optimum: 8.6.
Storage: 4 °C
For one reactor, 400 μL of suspension (equal to 435 μg of protein or 50 Units SOx) is immobilized on ca. 40 mg Eupergit®.

Marine sulfite oxidase
From the marine bacterium *Sulfitobacter pontiacus* [5]. Molar Mass 45000 Da
Temperature optimum: 33 °C
pH-optimum: 4.6–8.5
For one reactor: 5.7 mL = 48 mg Protein = 51 U is immobilized on ca. 40 mg Eupergit®.

2.2.3.2. Enzyme Immobilization

In order to use and re-use enzymes for analytical application in HPLC-IMER, they have to be immobilized on a carrier material. This immobilization step leads to a stabilization of the enzymes, allowing for long standing times and good performance.

In our work, Eupergit C 250 L® was used as carrier material. Eupergit C 250 L® are microporous (r = 100 nm), epoxy-activated acrylic beads with a diameter of approximately 190 μm. The material is a copolymer of methacrylamide, N,N'-methylene-bis-(methacrylamide), and two monomers containing oxirane groups. According to the producer, the oxirane content of Eupergit C 250 L® is at least 0,36%. The end-standing oxirane groups of the carrier material (density of 300 μmol/g dry beads) react with the endstanding amino groups of the enzyme in a two-step binding mechanism, leading to a strong covalent linkage between the enzyme and the carrier material with minimal chemical modification of the enzyme [64]. Figure 2.1 illustrates the binding mechanism.

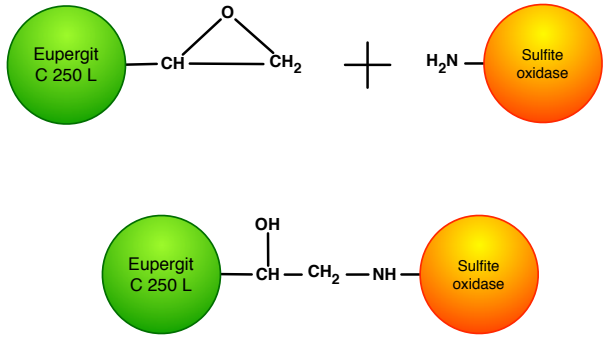


Figure 2.1.: Mechanism of covalent immobilization of sulfite oxidase on Eupergit® as carrier material (Information by Degussa®).

The immobilization procedure induces a partial loss of enzyme activity. However, the immobilized enzyme still provides sufficient conversion rates, and also a good long term stability.

Preparation of the enzyme reactor

The preparation procedure for the enzyme reactors was the same for all three different enzymes (animal, plant and marine bacterial sulfite oxidase).

The desired amount of enzyme is dissolved in 1 mL of phosphate buffer (pH 8.0), and 40 mg of the carrier material Eupergit C 250 L® are added to 1 mL of the same phosphate buffer. Both solutions are then combined and allowed to react for 24–48 hours at room temperature. During that time, the suspension is constantly shaken at a slow pace for homogenous mixing of the enzyme with the carrier material.

Afterwards, the suspension is kept in the refrigerator at 4°C until it is filled into the cartridge.

For the filling, a polycarbonate cartridge is connected to a vacuum box and provided with an HDPE frit to retain the material inside the cartridge. The carrier beads are filled into the space inside the cartridge and flushed several times with phosphate buffer (pH 7.5). All liquids are removed by applying low pressure to the vacuum box with a water-jet pump. After the filling is completed, the cartridge is sealed with another HDPE frit and tested for leaks.

The filled reactor is then connected to the HPLC and flushed thoroughly to remove unbound enzyme material, before the outlet of the reactor is connected via capillary to the detector cell of the amperometric detector.

2.2.4. Destillation Method IFU 7a

Infrared Heater	IRB1 230 V, Bühler (Tübingen, Germany)
Nitrogen gas	Praxair (Düsseldorf, Germany)
Lieb-Zacherl apparatus (see figure 2.2)	VWR (Darmstadt, Germany)

Principle:
The destillation is done in a Lieb-Zacherl apparatus (see figure 2.2). Bound sulfites are released as gaseous SO_2 by high temperature and low pH. The entire sulfur dioxide is carried by a nitrogen stream into a neutralized H_2O_2 solution, where SO_2 is oxidized to sulfate. The resulting sulfuric acid is quantified by titration.

Preparation:
Water cooling is turned on. The dropping funnel is filled with 15 mL of phosphoric acid and the receiver flask is filled with 3 mL of hydrogen peroxide solution (0.3%, pH- and redox-indicator are added and the solution is neutralized with sodium hydroxide solution c = 0.01 mol/L). 50 mL (or 50 g, respectively) of the sample and a few boiling chips are filled into the 250 mL flat bottom flask. All flasks are returned to the apparatus and all ground necks are checked for leak tightness.

Distillation:
15 mL of phosphoric acid are added through the valve of the dropping funnel to the sample, immediately closing the valve after all acid is added. The gas flow is turned

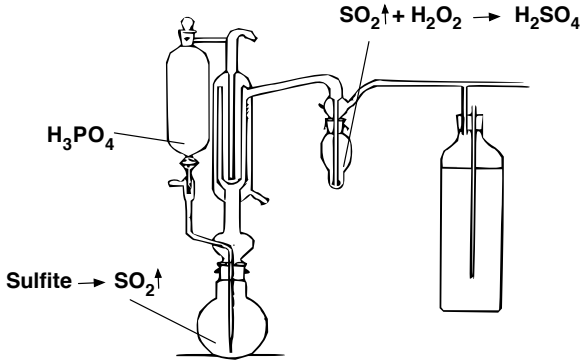


Figure 2.2.: The Lieb-Zacherl apparatus.

on and the heater is started. As soon as the liquid starts to boil, the destillation continues for exactly 15 minutes. After 15 minutes, the flask is removed from the apparatus, remaining drops are flushed into the flask with distilled water, and the fluid is then titrated with sodium hydroxide solution (0.01 mol/L) to the point of colour change.

Quantification:

1 mL of 0.01 N NaOH is equal to 0.32 mg of SO_2.
Thus,

$$\text{mg/L } SO_2 = a * 6.4 \tag{2.1}$$

(with a = mL NaOH, used for titration)

Reliability of the method:

Repeatability (r): r = 0.8 mg/L; $s_r = \pm$ 0.297 mg/L

Reproducibility (R): R = 3.5 mg/L; $s_R = \pm 1.246$ mg/L

2.2.5. DE-HPLC-IMER

DE-HPLC-IMER is the abbreviation for destillation (DE) method, coupled with the HPLC-IMER.

Principle:
Bound SO_2 is set free by destillation as described above for the IFU 7a. Gaseous SO_2 is introduced into alkaline carbonate buffer where it turns into the sulfite (SO_3^{2-}) form. Part of this solution is then injected into the HPLC-IMER in order to quantify the amount of sulfite.

Execution:
The dropping funnel is filled with 15 mL of phosphoric acid. The receiver flask is filled with 10.0 mL of carbonate buffer (pH = 10.6). 50 mL (or 50 g, respectively) of the sample and a few boiling chips are filled into the 250 mL flat bottom flask. All flasks are returned to the apparatus and all ground necks are checked for leak tightness. 15 mL of phosphoric acid are added through the valve of the dropping funnel to the sample, immediately closing the valve after all acid is added. The gas flow is turned on and the heater is started. As soon as the liquid starts to boil, the destillation continues for exactly 15 minutes. After 15 minutes, the receiver flask is removed from the apparatus. The solution is mixed by gently shaking the flask and then injected into the HPLC. The HPLC-IMER conditions are the same as described above.

2.2.6. HPLC-UV

Pump	System Gold Programmable Solvent Module 125, Beckman (Unterschleißheim, Germany)
Degasser	Degasys DG-1210, Uniflows (Tokyo, Japan)
Autosampler	LC-Triathlon for Beckman (Unterschleißheim, Germany) no. 507, Spark Holland Inc. (Emmen, Netherlands)
Column oven	W. O. electronics (Langenzersdorf, Austria)
Guard column	RP-18 Security Guard, 4 mm x 2 mm i. d., Phenomenex (Aschaffenburg, Germany)
Detector	System Gold Scanning Detector Module 167, Beckman (Unterschleißheim, Germany)
Column 1	Aqua RP-18, 3 μm, 125 A, 150 x 4,6 mm, Phenomenex (Aschaffenburg, Germany)
Column 2	Synergi Fusion-RP, 4 μm, 80A, 150 x 4,6 mm, Phenomenex (Aschaffenburg, Germany)
Software	Beckman Gold version 711U, SCC 2.000, Beckman (Unterschleißheim, Germany)

Chromatographical parameters:
STTRA55

Flow rate	1 mL/min
Solvent A	water, 0.2% TFA
Solvent B	acetonitrile, 0.2% TFA
Gradient	0 min: 5% B; 60 min: 35% B; 70 min: 100% B; 80 min: 5% B
UV detection	520 nm
Column temp.	20 °C
Injection volume	5-25 μL

STTRA25

Flow rate	1 mL/min
Solvent A	water, 0.2% TFA
Solvent B	acetonitrile, 0.2% TFA
Gradient	0 min: 5% B; 60 min: 35% B; 70 min: 100% B; 80 min: 5% B
UV detection	280 nm
Column temp.	20 °C
Injection volume	5-25 μL

2.2.7. HPLC-CEAD

Degasser	Degasys DG-1210, Uniflows (Tokyo, Japan)
Pump	System Gold Programmable Solvent Module 125, Beckman (Unterschleißheim, Germany)
Autosampler	LC-Triathlon for Beckman (Unterschleißheim, Germany)
Column oven	W. O. electronics (Langenzersdorf, Austria)
Column	CarboPac PA-100 Guard, 4 x 50 mm, Dionex (Idstein, Germany)
Detector	CoulArray 5600 with one electrode, ESA (Chelmsford, MA, USA)
Software	CoulArrayWin, Version 1.02, ESA (Chelmsford, MA, USA)

2.2.8. HPLC-MS/MS

HPLC Summit, all devices by Dionex (Idstein, Germany) if not otherwise indicated.

Degasser	Degasys DG-1310, Uniflows (Tokyo, Japan)
Pump	P-580A HPG
Autosampler	ASI 100 T
Column oven	STH-585
Guard column	RP-18 Security Guard, 4 mm x 2 mm i. d., Phenomenex (Aschaffenburg, Germany)
Column	Synergi Fusion-RP, 150 mm x 2 mm i. d., particle size 4 μm, Phenomenex (Aschaffenburg, Germany)
Detector	UVD-340S UV/Vis detector, equipped with a capillary cell
Software	Chromeleon, last version 6.8 SP1 Build 2238
Eluents	A: 0.2% TFA, 5% Acetonitrile, 94.8% water; v+v+v B: 0.2% TFA, 45% Acetonitrile, 54.8% water; v+v+v
Gradient	0 min: 0% B; 60 min: 75% B; 61 min: 100% B; 70 min: 100% B; 71 min: 0% B; 80 min: end
Injection volume	10 μL
Flow rate	0.2 mL/min

MS parameters LCQ classic ion-trap mass spectrometer, Thermo Finnigan (Dreieich, Germany) with an electrospray interface and a metal needle kit.
In order to optimize the ionisation of the analyte, 100 μl/min of methanol was added to the HPLC flow by a System Gold Programmable Solvent Module 116, Beckman (Unterschleißheim, Germany), before entering the ion source.
Software: Excalibur version 1.2 SP1.

Capillary Temperature	310 °C
Sheath Gas Flow	90
Aux Gas Flow	5
Source Type	ESI

Ionisation

Positive Polarity

Source Voltage	4 kV
Source Current	80 μA
Capillary Voltage	10 V
Tube Lens Offset	−20 V
Multipole RF Amplifier	850 Vp-p
Multipole 1 Offset	−4 V
Multipole 2 Offset	−10 V
InterMultipole Lens Voltage	−30 V
Trap DC Offset Voltage	−10 V

Negative Polarity

Source Voltage	4 kV
Source Current	80 μA
Capillary Voltage	−10 V
Tube Lens Offset	20 V
Multipole RF Amplifier	850 Vp-p
Multipole 1 Offset	4 V
Multipole 2 Offset	10 V
InterMultipole Lens Voltage	30 V
Trap DC Offset Voltage	10 V

Scan events	3
thereof	(1) Positive, mass range 150-2000 m/z (2) Positive, Dep MS/M Most intense ion from (1) (3) Positive, Dep MSn Most intense ion from (2)
Isolation width	2.8 u
Normalized Collision Energy	40%
Activation Q	0.25

2.2.9. Comparison of Three Different Methods for SO_2 Analysis

The comparison of the HPLC-IMER with the distillation method for fruit juices (IFU 7a), and with the DE-HPLC-IMER was conducted with two different grape juices.

Red grape juice:
1 litre carton packages (Pure-Pak®) by rio d'oro, 100% juice, not from concentrate

White grape juice:
1 litre carton packages (Pure-Pak®) by rio d'oro, 100% juice, not from concentrate

Both juices were produced by Jacobi Scherbening GmbH and kindly provided by Faethe Laboratory.

All packages of each sort were from the same batch. All juices were stored at 4°C and allowed to warm to room temperature before analysis. For each analysis, one package was freshly opened after thorough shaking. All analyses were performed three times as duplicates.

2.2.10. Statistical Analysis

All calculation of statistical parameters was done with Laborvalidate software:
LaborValidate®
Labormethoden Validierung
Version 2.5.5
Copyright: Hoffiesoft, 2006

The following abbreviation terms are used:

$\bar{x}$ = arithmetic mean value
N = number of measured values
x_i = single value
s_x^2 = variance
s_x = standard deviation
V = relative standard deviation

The calculations are based on the following equations:

$$\bar{x} = \sum_{i=1}^{N} x_i \tag{2.2}$$

$$s_x^2 = \frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1} \tag{2.3}$$

$$s_x = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}} \tag{2.4}$$

$$V = \frac{s_x}{\bar{x}} * 100\% \tag{2.5}$$

3. Results and discussion

3.1. Addition Reactions of Sulfites with Anthocyanins

The analysis of sulfur dioxide with the HPLC-IMER method has been described to perform well for some food matrices, while exhibiting difficulties for others. Especially products like grape juices or red wines, that contain large amounts of anthocyanins and other polyphenols, have been described to lead to results, that are too low and sometimes irreproducible [78]. Therefore, the influence of anthocyanins on the recovery of sulfites with the HPLC-IMER was to be examined. The following experiments are designed to reveal the anthocyanin contents of certain grape products, and to give information on the stabilities of the anthocyanin-sulfite adducts that may account for some of the irregularities in HLC-IMER analysis.

In order to identify the anthocyanins of different grape juices that react readily with sulfites to form adducts, a method for a chromatographic separation and identification of anthocyanins was developed.

An unsulfurized grape juice (produced at the Forschungsanstalt Geisenheim) was analyzed with HPLC-UV for its content of anthocyanins and other polyphenols. Samples of the same juice were then treated with different amounts of sodium sulfite. The idea was, to compare the chromatograms of the pure grape juice with those of the treated juices, in order to make the adducts visible by an anthocyanin peak reduction.

The red colored anthocyanins are detected at a wavelength of 520 nm, but the colorless and uncharged adducts will have different retention times and absorption maxima. Therefore, the sulfurized juices are expected to lead to smaller anthocyanin peaks in the chromatogram. Theoretically, a juice that is treated with sulfites to complete decolorization will show no anthocyanin peaks in the chromatogram.

A grape juice of the variety Blauer Portugieser was chosen for these experiments. The grape belongs to the species *Vitis vinifera* of the botanical family *Vitaceae*. Produced from this grape, unsulfurized juices were available, as well as two juices with different sulfur dioxide contents (addition of 75 mg/L and 150 mg/L SO_2). All juices were produced at the Forschungsanstalt Geisenheim (for details see appendix B.5).

Figure 3.1 shows a chromatogram of a red grape juice without added sulfites at 520 nm.

All anthocyanin peaks were analyzed by UV detection and mass spectrometry. The anthocyanins were identified by comparison of retention times, masses and fragment spectra with data from the literature [116, 6, 66]. For MS^n analysis, the chromatographic conditions had to be slightly modified (for details see chapter

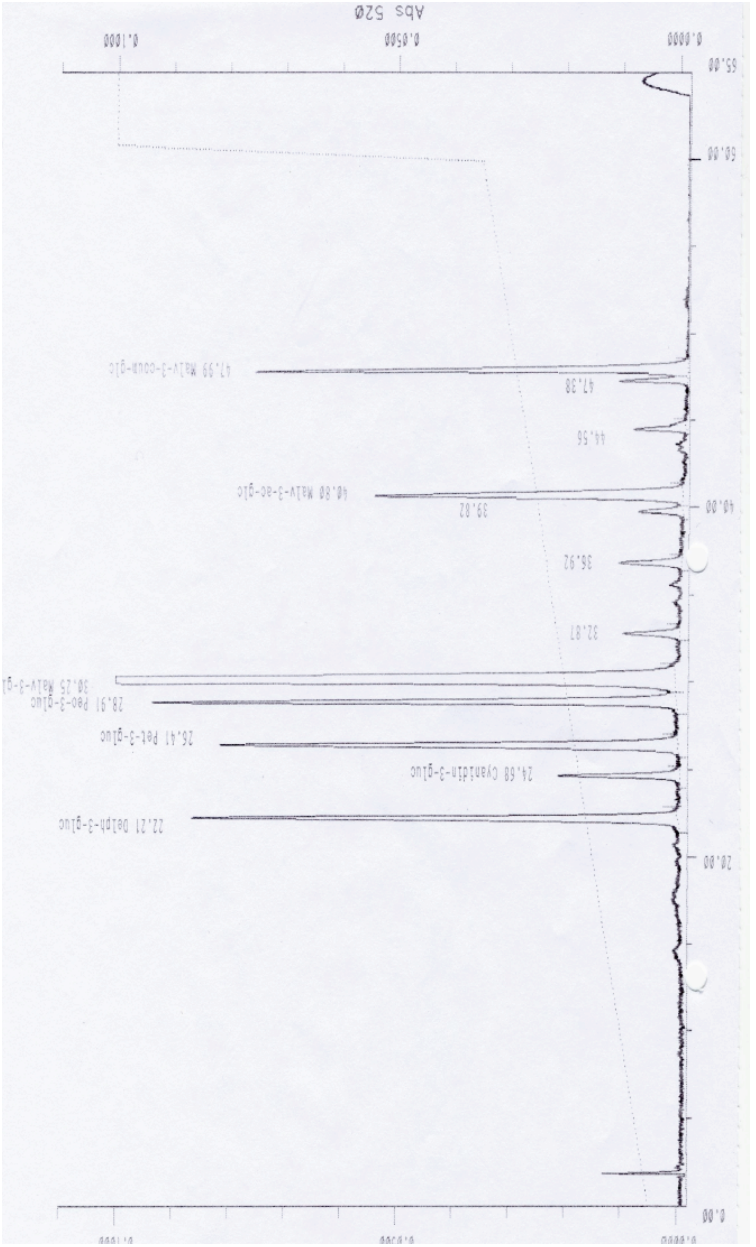


Figure 3.1.: Chromatogram of a grape juice with HPLC-UV at 520 nm with eluent containing 0.2% TFA.

2.2.8 on page 45). A chromatogramm under HPLC-UV-MS conditions therefore is not identical to that obtained under HPLC-UV conditions. Figure 3.2 shows a chromatogram of a Blauer Portugieser grape juice with the modified HPLC-UV-MS method. The peak numbers are explained in table 3.1.

The calculations of the amounts displayed in table 3.1 are based on the malvidin 3-glucoside standard solution, as standard substances were not available for all anthocyanins.

Table 3.1.: Anthocyanins identified in Blauer Portugieser red grape juice; calculation of amounts is based on a malvidin 3-glucoside standard solution.

Peak no.	**Substance**	**Amount in [mg/L]**
1	Delphinidin 3-glucoside	120
2	Cyanidin 3-glucoside	100
3	Petunidin 3-glucoside	100
4	Peonidin 3-glucoside	140
5	Malvidin 3-glucoside	**450**
6	Del 3-(6″-acetyl-)glucoside	40
7	Cy 3-(6″-acetyl-)glucoside	10
8	Pet 3-(6″-acetyl-)glucoside	25
9	Peo 3-(6″-acetyl-)glucoside	15
10	Malv 3-(6″-acetyl-)glucoside	100
11	Del 3-(6″-coumaryl-)glucoside	15
12	Cy 3-(6″-coumaryl-)glucoside	2
13	Pet 3-(6″-coumaryl-)glucoside	10
14	Peo 3-(6″-coumaryl-)glucoside	10
15	Malv 3-(6″-coumaryl-)glucoside	80

An example of the identification of malvidin 3-glucoside, which is the most prominent anthocyanin in most grape juices of *vitis vinifera*, is given in figure 3.3.

All identified anthocyanin peaks are listed in figure 3.4, ion masses and fragment masses are given in table 3.2.

Out of the six most common anthocyanidins, five were detected in the grape juice. Pelargonidin was not present in Blauer Portugieser.

The elution order is eye-catching: the five aglycons elute in the order delphinidin - cyanidin - petunidin - peonidin - malvidin. This order stays the same for all different glucosides. These findings are in accordance with literature on the grape species *Vitis vinifera* [23, 51].

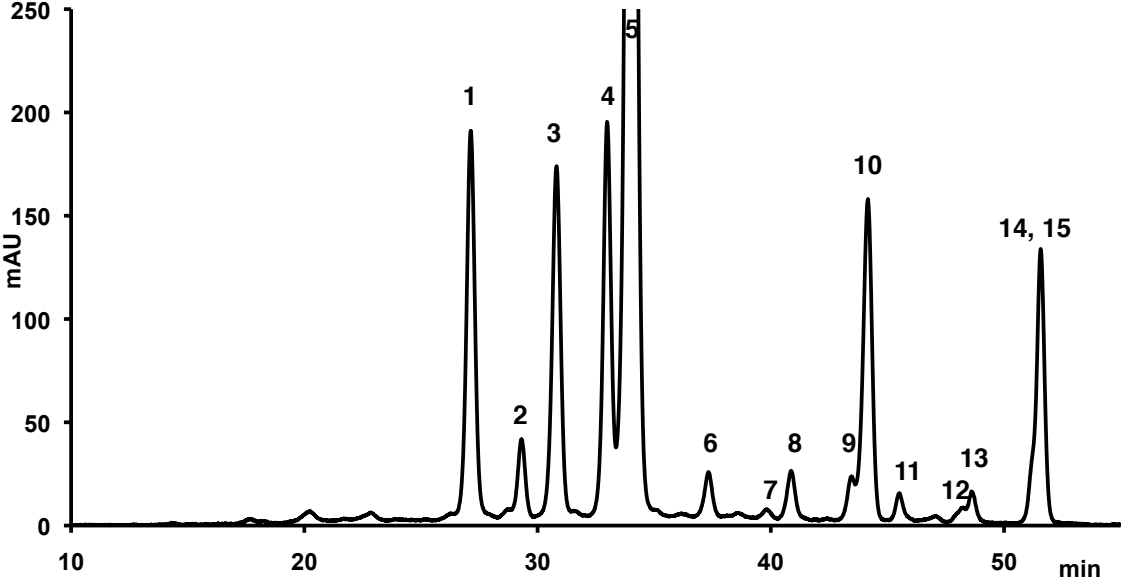


Figure 3.2.: Chromatogram of a grape juice (Blauer Portugieser) with HPLC-UV-MS method; detection at 520 nm.

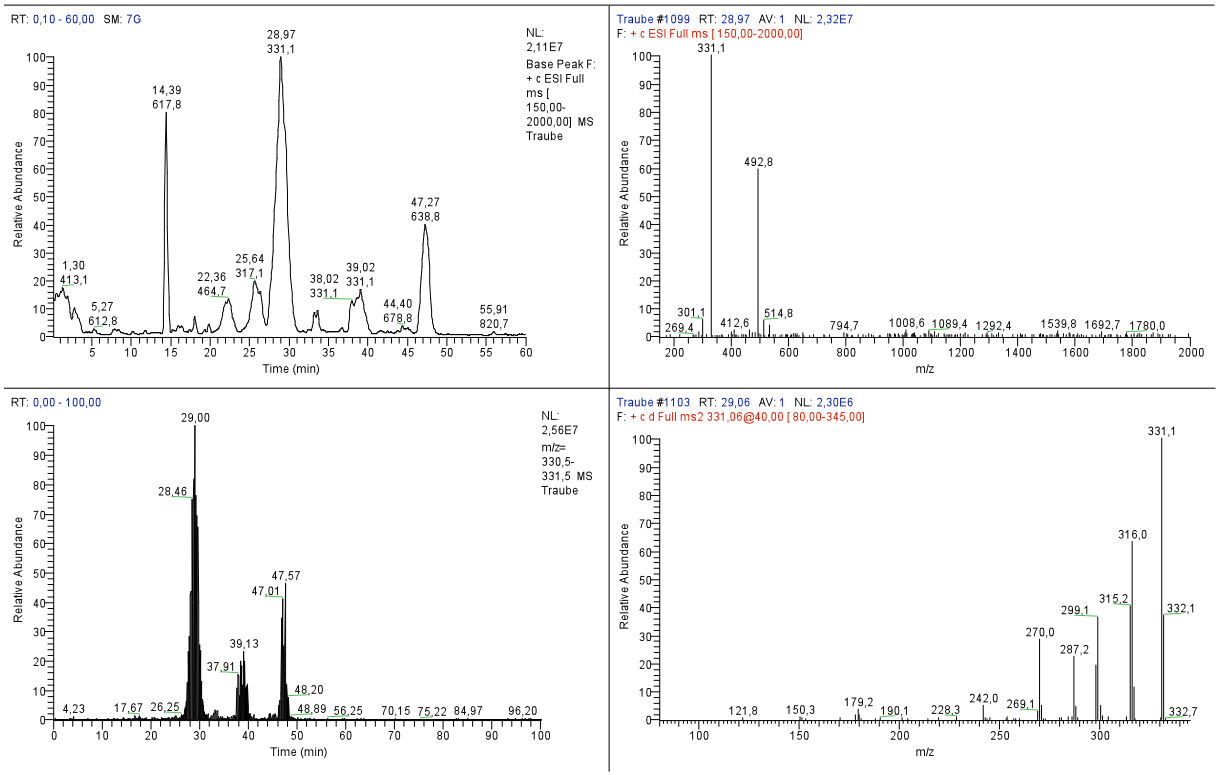


Figure 3.3.: Identification of malvidin-3-glucoside with MS^n.
Upper left: base peak, m/z 150-2000, malvidin-3-glucoside at 28.97 min; lower left: m/z 331 (malvidin); upper right: mass of malvidin-3-glucoside (m/z 493) and mass of malvidin fragment (m/z 331); lower right: fragment spectrum of malvidin.

1	Delphinidin 3-glucoside
2	Cyanidin 3-glucoside
3	Petunidin 3-glucoside
4	Peonidin 3-glucoside
5	Malvidin 3-glucoside
6	Delphinidin 3-(6"acetyl-)glucoside
7	Cyanidin 3-(6"acetyl-)glucoside
8	Petunidin 3-(6"acetyl-)glucoside
9	Peonidin 3-(6"acetyl-)glucoside
10	Malvidin 3-(6"acetyl-)glucoside
11	Delphinidin 3-(6"-coumaryl-)glucoside
12	Cyanidin 3-(6"-coumaryl-)glucoside
13	Petunidin 3-(6"-coumaryl-)glucoside
14	Peonidin 3-(6"-coumaryl-)glucoside
15	Malvidin 3-(6"-coumaryl-)glucoside

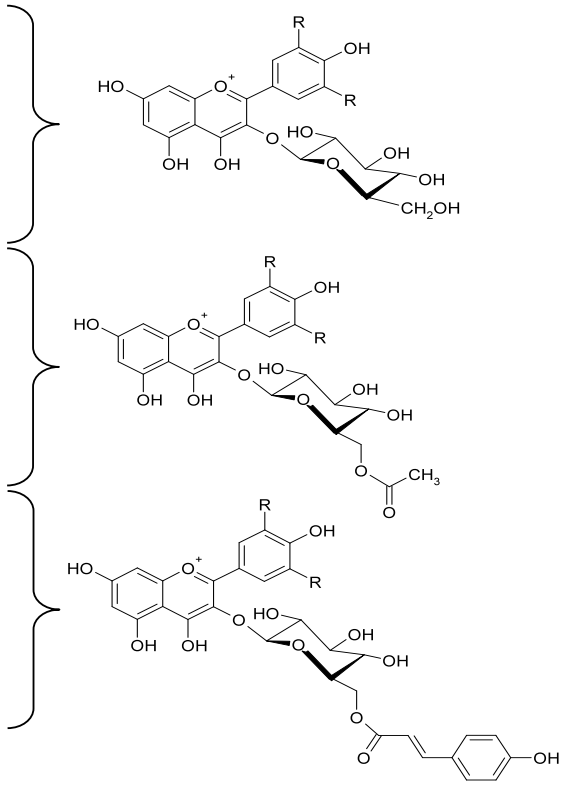


Figure 3.4.: Elution order of anthocyanins in Blauer Portugieser grape juice. Structural formulae of the anthocyanidin 3-glucoside, 3-(6″-acetyl-)glucoside and 3-(6″-coumaryl-)glucoside forms.

Table 3.2.: Retention times, ion masses and main fragments of the anthocyanins found in Blauer Portugieser grape juice.

Min	Anthocyanin	Ion mass	Fragments
22	Delphinidin 3-glucoside	465	303
24.5	Cyanidin 3-glucoside	449	287
25.87	Petunidin 3-glucoside	479	317
28	Peonidin 3-glucoside	463	301
29	Malvidin 3-glucoside	493	331
32	Del 3-(6”-acetyl-)glucoside	507	303
35.14	Cy 3-(6”-acetyl-)glucoside	491	287
36.35	Pet 3-(6”-acetyl-)glucoside	521	317
38.3	Peo 3-(6”-acetyl-)glucoside	505	301
39.2	Malv 3-(6”-acetyl-)glucoside	535	331
41.6	Del 3-(6”-coumaryl-)glucoside	611	303
44	Cy 3-(6”-coumaryl-)glucoside	595	287
44.5	Pet 3-(6”-coumaryl-)glucoside	625	317
47	Peo 3-(6”-coumaryl-)glucoside	609	301
47.17	Malv 3-(6”-coumaryl-)glucoside	639	331

After the successful characterization of the anthocyanin profile of the grape juice, in a next step, chromatograms of different sulfurization levels were compared.

As mentioned before, two grape juices were produced at the Forschungsanstalt Geisenheim with 75 mg/L and 150 mg/L SO_2, respectively. However, comparing the unsulfurized grape juice with the two sulfurized ones in order to determine the influence of sulfite addition on the anthocyanin profile, does not lead to valid results. All three grape juices were not produced under identical conditions, nor out of the same batch of grapes. Therefore, variations in the chromatograms may be due to the addition of sulfur dioxide, but they may also be a result of small differences in the production procedure or in the grape quality.

In order to overcome these uncertainties, the unsulfurized red grape juice (Blauer Portugieser) was sulfurized in the laboratory with different amounts of sodium sulfite. As expected, a decrease in color was observed with increasing amounts of added sulfites.

Two chromatograms of the juice, containing 500 mg/L and 1000 mg/L SO_2, respectively, are presented in figure 3.5.

Both chromatograms are nearly identical, even though the discoloration level of the juices before injection was different. These findings were the same for all examined sulfurization levels. Also, there is no distinction to the chromatogram without added sulfites.

These findings lead to the conclusion, that during chromatography all anthocyanin-bound sulfites are released, thus setting free all of the anthocyanins formerly present in the juice sample. The goal of identifying the binding characteristics of anthocyanins and sulfites was not achieved under the chosen conditions.

Two mechanisms can be considered to lead to the cleavage of the sulfoanthocyanins. The pH value of the eluent is very low (about 1.8), and might therefore result in a reaction shift back to the reactants. However, in our experiments outside the HPLC apparatus, an acidification of the sulfurized grape juice to pH 1.8 did not lead to complete recolorization. Therefore it is unlikely, that the acidic medium is the only cause of the equilibrium shift. Only in conjunction with the strong dilution of the sample during an HPLC run, a shift in chemical equilibrium back to the reactants is conceivable.

Experiments with different HPLC conditions, for example with higher pH values of the eluent, in order to prevent the cleavage of the anthocyaninsulfonates, did not lead to improvements in this issue. A decrease in the acidity of the eluents rapidly leads to chromatograms with much broader and poorly separated peaks.

In spite of the chromatographic difficulties in detecting addition products of anthocyanins and sulfites, a valuable conclusion can be drawn from these experiments. It was shown, that the binding between anthocyanins and sulfites is easily broken

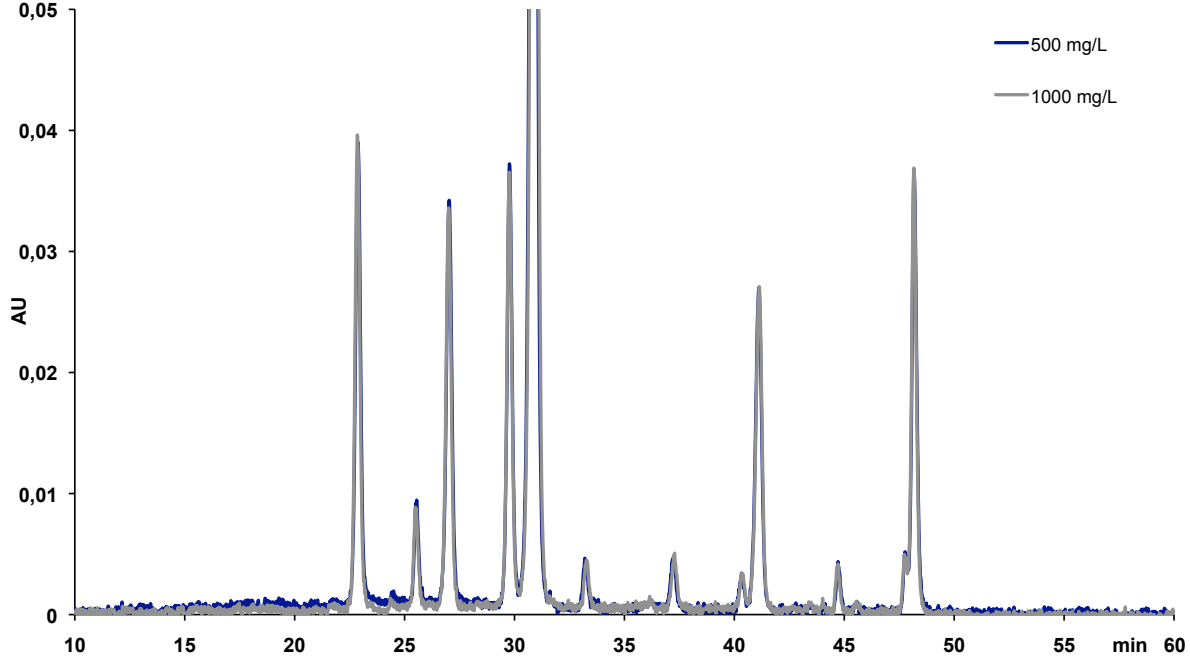


Figure 3.5.: Chromatograms of two Blauer Portugieser grape juices with 500 and 1000 mg/L SO_2; UV detection at 520 nm.

under acidic conditions in combination with dilution of the sample. It has been reported, that an alkaline medium is even more effective in breaking these bonds. Therefore, it is very likely, that the connection between anthocyanins and sulfites is not stable under alkaline conditions, either. If this consideration is true, the typical anthocyaninsulfonates are not the molecules responsible for the insufficient findings of sulfites in HPLC-IMER analysis.

With these considerations, it has to be taken into account, that the sulfurization, which has been performed in the laboratory, cannot be compared to that of a typical production process in the grape juice industry. In the industrial production of grape juices, sulfites are added to the grape mash. This step is followed by many production steps, typically including the desulfurization procedure. This further treatment may lead to different, and possibly to more stable bonds between sulfites and matrix compounds. These addition products, that are not simply just anthocyanin-sulfite adducts, may account for the difficulties in the recovery of sulfites from grape products.

3.2. Parameters and Optimization of the HPLC-IMER

Sulfite analysis by the coupling of an ion exchange HPLC with an enzyme reactor has been described before [78, 113, 75, 76]. In these works, the method was applied mainly on just a few food samples as i. e. beer, grape juice and wine. Especially with wine and grape juices, problems have been described regarding matrix effects of certain metal ions and polyphenols. One goal of this work was to establish adequate parameters for this analytical method. Furthermore, the sample preparation was to be adjusted to different matrices of liquid, semi-liquid and solid foods. One optimized method of preparation for all samples was desired, in order to gain a simple and fast analysis applicable to all foodstuffs containing sulfites. In the following paragraphs, the various method parameters will be discussed.

3.2.1. Column, Flow Rate

The use of an ion exchange column for the separation of sulfites from other compounds of the sample matrix has been established by Weßels [112]. The separation capacity of the short pre-column is sufficient. The use of a normal anion exchange column as opposed to the pre-column did not improve the separation performance of this method to an extend that would compensate for the much longer times for one HPLC run. With the normal column one run takes 30 minutes, whereas with the pre-column it is finished within 8 minutes.

3.2.2. Enzyme Reactor

The amount of immobilized sulfite oxidase that is filled into a reactor may vary due to the degree of successful immobilization on the carrier material. The purity of the enzyme is one important factor for a good immobilization. Also, allowing sufficient time for the binding reaction between the enzyme and the carrier material is essential.

A tight packing of the carrier material is important for two reasons: the more enzyme is immobilized in the reactor, the more sulfite per sample can be transformed. Secondly, a tighter packing leads to less dead volume in the HPLC and therefore to more narrow peak forms.

3.2.3. Detector

The performance of four different detector models was compared. All models were products of TRACE®. Two of the detector models were of the same type, TED

2020, with a block-shaped wall-jet cell; one was of the same type, but with an older, circular shaped cell model, and the fourth detector was a type TED 4020 detector with block-shaped cell.

The influence of the detector type on the sensitivity of this method was to be examined, in order to choose the best type for the following experiments. Differences between the four detectors were determined by comparing the limits of detection (LOD) for sulfite standard solutions by the signal to noise ratio.

Experimental setup:
The same block-shaped cell with platinum electrode was used for three detector models, the circular shaped cell for the fourth model. Each detector was connected to the same HPLC apparatus with integrated enzyme reactor. A sulfite standard solution (c = 0.4 mg/L) was prepared and injected into the HPLC at least four times. The resulting chromatograms were compared, and the LOD was determined as follows: the baseline noise was identified with the Chromeleon® software, and the sulfite concentration corresponding to a peak of thrice that noise was calculated. Therefore, the more calm the baseline and the larger the peak area, the more sensitive is the detection of sulfite.

A summary of the results is shown in table 3.3.

Table 3.3.: Comparison of four different detector models. cc = circular-shaped cell; bc = block-shaped cell; LOD limit of detection (3 ∗ signal/noise); LOQ limit of quantification (10 ∗ signal/noise).

	TED 2020 cc	**TED 4020 bc**	**TED 2020 bc1**	**TED 2020 bc2**
peak height [mV]	23.7	14.4	20.7	20.5
baseline noise [mV]	0.173	0.027	0.538	0.191
LOD [mg SO_2/L]	0.009	0.002	0.031	0.011
LOQ [mg SO_2/L]	0.029	0.008	0.104	0.037

Out of the four tested detectors, the model TED 4020 showed the baseline with the lowest noise level and, despite of the comparatively low peak height, the lowest LOD. With the calm baseline, a correct peak integration is more easy and more reproducible, as beginning and end of a peak shape are well defined. That is especially of importance, since the peak shape of the hydrogen peroxide peak in this method is very broad, and its slope is rather flat in the beginning.

In figure 3.6, chromatograms of TED 4020 and of TED 2020 bc1 are shown.

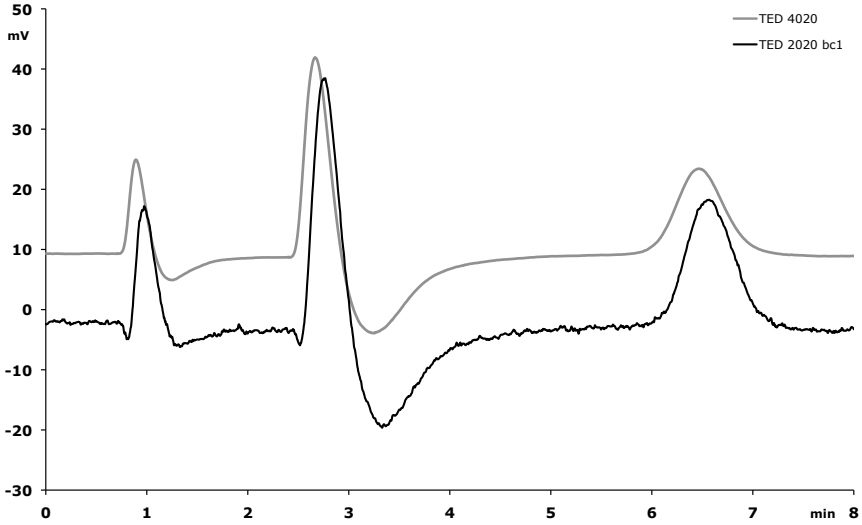


Figure 3.6.: Chromatograms of sulfite standard solution (c = 0.4 mg/L) with two different detector models. Upper chromatogram: TED 4020; lower chromatogram: TED 2020 bc1.

The detector model TED 4020 was chosen for all further experiments, due to the very low level of baseline noise and the resulting sensitivity of the detector.

3.2.4. Platinum Electrode

For the electrochemical detection of hydrogen peroxide, the use of a platinum electrode is well established [25]. After some time of usage, the performance of the electrode decreases. Matrix compounds can cause electrode fouling, leading to a slightly more rugged baseline and increased peak areas for certain substances, i. e. for the two carbonate peaks, but not for the hydrogen peroxide peak. Chromatograms of a standard sulfite solution with a used and a new electrode are shown in figure 3.7.

With the used electrode, the integration of the analyte peak is aggravated. Behind the carbonate peaks, the baseline is returning more slowly to its normal level. Thus it is more difficult to determine the starting point of the sulfite peak area.

Besides the effects on the baseline, there is another observation with used electrodes. A new and clean electrode shows almost no sulfite peak, when chromatography is performed without an enzyme reactor. The electrode potential of 0.2 V is not strong enough to oxidize the sulfite ion itself. Only the hydrogen peroxide resulting from the interaction between sulfite and the enzyme is detected. An electrode that has been used in the HPLC-IMER for a long time does lead to a small sulfite peak even without an enzyme reactor prior to detection. There are two possible explanations for this effect: either the platinum cell does get more sensitive for sulfite ion with extended use (as it does for carbonate as well), or part of the sulfite oxidase was washed out of the reactor and got immobilized on the surface of the detector electrode. As in a typical biosensor construction, the enzyme then produces H_2O_2 right on the surface of the electrode, where it gets oxidized immediately, leading to the sulfite peak in the chromatogram.

Either way, a sulfite peak without the enzyme reactor attached is undesired. For the validation of a designated sulfite peak, it is essential that there is no sulfite peak without the enzyme reactor. Only so, underlying peaks of other substances than sulfite can be detected (see figure 3.8). A clean electrode is therefore essential for valid results. The cleaning is done either by applying alternately very high and very low potentials to the electrode, or by gently rinsing and polishing the electrode surface with water and methanol.

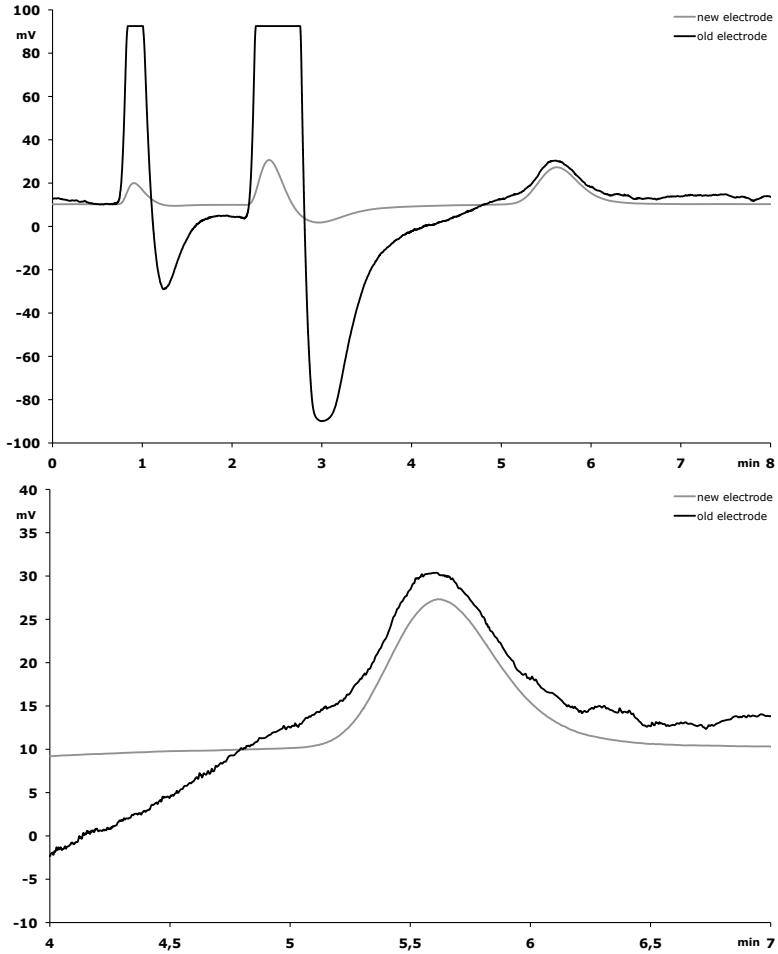


Figure 3.7.: Chromatograms of a sulfite standard solution (c = 0.4 mg/L). Detection with a used platinum electrode (black line, more rugged, larger carbonate and bicarbonate peaks) and a new platinum electrode (grey line, smooth, small carbonate and bicarbonate peaks). The lower figure shows the peak in detail and enlarged.

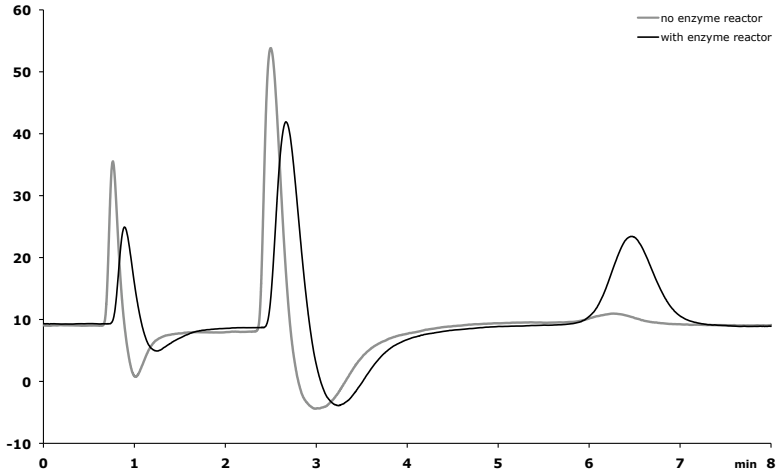


Figure 3.8.: Chromatograms of a sulfite standard solution (c = 0.4 mol/L) with and without enzyme reactor. The platinum electrode of the detector has been in use for a few weeks, a small peak appears even without the enzyme reactor. The shift in retention times is due to more dead volume in the HPLC with the enzyme reactor.

3.2.5. Sample Preparation

An optimized sample preparation is the precondition for precise and correct results of an analytical method. In sulfite analysis, the main purpose of the sample preparation, besides bringing the sample into a liquid form, is to bring all sulfites, bound and free, into the same ionic state, that is sulfite (SO_3^{2-}).

The alkaline pH value of the buffer leads to the conversion of all free sulfites into the desired form. Some of the strongly bound sulfites require more extreme conditions and more exposure time in order to get released. Addition of excess sodium hydroxide solution to the sample provides an alkaline pH value of 14 and leads to the decomposition of sulfite bonds without interfering the HPLC conditions.

The purpose of the following experiments was to determine the rate of sulfite release and to evaluate whether there are any other effects that support or counteract this release.

A manual sample preparation for HPLC-IMER, as described in the work of Weßels, includes the addition of 2 mL of sodium hydroxide solution to 1 mL of the sample, allowing a certain time for reaction, and a dilution of this solution with carbonate buffer (pH 10.6), typically 3:25 (v+v).

For the following experiments, the manual sample preparation was replaced by an automated sample preparation. Thus, it was possible to execute experiments over long time periods without interruptions, but with very high reproducibility.

An autosampler was programmed to do the entire sample preparation, consisting of the addition of sodium hydroxide solution to the sample, mixing, waiting, addition of buffer, mixing, and injection (the complete programming code is given in appendix B.3). Figure 3.9 shows the automated sample preparation in a flow diagram.

800 μL of sodium hydroxide solution (or other solutions like water or carbonate buffer, respectively) is filled from reservoir C into an empty vial (A), and then 400 μL of the sample solution is added. Both liquids are mixed thoroughly. From this vial, now containing 1200 μl of the treated sample, an aliquot of 100 μL is pipetted into a new vial (B), where further dilution with carbonate buffer is performed. The diluted sample is then injected into the HPLC. This action, pipetting alkalized sample solution from vial A to a new vial, followed by dilution and injection can be repeated several times. Thus, the hydroxide solution is allowed different amounts of time to react with the sample matrix.

The resulting chromatograms show the effects of sulfite release against reaction time with sodium hydroxide solution.

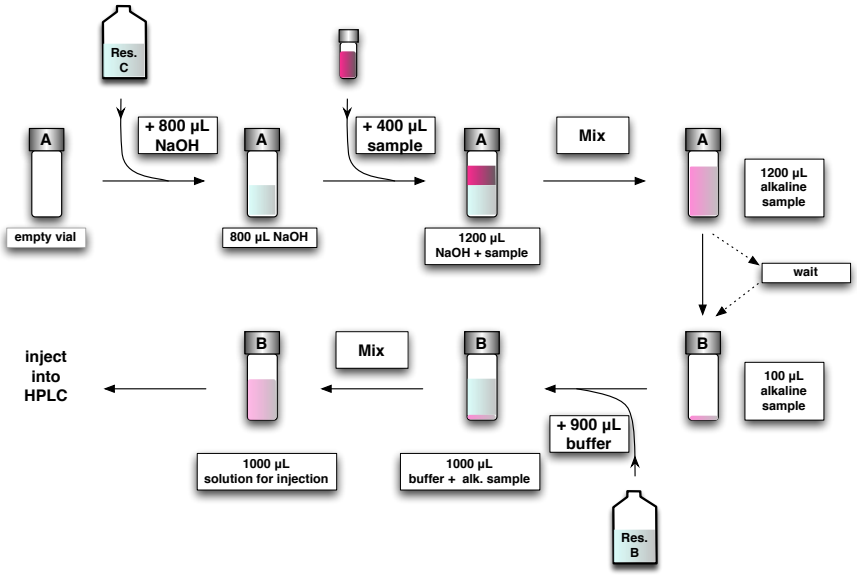


Figure 3.9.: Automated sample preparation with AS 50.
Reservoir B: carbonate buffer, c = 0.06 mol/L;
Reservoir C: sodium hydroxide solution, c = 1 mol/L.

Compared to the manual preparation described before, there is a slight difference in the concentration of the sample at the time of the injection. In the manual sample preparation the liquid sample is diluted 1:25 (v+v), however, the automated sample preparation yields a sample dilution of 1:27 (v+v). This needs to be considered in calculating the sulfite contents of the sample.

In another method, the autosampler was programmed to perform a dilution of 1:100 (v+v) with several different diluents, like water, buffer, or sodium hydroxide solution. This method was used for the following experiments (see appedix B.3).

In a standard sulfite solution, there are no bound sulfites that need to be released. As expected, for a standard sulfite solution, the reaction time with water or carbonate buffer has no influence on the amount of sulfites detected with the HPLC, as shown in figure 3.10. This figure shows the peak area of a sulfite standard solution that has been diluted 1:100 (v+v) with water[1] and was then further di-

[1] containing EDTA and fructose for stabilization

luted with either water oder buffer. Each solution was then injected seven times, covering a total time span of 11 hours. This experiment was intended to show the influence of water addition or buffer addition to sulfite in a standard solution over time. Water or buffer do not have significant effects on the recovery of sulfites from a standard solution.

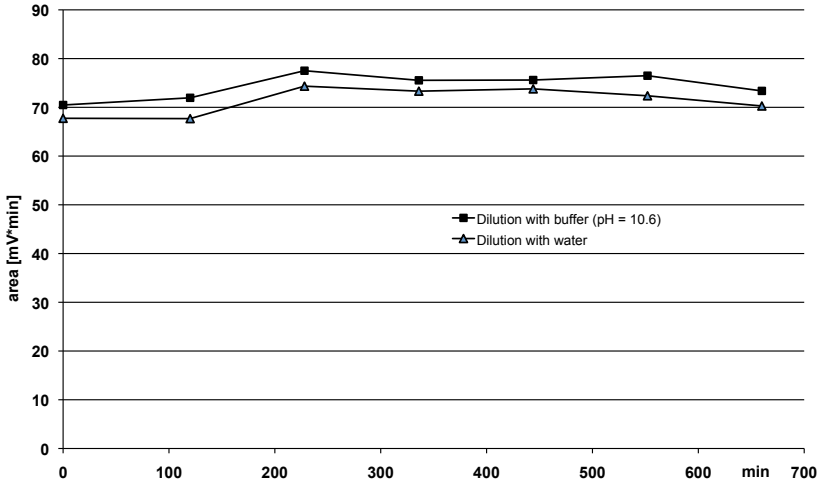


Figure 3.10.: Sulfite amounts of a standard solution that was diluted by the autosampler with either water or carbonate buffer (pH = 10.6). Each mark reflects the average value of 5 injections.

For a food sample, like grape juice, the situation is different. The juice examined in the following experiments was again a Blauer Portugieser produced in the Forschungsanstalt Geisenheim. The initial amount of sulfur dioxide was 75 mg/L. This sample was diluted 1:100 (v+v) with carbonate buffer by the autosampler. The mixture was injected nine times over a timespan of 876 minutes. Each result presented in figure 3.11 is the average result of five sample injections.

From an initial measured amount of sulfur dioxide of about 60 mg/L, a rapid decrease set in. After 120 minutes, the recovery has dropped down to less than 25% of the initial value. Five hours after the first injection, more than 90% of the sulfur dioxide have disappeared. In contrast to the sulfite standard solution, there is a negative effect of the buffer solution on recovery of sulfites from the

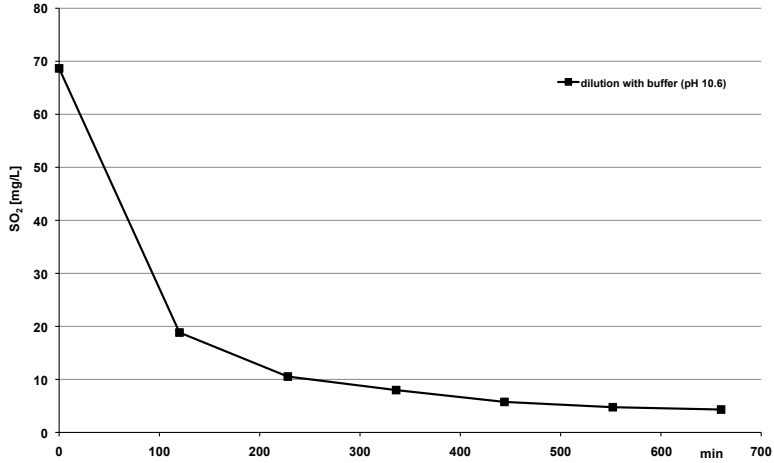


Figure 3.11.: Sulfite amounts in a red grape juice (Blauer Portugieser) that was diluted 1:100 (v+v) by the autosampler with carbonate buffer (pH 10.6).

Sample:	red grape juice
Sample preparation:	by Autosampler AS 50
Method:	1)1zu100aus11mitB 2) 0,6ml_min_valve2
Preparation steps:	1) dilution 1:100 (v+v) with buffer (pH 10.6) 2) injection after waiting time
Data	each point is the average out of five samples

grape juice. The longer the grape juice was exposed to the buffer solution, the less sulfites are found in the sample.

In order to find out whether this effect is solely due to the alkaline pH value of the buffer solution, the same experiment was performed with sodium hydroxide solution of a similar pH value (pH = 10.3). As shown in figure 3.12, the decrease after dilution with sodium hydroxide solution is less strong. For the first four hours, no change is visible. Afterwards, there is a rather slow decrease down to 80% of the initial value 14.6 hours after the dilution step. Immediately after the dilution step, both solutions gave the same results for sulfite concentration: 60 mg/L.

There is a serious difference between the sulfite recoveries. Sulfite seems to vanish when the juice is diluted with carbonate buffer. In order to examine the reasons for these findings, further experiments were performed. An oxidation of sulfite to sulfate may be a reason, as well as the formation of irreversibly bound sulfite-adducts, possibly including carbonate ions.

An apple must[2] gives results similar to the grape juice (see figure 3.13). The dilution with carbonate buffer leads to a fast and dramatic decrease in measurable sulfites. After two hours, sulfite is not detected in the sample solution anymore. Dilution with alkaline sodium hydroxide solution, instead of the alkaline buffer, only leads to a slow and a lot less intense decrease of sulfite detection.

However, a combination of both diluents (buffer and NaOH) leads to an improved recovery of sulfites (see figure 3.14). There is an increase in detected sulfites over time, which is stronger with 20% NaOH than with 10% at first, but after about 550 minutes both lead to the same results, with the sulfite peak areas continuously rising. Thus, a combination of NaOH and carbonate buffer is much more effective in releasing sulfites than either one by itself. Not only does the mixture lead to higher results, it also inhibits the decrease over time that was seen with pure buffer solution.

Similar results are obtained for a grape juice (Blauer Portugieser), when tested with four different diluents. The juice was diluted 1:100 (v+v) with plain water, carbonate buffer (pH 9.1), carbonate buffer (pH 10.6) and a mixture of the buffer (pH 10.6) and 10% of sodium hydroxide solution (c = 1 mol/L). For results see figure 3.15.

The same experiment was repeated three times, always leading to the same results.

In another experiment, a different sort of grape juice, originally not containing any sulfites, was sulfurized in the laboratory and analyzed according to the procedure described in the preceding paragraph. This sample of “Dunkelfelder” showed

[2] Apple must (Apfelmost) is a German variant of cider made out of apples, containing 5.5–7% of alcohol.

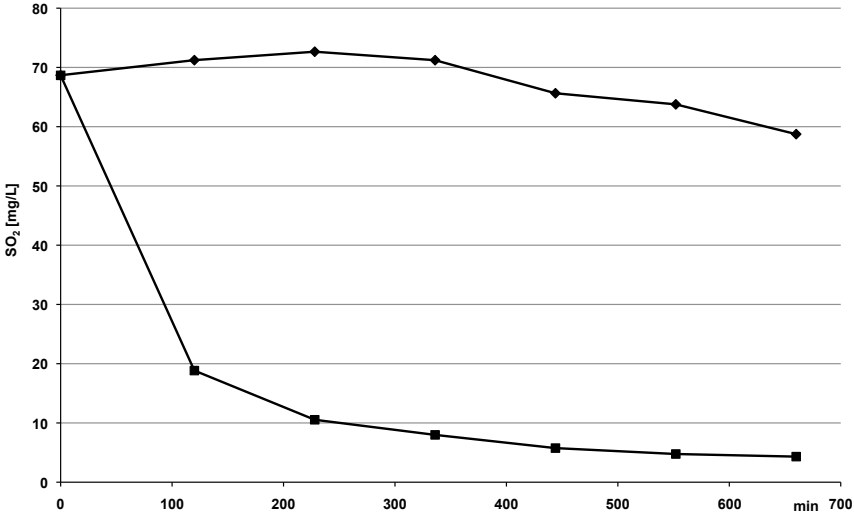


Figure 3.12.: Sulfite amounts of a red grape juice (Blauer Portugieser), diluted 1:100 (v+v) by the autosampler with:
♦ sodium hydroxide solution of (pH 10.3)
■ carbonate buffer (pH 10.6).

Sample:	red grape juice
Sample preparation:	by Autosampler AS 50
Method:	1)♦ 1zu100aus11mitA 1)■ 1zu100aus11mitB 2) 0,6ml_min_valve2
Preparation steps:	1)♦ dilution 1:100 (v+v) with NaOH (pH 10.3) 1)■ dilution 1:100 (v+v) with buffer (pH 10.6) 2) injection after waiting time
Data	each point is the average out of five samples

decreasing sulfite peak areas even with the mixture of NaOH and carbonate buffer. The decrease, however, was less strong than it was with pure buffer dilution.

The results obtained from the previously discussed measurements lead to the conclusion that, of the tested materials, a mixture of sodium hydroxide solution and carbonate buffer is most effective for the analysis of sulfites with the HPLC-IMER method.

The following step was to investigate the effect of the order of addition. Does it make a difference whether NaOH is added first, followed by the buffer, or should both be added to the sample at the same time?

Different samples (white, rosé and red wines as well as red and white grape juices) were prepared by the autosampler, each in two different ways:

1. the sample liquid was added to twice its amount of sodium hydroxide solution and mixed. From this mixture, a small amount was taken up and mixed with carbonate buffer immediately before injection into the HPLC. The second step was repeated several times, allowing the NaOH to react with the sample matrix for varying amounts of time (sample preparation as described in figure 3.9).
2. the sample was mixed with sodium hydroxide solution and carbonate buffer (concentrations and amounts as described above) at the same time, and this mixture was then injected into the HPLC several times over a certain timespan.

The preparation parameters and results are shown in figure 3.16.

For each of the four different samples there is a clear difference between both preparation methods. The immediate addition of NaOH and buffer leads to a constant level of sulfites that does not significantly rise or decrease over the tested time span of about one hour.

In contrast, the addition of pure NaOH solution leads to ascending sulfite amounts over time (the time span of the experiment was up to 224 minutes). The degree of increase varies between the samples (refer to table 3.4). For all samples, neither the absolute increase, nor the relative increase are concordant (as presented in table 3.5). The absolute increase in sulfite findings for the sodium hydroxide treatment varies from 0.2 mg/L (white grape juice) to 21.1 mg/L (red wine B). The relative increase after the time span of 224 minutes in relation to the first injection was in the range of 2% (white grape juice) up to 39% (both red wines). So the ability of the alkaline solution to set free bound sulfites is dependant on the type of sample, the absolute amounts of sulfites and probably even on the specific production procedure of a juice.

Table 3.4.: Sulfur dioxide recovery after different reaction times with NaOH.

Sample	**Reaction time with NaOH** [mg/L SO_2]		
	0 min	**60 min**	**224 min**
Standard solution	18.5	18.5	18.8
Red grape juice	7.5	9.0	9.3
White grape juice	9.0	8.8	9.2
White wine	56.7	61.8	64.3
Red wine A	39.7	48.3	55.3
Red wine B	53.9	68.1	75.0
Rosé wine	91.9	106.8	110.0

Table 3.5.: Sulfur dioxide recovery after 60 min and after 224 min of reaction with NaOH, expressed as absolute and relative difference to the initial values with no reaction time.

Sample	**Absolute difference** [mg/L]		**Relative difference** [%]	
	60 min	**224 min**	**60 min**	**224 min**
Standard solution	0.0	0.3	0	2
Red grape juice	1.5	1.8	21	25
White grape juice	-0.2	0.2	-2	2
White wine	5.1	7.6	9	13
Red wine A	8.6	15.6	22	39
Red wine B	14.2	21.1	26	39
Rosé wine	14.9	18.1	16	20

For the white grape juice with about 9 mg/L SO_2, for example, the relative increase was only 2%, whereas for a red grape juice with approximately the same amount of sulfites, the increase over time was 25%.

The initial thesis, that anthocyanins cause problems in the HPLC-IMER analysis of sulfites is supported by the data presented. With insufficient reaction times for sodium hydroxide solution, not all bound sulfites are released, this leading to lower sulfite findings. The red wines show higher relative increases in sulfite release than the rosé wine, which again rises more than the white wine. Also, the relative increase in sulfite findings of the red grape juice was a lot stronger than that of the white grape juice.

These analyses were possible with automatic sample preparation only. Absolute reproducibility was very important, and the long timespans required for one entire run of several sample preparation and analysis steps (up to 14 hours) was impossible to be achieved by one person executing the preparation manually.

Conclusion:
The best sample preparation method for the HPLC-IMER includes an addition of sodium hydroxide solution to force the release of combined sulfites, as well as dilution with carbonate buffer in order to ensure a similar pH value for all samples for stable retention times of the sulfites.

The waiting step between the addition of NaOH and the addition of carbonate buffer is relevant for most samples, however not for all. For some samples (i. e. wines), a long reaction time is the best choice, for other samples, like the white grape juice, a delay is not necessary at all. Therefore, there is not one ideal solution for all samples.

To provide a guideline, even for samples of unknown character, one hour of delay is suggested, in order to keep the time for one analysis as short as possible without unjustifiable minor findings. As shown in table 3.5, after allowing one hour of reaction time, most samples have released large amounts of sulfites.

For samples that are exceptionally rich in polyphenols (e. g. anthocyanins), a longer reaction time may be recommended. In order to release as many sulfites as possible, up to four hours may be required, considering the slow release of sulfite in wines (see figure 3.16).

However, considering possible adverse health effects of sulfites in food, it is questionable, whether sulfites that are only released after one hour (or more) of alkaline treatment, are after all of relevance in toxicological aspects.

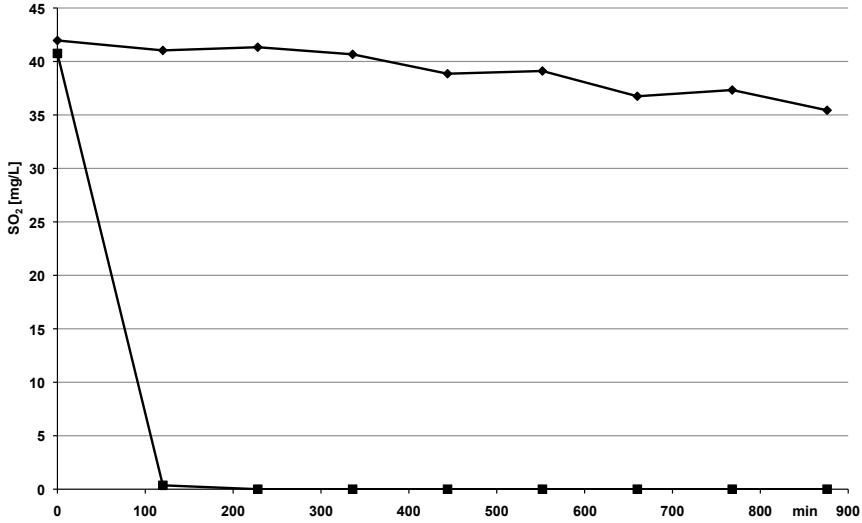


Figure 3.13.: Sulfite amounts of an apple must, diluted 1:100 (v+v) by the autosampler with:
♦ sodium hydroxide solution (pH 10.3)
■ carbonate buffer (pH 10.6).

Sample:	apple must
Sample preparation:	by Autosampler AS 50
Method:	1)♦ 1zu100aus11mitA 1)■ 1zu100aus11mitB 2) 0,6ml_min_valve2
Preparation steps:	1)♦ dilution 1:100 (v+v) with NaOH (pH 10.3) 1)■ dilution 1:100 (v+v) with buffer (pH 10.6) 2) injection after waiting time
Data	each point is the average out of five samples

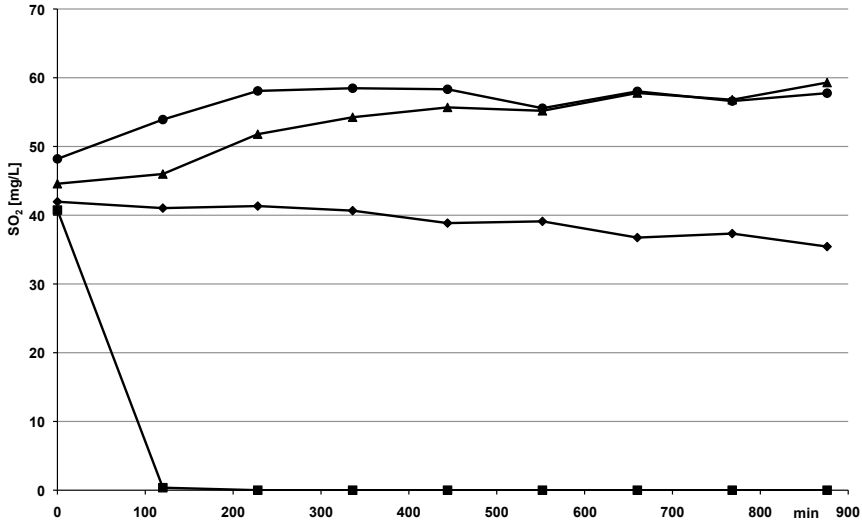


Figure 3.14.: Sulfite amounts over time of an apple must, diluted 1:100 (v+v) by the autosampler with:
♦ sodium hydroxide solution (pH 10.3)
■ carbonate buffer (pH 10.6)
▲ carbonate buffer with 10% NaOH (1 mol/L)
• carbonate buffer with 20% NaOH (1 mol/L).

Sample:	apple must
Sample preparation:	by Autosampler AS 50
Method:	1)♦/▲ 1zu100aus11mitA 1)■/• 1zu100aus11mitB 2) 0,6ml_min_valve2
Preparation steps:	1)♦ dilution 1:100 (v+v) with NaOH (pH 10.3) 1)■ dilution 1:100 (v+v) with buffer (pH 10.6) 1)▲ dilution 1:100 (v+v) with 10% NaOH in buffer 1)• dilution 1:100 (v+v) with 20% NaOH in buffer 2) injection after waiting time
Data	each point is the average result out of five samples

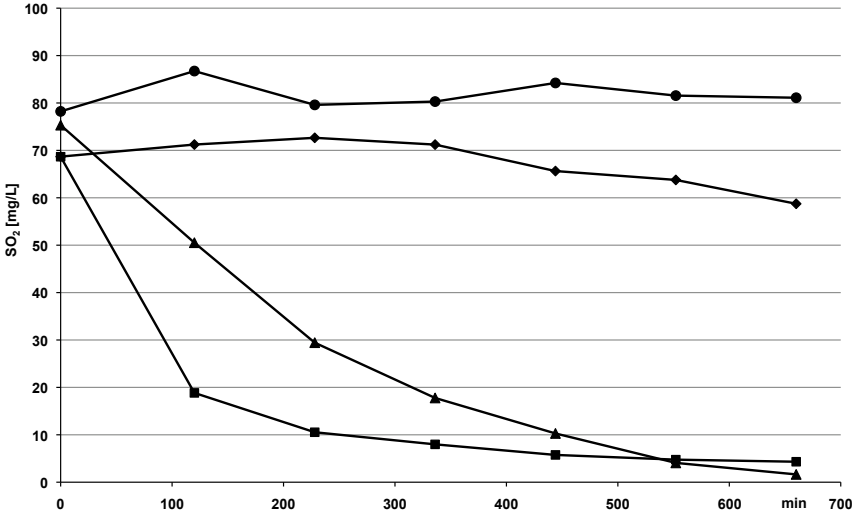


Figure 3.15.: Sulfite amounts of a Blauer Portugieser, diluted (1:100; v+v) with four different solutions:
♦ NaOH (pH 10.3)
▲ carbonate buffer (pH 9.1)
■ carbonate buffer (pH 10.6)
• carbonate buffer (pH 10.6) with 10% NaOH (1 mol/L).

Sample:	Blauer Portugieser
Sample preparation:	by Autosampler AS 50
Method:	1)♦/■ 1zu100aus11mitA 1)▲/• 1zu100aus11mitB 2) 0,6ml_min_valve2
Preparation steps:	1)♦ dilution 1:100 (v+v) with NaOH (pH 10.3) 1)▲ dilution 1:100 (v+v) with buffer (pH 9.1) 1)■ dilution 1:100 (v+v) with buffer (pH 10.6) 1)• dilution 1:100 (v+v) with 10% NaOH in buffer 2) injection after waiting time
Data	each point is the average result out of five samples

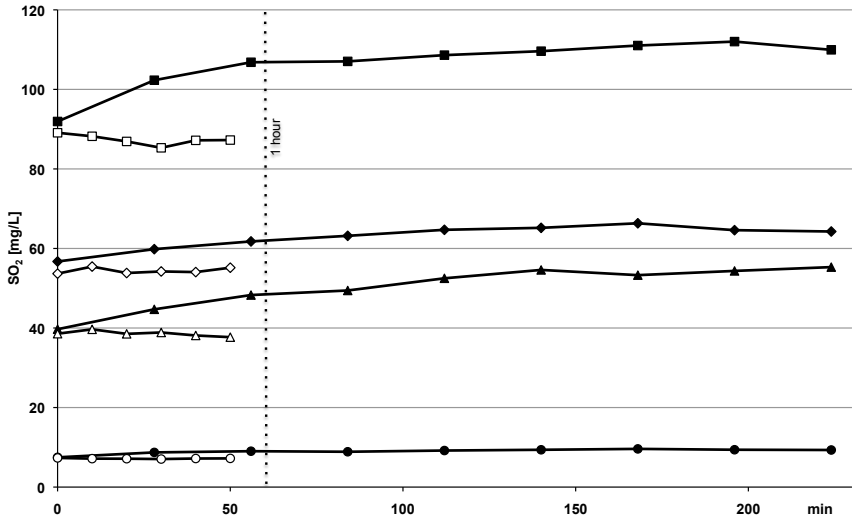


Figure 3.16.: Comparison of sample preparation with two different methods.
1) addition of NaOH (1 mol/L) to sample, wait, dilution with carbonate buffer (pH 10.6) immediately before injection
■ Rosé wine
♦ White wine
▲ Red wine
● Grape juice.
2) addition of NaOH (1 mol/L) and buffer at the same time, wait, injection
□ Rosé wine
◊ White wine
△ Red wine
○ Grape juice.

3.3. Comparison of Sulfite Oxidases

The HPLC-IMER was first developed by applying the commercially available sulfite oxidase (SOx) derived from chicken liver (EC 1.8.3.1). In 2001, Eilers et al. demonstrated the existence of sulfite oxidase in plants for the first time. They succeeded in cloning a plant sulfite oxidase gene from *Arabidopsis thaliana* and characterized the encoded protein [28].
As presented earlier, both types of sulfite oxidase, animal and plant, are molybdenum enzymes with molybdopterin as an organic component of the molybdenum cofactor. X-ray crystal structures of both enzymes show nearly identical square pyramidal coordination around the Mo atom, but there is one major difference between the two: In contrast to the sulfite oxidase of mammals and birds, plant SOx lacks the heme domain. The molecular weight of the mammalian and avian SO dimer is 110 kDa, it is therefore bigger than the enzyme from *Arabidopsis thaliana* with 90 kDa.

Both enzymes catalyze the same reaction:

$$SO_3^{2-} + H_2O + O_2 \xrightarrow{SOx} H_2O_2 + SO_4^{2-}$$

As the animal SOx has proven its potential for use in HPLC-IMER, in the following experiments the performance of the plant sulfite oxidase was examined. For these experiments, the HPLC-IMER was equipped with a switch valve (see figure 3.17). This setup allows the use of two different enzyme reactors under equal conditions. By injecting each sample twice, and switching from one reactor to the other between the analyses, both enzyme reactors are equally stressed. Thus, a direct comparison of both enzymes under identical conditions is possible. Also, a comparison of the results with and without enzyme reactor is possible by replacing one reactor with a "dummy" reactor (without enzyme), or by omitting one enzyme reactor.

3.3.1. Linear Range

The linear range of two sulfite oxidase reactors, one with animal and one with plant sulfite oxidase, was examined. Two enzyme reactors were prepared freshly, each with about 50 units of sulfite oxidase. The immobilization procedures were identical for both enzymes (see chapter 2.2.3.2 on page 41).

Sulfite standards with the following concentrations were prepared: 0.04 mg/L; 0.08 mg/L; 0.2 mg/L; 0.4 mg/L; 0.8 mg/L; 2 mg/L; 4 mg/L; 8 mg/L; 10 mg/L. Each standard was analyzed with both enzyme reactors in the same sequence. The switch valve was programmed to switch after each run, so that both enzymes were

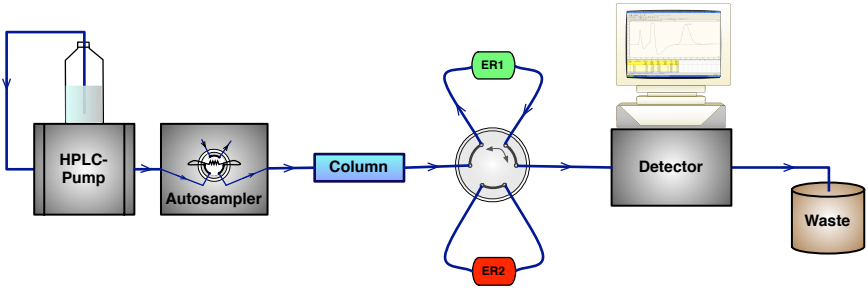


Figure 3.17.: Setup of the HPLC-IMER with switch valve and two different enzyme reactors (ER1 and ER2).

used under the same conditions regarding standing times, temperatures, flow rates and number of injected samples. Figure 3.18 shows the calibration curves of both enzymes.

The plant sulfite oxidase shows an almost perfectly linear range from 0.04 mg/L to 2 mg/L SO_2. In contrast, the results with animal SOx do not show a linear correlation, but rather form a curve in the same concentration range. Thus, the plant SOx seems to have either a greater capacity for the reaction with sulfite, or its reaction time (in the immobilized state) is significantly faster than that of the animal SOx.

It will be shown in chapter 3.5.1, that the linear range of a plant sulfite oxidase reactor continues far beyond 2 mg/L SO_2. The limiting factor in this experimental setup is the electrochemical detector, which reaches its detection limit at H_2O_2-concentrations little above the equivalent of 2 mg/L SO_2.

3.3.2. Stability

Enzymes are stable only under certain conditions. In their physiological environment they can be active for severals weeks, whereas the isolated enzyme is not stable at room temperature for a long time. As enzymes are proteins, they are very sensitive to changes of temperature and pH value of their surroundings.

The stability of two different enzyme reactors, one with chicken liver sulfite oxidase and one with plant sulfite oxidase, was compared. Both reactors were prepared the same day with approximately the same amount of sulfite oxidase enzyme. Storage conditions were exactly the same for both enzyme reactors at all times. The reactors were applied in the analysis of standard sulfite solutions, as well

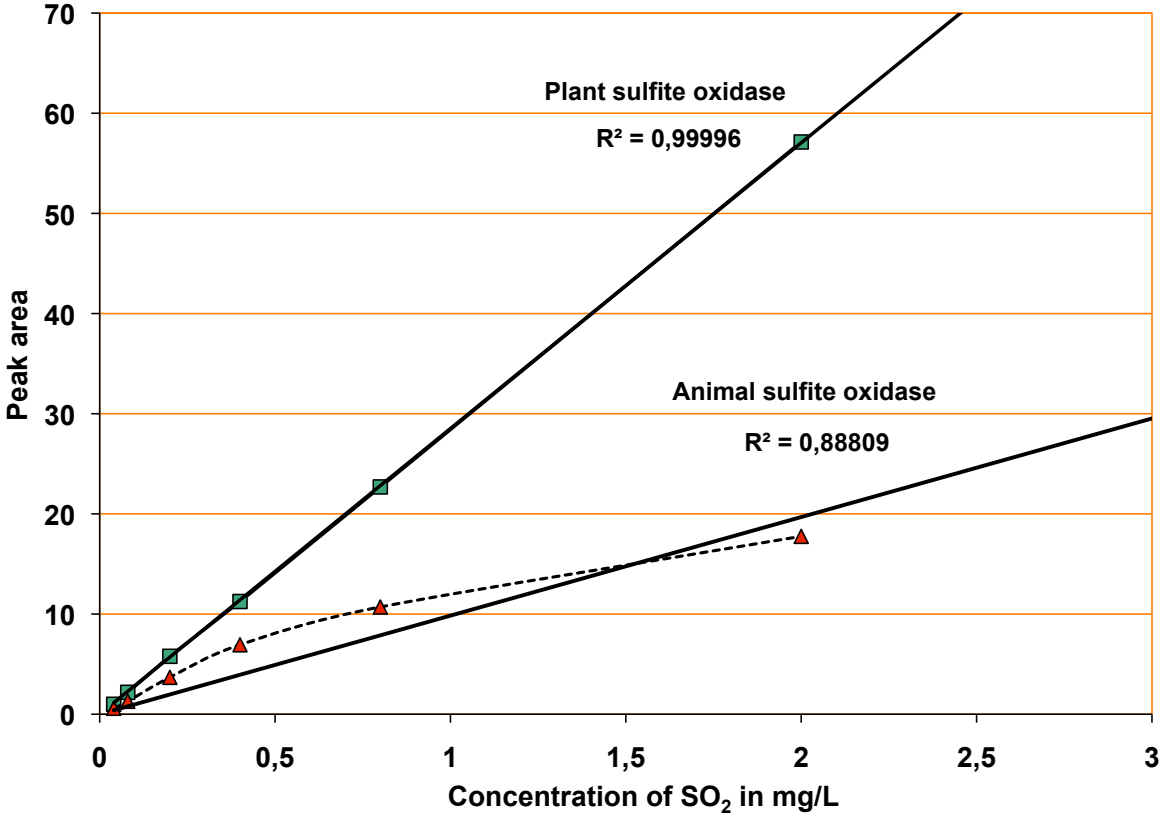


Figure 3.18.: Calibration curves in the concentration range of 0.04 – 2 mg/L SO_2. Comparison between plant and animal sulfite oxidase in HPLC-IMER.

as of food samples. Each sample was injected two times consecutively, with the switch valve changing between reactors after each sample. Thus, both reactors were exposed to the same conditions at all times.

In table 3.6, some data of this experiment is presented. Within two months, both reactors have been extensively stressed with many different grape juice samples and standard solutions.

Table 3.6.: Comparison of animal and plant sulfite oxidase.

Day	**# Injections Sulfite std. sol.**	**Area of sulfite standard**		**Ratio animal/plant SO_2**
		Plant SOx	**Animal SOx**	
0	1	12.7	9.75	0.77
1	50	11.5	8.9	0.77
6	100	11.2	6.8	0.61
14	150	8.3	2.7	0.33
42	200	12	3.9	0.33
58	250	9.3	1.3	0.14

Figure 3.19 shows the comparison of the obtained peak areas relative to each other. Out of 250 different samples and standard solutions analyzed, only the values for the standard solutions are displayed for reasons of better comparability. Food samples generally contain a lot more known and unknown substances than a standard solution, which might influence the performance of either one or both of the enzymes and therefore lead to incommensurable results. The results, however, were found to be very similar for food samples.
At the beginning of the experiment, the reactor with animal sulfite oxidase showed an activity level of 77% compared to the plant sulfite oxidase reactor. After 50 injections, the ratio was still about the same. From thereon, the activity of the animal SOx decreased compared to that of the plant sulfite oxidase. After 200 injections, the peak areas of a standard solution with the animal sulfite oxidase reactor were only 34% of that of the plant SOx, after 250 injections the ratio decreased down to 14%.

It is necessary to compare both enzyme reactors relative to each other, as the absolute peak area of a reactor may vary due to different factors (see chapter 3.2). It is not possible to determine the exact decrease of one sulfite oxidase reactor's reactivity.

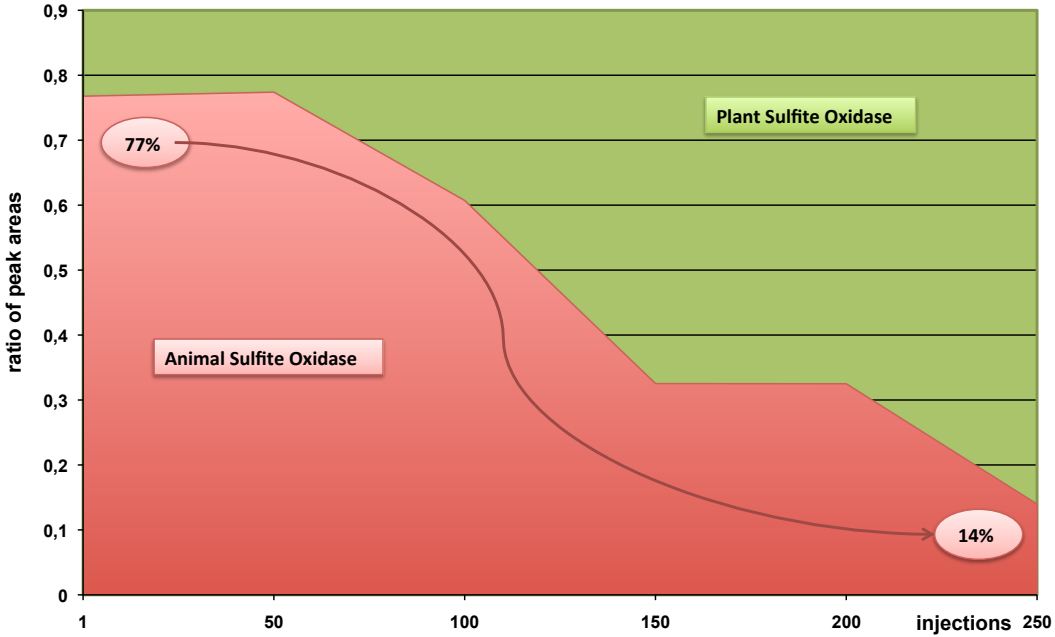


Figure 3.19.: Comparison of sulfite oxidase performances. Both enzymes were used under the same conditions. Peak areas of standard solutions are presented, the area of plant sulfite oxidase is set at 100% for better comparison.

After two months and 250 injections of standard sulfite solutions and food samples, the performance of the animal sulfite oxidase reactor became too weak for sensitive sulfite analysis with HPLC-IMER. The peak area of a standard solution with 0.4 mg/L SO_2 was only 13% of its initial value. In comparison, the plant sulfite oxidase reactor still showed about 73% of its initial peak area after the same amount of time and stress.

From both experiments, it can be concluded that for application in the HPLC-IMER, the plant sulfite oxidase is better suited than the sulfite oxidase from chicken liver.

The broad linear range of the plant sulfite oxidase allows for a one point calibration for all samples within the detection range of the electrochemical detector. With the animal sulfite oxidase reactor this is not possible, as the linear range is a lot smaller. One would either have to accept not very exact results, or a calibration curve would be necessary for every sample. The latter is an inefficient and time consuming step. As described earlier, the response of a reactor may change within an hour. Therefore, a calibration curve for every sample appears not at all practical for the HPLC-IMER.

The better immobilization stability of the plant oxidase is a further reason to prefer this enzyme over the animal sulfite oxidase. In most cases, the plant SOx reactor leads to higher responses from the start, and shows a much better stability compared to the animal sulfite oxidase. Some plant SOx reactors were still applicable after two years of moderate use, whereas the animal SOx reactors showed either very small peaks or no signals at all after the same period of time.

Conclusively, both sulfite oxidase enzymes are suited for use in the HPLC-IMER. Compared to the chicken liver SOx, the plant SOx shows two major advantages: The immobilized plant sulfite oxidase is more stable, and it can be used over a longer period of time than the animal sulfite oxidase. The linear range of the plant sulfite oxidase reactor is a lot bigger than that of the animal sulfite oxidase, limited only by the maximum load of the electrochemical detector.

The application of a marine sulfite oxidase in HPLC-IMER analysis was not successful. A sulfite detection after immobilization of the marine sulfite oxidase on the carrier material was not achieved.

3.4. Different Matrices

An analytical method for the determination of sulfites in foodstuffs will only be used in routine analysis, if it is applicable to a wide range of different matrices without complicated adaptions in sample preparation.

In most cases, the HPLC-IMER needs only minimal sample preparation procedures. The general purpose of a sample preparation is to make the analyte available for analysis (usually by removing potentially interfering substances), adjusting the concentration of the analyte, and bringing it into the desired chemical form.

In the case of the HPLC-IMER, the desired chemical form is the free sulfite anion (SO_3^{2-}). Hydrogen sulfite and sulfur dioxide are turned into the desired form easily at alkaline pH value, which is achieved by the addition of sodium hydroxide solution to the sample. Reversibly bound sulfites are easily liberated in alkaline medium and turn into free sulfites as well. Therefore, the addition of excess sodium hydroxide solution to the sample ensures the desired state of the analyte irrespective of its prior state within the food.

The adjustment of sulfite concentration is, if needed, easily done by a dilution with sodium carbonate buffer. The detection limit of the method is extremely low, so concentration of the analyte is not necessary for the majority of samples.

Interferences with other substances can preclude correct analytical results. In HPLC-IMER, potential interferences are minimized by four different mechanisms:

1. the ion-exchange column: negative ions are separated from each other, leading to a specific retention time for sulfites.
2. the enzyme reactor: the enzyme sulfite oxidase is very specific; it reacts exclusively with sulfite (SO_3^{2-}), yielding equivalent amounts of hydrogen peroxide.
3. the electrochemical detector: the platinum electrode is requiring very low voltage for the very sensitive oxidation of hydrogen peroxide. Only very few other substances will be oxidized and lead to a measurable output on the detector.
4. the comparison of results with and without the enzyme reactor: a designated sulfite peak in a chromatogram must disappear when the sample is analyzed without the enzyme reactor. If the peak remains, the substance detected is not sulfite.

Considering all arguments mentioned above, one can expect the HPLC-IMER to work well with food samples of any kind. In order to prove this thesis and to find evidence, a lot of different food samples were tested with this application. The

focus in this work is on liquid samples, but some solid matrices have been tested as well.

The following table gives an overview of the tested food samples, of which some will be discussed below.

Food matrix	Number of samples
Fruit juices	>50
Fruit nectars	20
Smoothies	19
Sirups	>100
Wine	>10
Cinnamon	3
Onion powder	1
Asparagus	>10
Mustard	3
Jelly	1

To show a general applicability of the HPLC-IMER for the tested matrices, all liquid samples were equally treated and the resulting chromatograms compared. The chromatograms were checked for electrochemical interferences that might impair the quantification of sulfite. Whether a peak is caused by hydrogen peroxide derived from sulfite or by another substance of the sample, was determined by performing the same HPLC run twice, i. e. with and without the enzyme reactor.

All samples with liquid matrices were analyzed following the same scheme. 1 mL of the sample was added to 2 mL of sodium hydroxide solution (1 mol/L), after a waiting period of 1 hour the mixture was diluted with carbonate buffer (1:25 (v+v); wine: 1:100 (v+v)), filtrated if necessary, and then injected into the HPLC. Of all samples with semifluid or solid matrices, 1 g was weighed into a flask, and then treated like the liquid samples. The asparagus was pressed through a garlic press immediately prior to weighing.

Figures 3.20 and 3.21 show example chromatograms of a grape juice and a glucose sirup, respectively.

3.4.1. Results

The chosen conditions for the chromatography of the juices, nectars, sirups and wine samples resulted in chromatograms without interferences at or near the retention time of sulfite. If a peak appeared at the typical retention time of sulfite,

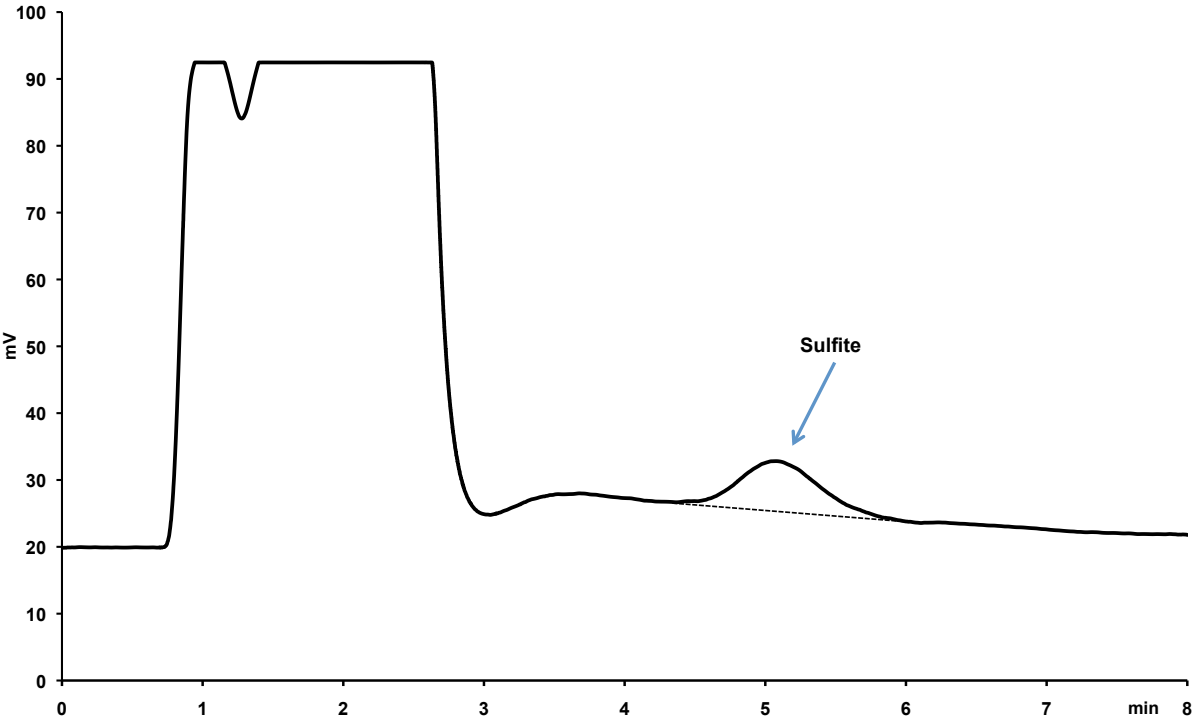


Figure 3.20.: Chromatogram of a grape juice containing SO_2.

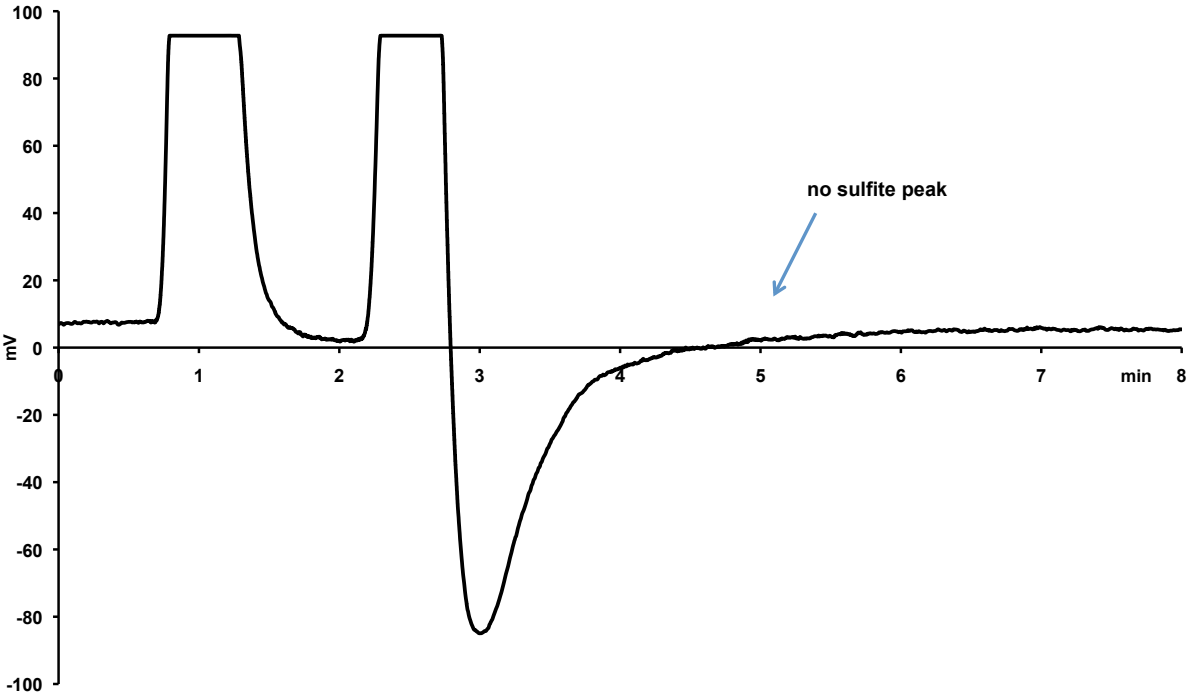


Figure 3.21.: Chromatogram of a glucose sirup without SO_2.

the sample was always re-analyzed without the enzyme reactor to prove its identity. Thus, the method and the chosen sample preparation can be concluded to work well for the liquid samples.

Some of the solid sample matrices caused more difficulties. That is partly because of the larger concentration of electrochemically active compounds, and partly because of the more complicated sample preparation procedure.

Cinnamon, for example, required extensive dilution in order to reduce interferences of other compounds in the chromatogram. After appropriate dilution, concentrations below 25 mg/kg were below the detection limit. However, the main advantage of HPLC-IMER, its sensitivity, was thus eliminated.

3.4.1.1. Fruit Juices and Alcoholic Beverages

More than 50 samples of fruit juices and other beverages have been analyzed with HPLC-IMER during this work. Most of these juices were randomly selected and bought from stores. In appendix B.7, these juices are listed with the names of the producers and relevant additional information. Some juices (i. e. juices prepared from grape, cherry, apple and pear) were obtained from the Forschungsanstalt Geisenheim. Of those, many were unsulfurized juices, and served as references for samples without added sulfites. Those, that were sulfurized, contained defined amounts of sulfites and were prepared in small scale according to common industrial procedures.

The results of HPLC-IMER analysis of 42 juices are presented in table 3.7. The grape juice that was produced in Geisenheim by intentionally adding very large amounts of sulfites was found to contain 72 mg/L SO_2.

All of the other products are legally not allowed to contain sulfite amounts above 10 mg/L (according to the ZZuLV). 17 out of the remaining 41 juices did not contain any detectable amounts of sulfites. Another 16 juices were found to contain sulfites between 0.3 and 9.7 mg/L calculated as SO_2. All of these are within the legal limit of 10 mg/L SO_2.

Eight juices, all of them red grape juices, were found to contain amounts of more than 10, and up to 24 mg/L SO_2. Therefore, some of the commercially available grape juices contain more than twice as much SO_2 as the legal limit allows for. Anyhow, these findings apply only for the results obtained with the HPLC-IMER. The results with the official methods may be different, as presented in chapter 3.7.

It is common practice, that grape juices are sulfurized during production steps in order to prevent undesired microbial damage and browning. Most of the added sulfites are removed before packaging, leaving residues mostly under 10 mg/L. However, there are cases with higher residues, as shown above.

Table 3.7.: Sulfur dioxide contents in different fruit juices analyzed with HPLC-IMER. For details on juices see appendix B.7. Juices with (G) were provided by the Forschungsanstalt Geisenheim.
'Grape juice' is red grape juice.

Type of juice	**SO_2** [mg/L]	**Type of juice**	**SO_2** [mg/L]
Grape juice (G)	72.0	Multivitamin juice (17)	0.5
Grape juice (5)	24.1	Grapefruit juice (22)	0.4
Grape juice (29)	22.0	Grape juice (G)	0.4
Grape juice (G)	18.0	Pear juice (G)	0.3
Grape juice (25)	16.9	Aronia berry juice (12)	0.0
Grape juice (4)	14.9	Sour cherry juice (10)	0.0
Grape juice (1)	14.5	Sour cherry juice (G)	0.0
Grape juice (G)	11.5	Red currant juice (9)	0.0
Grape juice (8)	11.0	Plum juice (11)	0.0
Grape juice, white (6)	9.7	Pear juice (G)	0.0
Grape juice, white (26)	7.5	Pear juice (G)	0.0
Grape juice (31)	7.3	Lime juice (34)	0.0
Lemon concentrate (32)	5.6	Lemon juice (33)	0.0
Grape juice (2)	5.6	Grape juice (30)	0.0
Grape juice (3)	5.6	Grape juice (G)	0.0
Elderflower juice (7)	4.4	Apple-grape juice (27)	0.0
Tomato juice (24)	2.5	Apple-grape juice (28)	0.0
Tomato juice (35)	2.4	Apple juice (13)	0.0
Pineapple juice (20)	2.1	Apple juice (14)	0.0
Multivitamin juice (16)	1.0	Apple juice (15)	0.0
Orange juice (23)	0.7	Apple juice (G)	0.0

A swabian must and a redcurrant wine contained 74 and 58 mg/L SO_2, respectively. More data on wines is presented in chapter 3.2.5.

3.4.1.2. Fruit Nectars

Fruit nectars are products prepared from fruit juices (or concentrates) with water and sugars. According to the FRUCHTSAFTVERORDNUNG, a nectar may contain up to 20% of sugars, including glucose and fructose sirups, sorbit, maltit and other sweeteners. An entry of sulfites into a nectar is possible not only through the fruit juices, but also through the added sugars. In table 3.8, the data of 23 different nectars is presented. About half of the nectars analyzed with HPLC-IMER did not contain any sulfites at all. Most of the others contained only very small amounts of less than 1 mg/L. Only two of the analyzed nectars, two peach nectars, contained more than 1 mg/L SO_2.

In comparison, with the official method of analysis, only trace amounts of sulfites were found in one of the peach nectars, the other one seemed to not contain any sulfites (see chapter 3.7).

With the very low findings of sulfites in some fruit nectars, these products are not critical in their sulfite amounts regarding the ZZULV (see appendix A.2). However, a declaration like "free of additives" would be misleading.

3.4.1.3. Smoothies

Smoothies have become more and more popular in the last couple of years. A smoothie is a beverage with a creamy consistency made out of whole fruits, vegetables and fruit juices, eventually containing also yoghurt, ice-cream, cream or the like. In the United States, smoothies first became available in the late 1960s, mainly at ice cream vendors and health food stores. In Germany, the pre-bottled versions sold in the supermarkets are predominating. Smoothies are supposed to be a healthy and convenient alternative to fresh fruit. Until today, there are no special legislative regulations for smoothies in Germany, the EU or even in the United States. Regarding sulfur dioxide, that means that the general legal limit of 10 mg/L SO_2 applies for all smoothies. Tabular 3.9 gives the results that were obtained by HPLC-IMER analysis of different commercial smoothies.

In total, 16 different smoothies were analyzed. As smoothies are viscous, they were weighed instead of pipetted, so results are given in mg/kg rather than mg/L. None of them contained more than 10 mg/kg SO_2, therefore, in respect to legislation regarding sulfites, all smoothies meet the requirement. Nevertheless, all of the samples were found to contain at least small amounts of sulfites. Five out of the 16 smoothies, almost a third, contained more than 5 mg/kg SO_2. All of the sulfite

Table 3.8.: Sulfur dioxide contents in different fruit nectars analyzed with the HPLC-IMER.

Type of nectar	**SO_2 [mg/L]**
Peach nectar	4.7
Peach nectar	2
Pineapple nectar	1
Mango-passion fruit nectar	1
Banana nectar	1
Orange nectar	0.9
Sea buckthorn nectar	0.6
Guava-redcurrant-lemon nectar	0.5
Passion fruit nectar	0.3
Banana nectar	0.3
Peach nectar	traces
Sea buckthorn nectar (organic)	traces
ACE Orange-carrot-lemon vitamindrink	–
Apple fruitdrink	–
Apple fruitdrink	–
Blood orange drink	–
Orange nectar	–
A-C-E Orange-carrot-lemon	–
Gooseberry nectar	–
Blackcurrant nectar	–
Sour cherry nectar	–
Cranberry nectar	–
Blackcurrant nectar	–

Table 3.9.: Sulfur dioxide levels in different commercial smoothies (detailed information on smoothies is presented in appendix B.6). The percentage of grape juice contained is presented, as grape juices are known to generally contain higher levels of sulfur dioxide.

Producer	**Type as declared by the producer**	**Grapes in the product**	**SO_2 in mg/kg**
Chiquita	Raspberry-pomegranate	>17.5%	7.6
VIVA VITAL	Banana-cherry	>31%	7.6
Knorr vie	Grape-banana-rhubarb	37%	6.0
VIVA VITAL	Raspberry-peach	<17.5%	5.4
VIVA VITAL	Grape-blackcurrant	25%	5.4
Chiquita	Coconut-mango	>15%	3.6
Schwartau	Strawberry-orange	17%	3.2
Knorr vie	Apple-carrot-strawberry	0%	2.7
Innocent	Blackberry-raspberry-boysenberry	7%	2.0
Valensina	Strawberry-banana-grape	12%	1.9
Chiquita	Strawberry-banana	0%	1.0
rio d'oro	Strawberry-apple-carrot	0%	0.9
Pro-X	Strawberry-apple-carrot	0%	0.8
VIVA VITAL	Mango-passion fruit	0%	0.7
Mövenpick	Blood orange-apple	3%	0.6
True fruits	purple (var. berries)	0%	0.6

amounts are too small to cause a preservative effect on the final product. It is likely that in most cases, one of the ingredients has been treated with sulfites prior to the production of the smoothie.

Grape products are commonly treated with sulfites. For the smoothies, an entry of sulfites into the product is likely to happen through grape juices or purees. Comparing the percentage of grapes in a smoothie with the amounts of sulfites found, a correlation of both becomes visible. The smoothies with high SO_2 amounts were prepared, in most cases, with more grape juice than those with very little SO_2. But an entry via other ingredients, i. e. other fruit components or pectines, is possible as well.

3.4.1.4. Sugar Sirups

For this work, over 120 samples of sugary sirups, mainly glucose sirups, were analyzed. The ZZulV (see appendix A.2) allows for up to 20 mg/L SO_2 to be added to a glucose sirup.

For the HPLC-IMER analysis of glucose sirups, an addition of sodium hydroxide solution was not necessary. An addition of NaOH did not lead to better recoveries of sulfites (as it did for many juices as shown in chapter 3.2.5), therefore the sample preparation was done simply by dilution with carbonate buffer.

None of the analyzed glucose sirups did contain more than trace amounts of sulfur dioxides. The majority of the glucose sirups did not contain any sulfites. Furthermore, none of the analyzed fructose sirups, fructose solutions or inverted sugar sirups contained SO_2.

For some samples, the chromatograms showed peaks that were not derived from sulfites. The peak area was not affected by the enzyme reactor, and the retention time was not identical with that of sulfite.

3.4.1.5. Samples with Solid Matrices

Cinnamon

In several samples of cinnamon (Ceylon cinnamon sticks, ground cinnamon) sulfites were not detected. The electrochemically very active matrix of all cinnamon samples made a very strong dilution necessary in order to get chromatograms without interferences. Due to the high dilution factor of 1:1250, the detection limit for sulfites in cinnamon rises to 25 mg/L.

Dried onion powder

The HPLC-IMER chromatogram of an onion power is not satisfying (see figure 3.25 on page 103). Onion powder contains electrochemically active substances that lead to a chromatogram with interferences. The sulfite peak is small, an amount of 2.7 mg/kg SO_2 is calculated. A combination of destillative separation and HPLC-IMER leads to better chromatograms and higher results, as presented in chapter 3.6.

Mustard

Different samples of mustard were analyzed. A sulfite peak was detected with the enzyme reactor, disappearing when analyzed without the reactor. Yet, the peak appearing to be sulfite may also be derived from isothiocyanates within the mustard seeds instead of sulfite. Further analysis needs to be performed, especially with pure mustard seeds, to confirm this suspicion.

Red wine jelly

A "Rotweingelee" (red wine jelly) was declared to be organic and without added preservatives. HPLC-IMER analysis revealed amounts of 28 mg/kg SO_2 in the jelly. This result shows the general applicability of the HPLC-IMER method for jelly and jam.
As an organic product without added preservatives, this product must not contain sulfites above the legal limit of 10 mg/kg. With HPLC-IMER as the official method for analysis in foodstuffs, this jelly would not be marketable.

3.5. HPLC-CEAD

3.5.1. Coulometric Electrode Array Detection (CEAD)

The amperometric detection of analytes after an HPLC separation is selective and very sensitive. For a lot of applications, a coulometric detection method offers even higher sensitivity than an amperometric detection. The coulometric electrode array detector consists of up to 16 consecutive cells with porous graphite material inside each flow through cell. A specific voltage applied to each cell, and each cell approaching 100% efficiency, allows to voltammetrically resolve even perfectly co-eluting peaks, as long as their half-wave potential differs by at least 60 mV. For the HPLC-IMER, a differentiation between sulfite-derived hydrogen peroxide and other potentially coeluting substances supersedes a second HPLC run without an enzyme reactor for validation. Therefore, even complicated and electrochemically active matrices cause less interference and generally need less sample preparation. Also, a 100% conversion compared to an estimated 10% conversion of the amperometric detector might possibly lead to a significantly lower limit of detection. A schematic illustration of the HPLC-IMER with a coulometric detector is shown in figure 3.22.

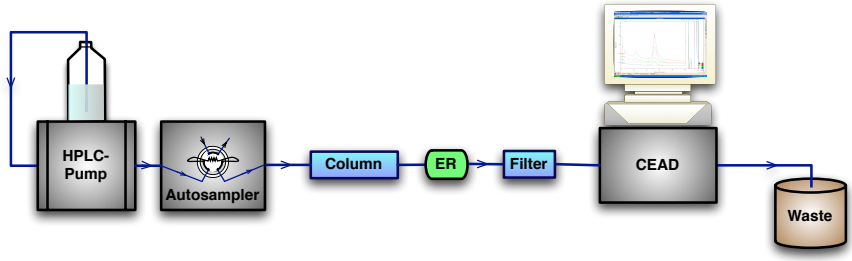


Figure 3.22.: HPLC-IMER with CEAD detection.

Experiments with standard sulfite solutions reveal the major drawback of this detection method. Graphite, as opposed to platinum, requires a higher potential for the oxidation of hydrogen peroxide. The higher voltage applied leads to reduced sensitivity, and also to a reduced selectivity as there are more compounds to be oxidized at higher voltages.

Figure 3.24 shows voltammograms of a standard sulfite solution with a concentration of 0.4 mg/L SO_2 at the voltages: 550 mV; 600 mV; 700 mV and 800 mV.

Voltammograms at four different potentials (200 mV; 400 mV, 600 mV and 800 mV) for a standard sulfite solution with c = 20 mg/L SO_2 are shown in figure 3.23.

It becomes clear, that a higher potential leads to better conversion of hydrogen peroxide at the coulometric graphite electrode. But even the most efficient potential of 800 mV does not lead to a sensitivity comparable to that of a platinum electrode. The peak size is small, whereas the interferences at 800 mV become too dominant, even with the standard solution (c=0.4 mg/L SO_2).
In table 3.10 examples of the limits of detection and quantification, respectively, are presented.

Table 3.10.: Limits of detection (LOD) and limits of quantification (LOQ) for coulometric (CEAD) detection at 550 mV and 600 mV and for amperometric detection (ECD) at 200 mV.

	CEAD 550 mV	**CEAD 600 mV**	**ECD 200 mV**
LOD	0.4 mg/L	0.1 mg/L	0.002 mg/L
LOQ	0.8 mg/L	0.2 mg/L	0.004 mg/L

The limit of detection with the platinum electrode of the electrochemical detector (ECD) is much lower that those obtained with the coulometric array detector. A coulometric cell out of platinum has not been realized yet. It is highly likely that such a cell would lead to a largely increased sensitivity of the method.

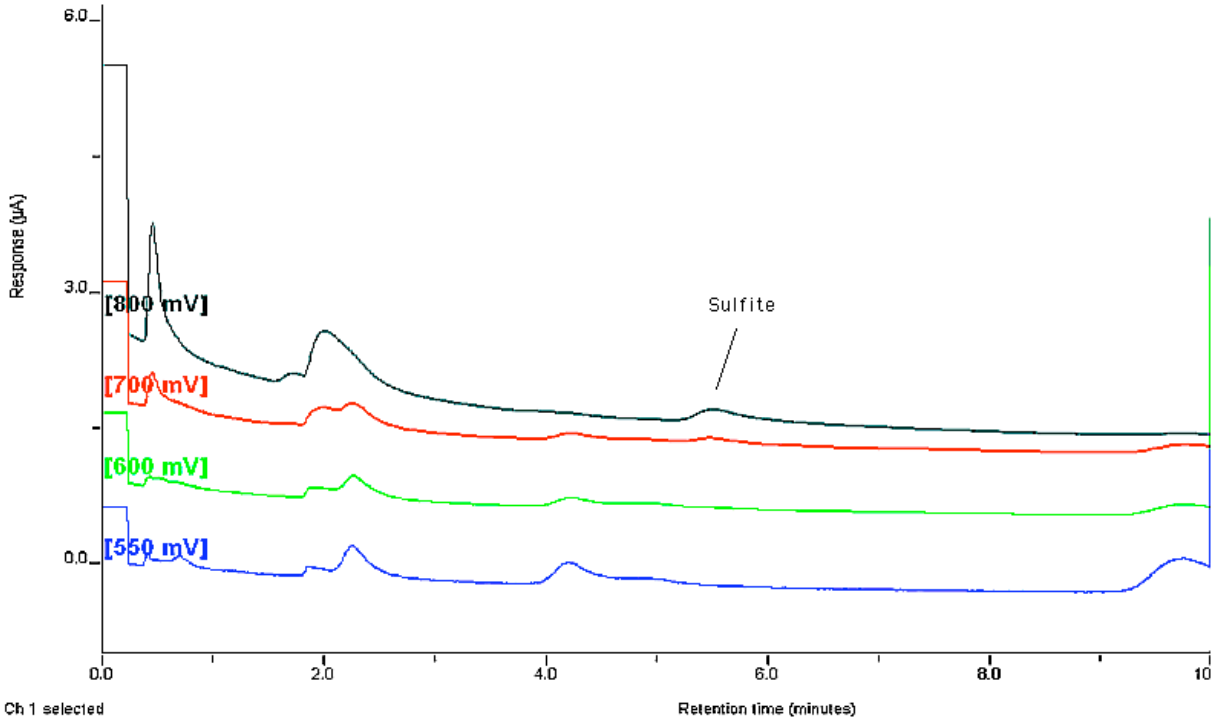


Figure 3.23.: Voltammogram of a standard sulfite solution (c = 0.4 mg/L SO_2) at different potentials.

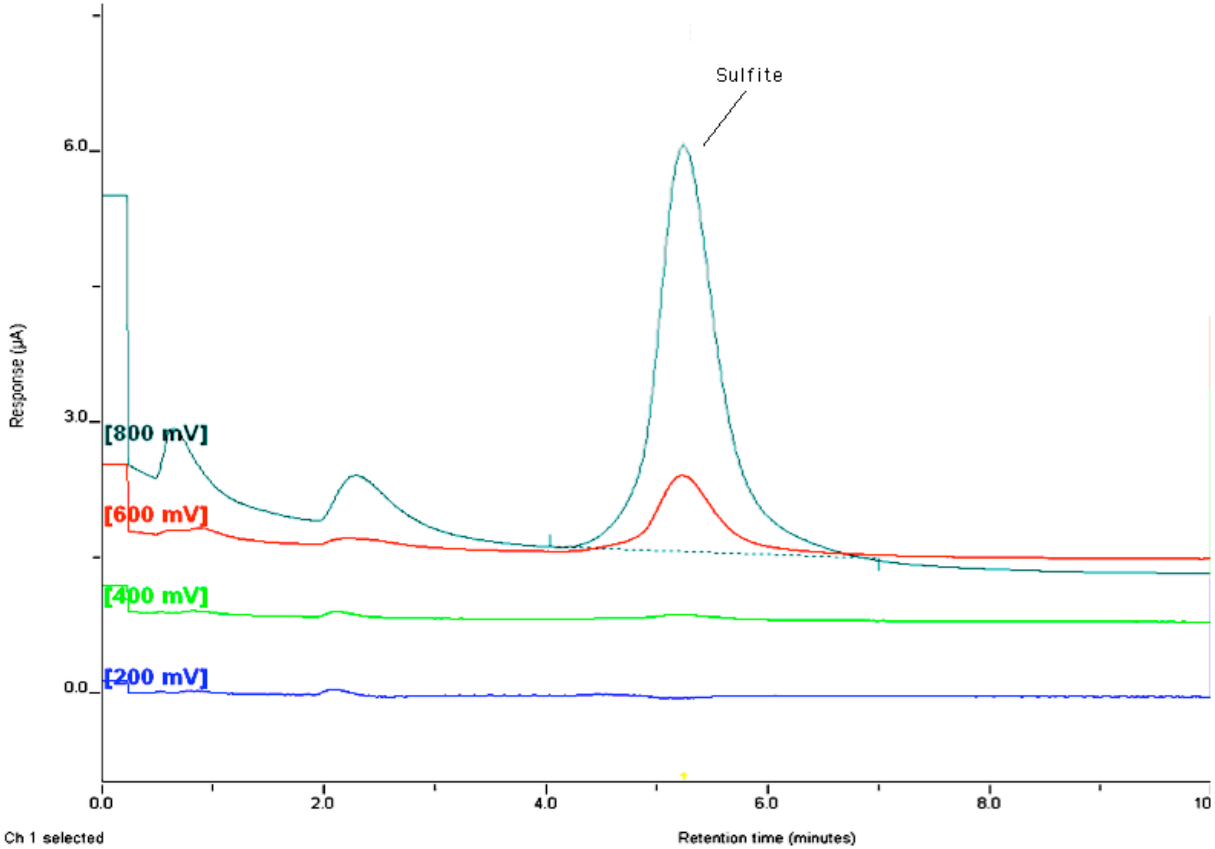


Figure 3.24.: Voltammogram of a standard sulfite solution (c = 20 mg/L SO_2) at different potentials.

3.6. DE-HPLC-IMER

A combination of destillation prior to the HPLC-IMER was developed to tackle more complex sample matrices. In the destillation step, sulfur dioxide is separated from other matrix components. The sulfur dioxide gas is introduced in carbonate buffer, forming ionic sulfite (SO_3^{2-}). This sulfite containing carbonate buffer is analyzed by HPLC-IMER, resulting in better chromatographic results.

Two chromatograms of an onion powder, one analyzed by HPLC-IMER and the other one by DE-HPLC-IMER, are displayed in figure 3.25. The sulfite peak in the HPLC-IMER chromatogram is not sufficiently separated from other electrochemically active substances of the onion powder. The peak area appears very small, calculation of the sulfite content yields 2.7 mg/kg SO_2. Applying the DE-HPLC-IMER, the chromatogram appears with less interferences. Here, the sulfite peak is clearly separated from other substances, and the calculation leads to a sulfite content of 6 mg/kg, calculated as SO_2.

The retention times of the sulfite peaks are not identical. This is due to the different pH values of the solutions that were injected into the HPLC. The sodium hydroxide solution used for sample preparation in HPLC-IMER leads to a higher pH value of 12.5, compared to a pH value of 10.6 in the distilled sample.

A red wine jelly was analyzed with both methods as well. Here, the chromatographic separation was likewise improved with the DE-HPLC-IMER (see figure 3.26). But the calculated amounts of sulfites were inverse to the last example. In this case, the HPLC-IMER yielded higher results: 28 mg/kg as opposed to 18 mg/kg with DE-HPLC-IMER.

For most samples, the HPLC-IMER resulted in higher sulfite findings than the DE-HPLC-IMER. This is in accordance with the assumption, that in alkaline media sulfites are more readily released. In onion powder, the lower findings of sulfites with HPLC-IMER compared to DE-HPLC-IMER are a consequence of too many interferences and the resulting incorrect peak integration.

Even though the coupling of a destillation with the HPLC is a lot more elaborate than applying just one of these methods, for certain samples, the combination may be the method of choice. The danger of false positives, as existent in destillation followed by titration, is thereby excluded and chromatographic difficulties are overcome.

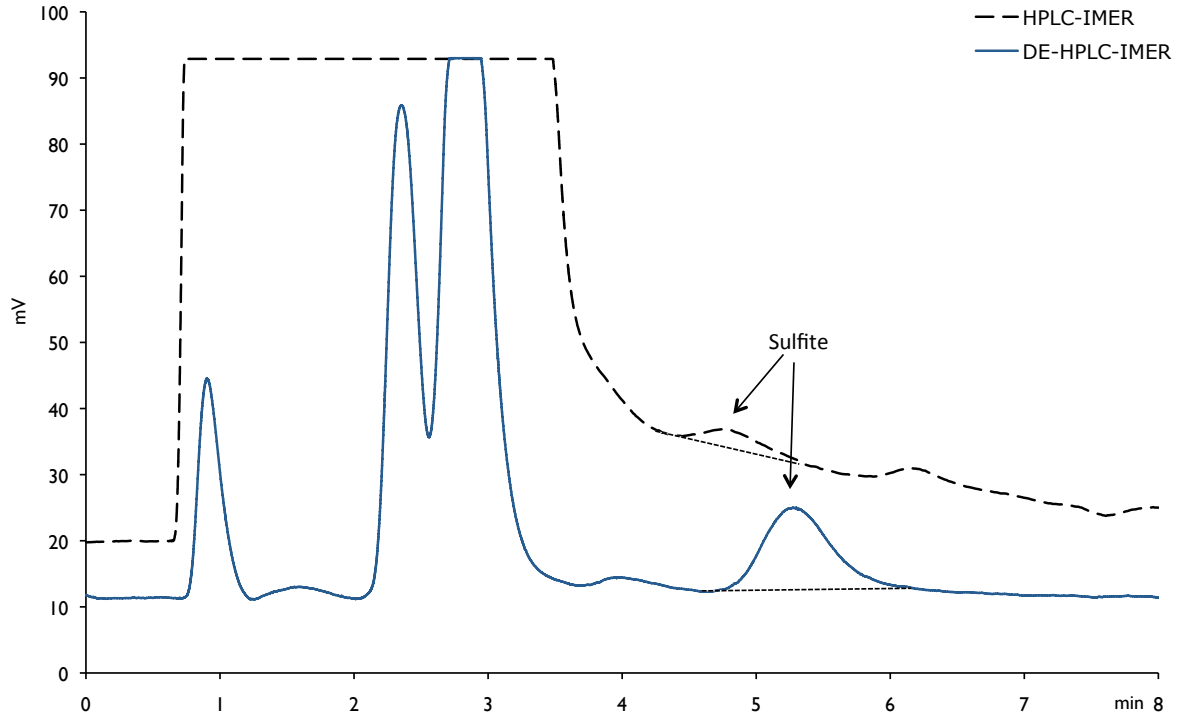


Figure 3.25.: Chromatograms of an onion powder, analyzed with HPLC-IMER and DE-HPLC-IMER.

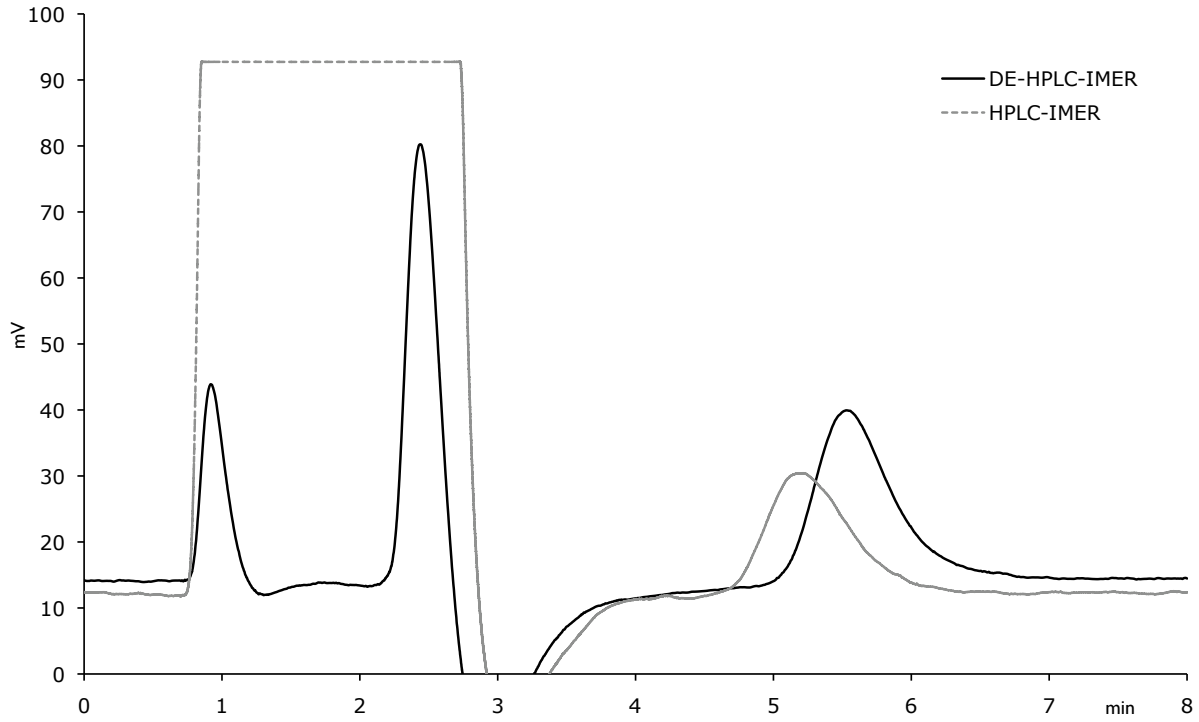


Figure 3.26.: Chromatograms of a red wine jelly. Top: analyzed with HPLC-IMER. Bottom: analyzed with DE-HPLC-IMER.

3.7. Comparison of HPLC-IMER with the Official Monier-Williams Method

The official method for the determination of SO_2 in juices is the IFU 7a , based on the destillation method by Monier-Williams [73]. As the HPLC-IMER is a comparably new method, it was compared to the well established method for determination of sulfites in fruit juices, the IFU 7a. 35 different juice samples from food stores were pre-tested for their sulfite residues. Of those containing sulfites, 15 were chosen for a comparison study. The results of both methods were compared and will be discussed.

3.7.1. Comparison Study

15 out of the 35 previously tested juices were chosen for the method comparison. All of these juices did contain sulfites, according to the HPLC-IMER pre-testing. About half of them had values of less than 10 mg/L, the other half more than 10 mg/L, up to about 20 mg/L. Especially those amounts of sulfur dioxide closely scattered around the legal limit of 10 mg/L were of interest, because wrong analytical results may lead to major and expensive consequences for the producer of the product.

All 15 juice samples were tested with HPLC-IMER, IFU 7a and DE-HPLC-IMER, respectively. All analyses were performed as duplicates. The average results are displayed in table 3.11.

There are significant differences in the results obtained by the different methods. In all but two samples (both products of the same producer), the HPLC-IMER method yielded higher results than the other two methods. In 9 juices, the result of the HPLC-IMER was more than 50% higher than that of the IFU 7a. The two tomato juices and the lemon concentrate contained low but well quantifiable amounts of sulfites, analyzed with HPLC-IMER. Yet, the destillation methods yielded only trace amounts or no sulfites at all. Three of the grape juices would not have been legally objectionable according to the results of the IFU 7a. However, considering the results of the HPLC-IMER, these juices actually contained more sulfites than officially allowed. All results of the HPLC-IMER have been supported by an analysis without enzyme reactor, which makes erroneously high results extremely unlikely.

The differences in the results of the three methods are not alike in respect to absolute or percentage amounts. Therefore no standard error for all samples can be concluded, but the differences in findings depend on the sample.

Table 3.11.: Results of method comparison, all amounts in [mg/L] SO_2.

	Method		
Juice	**HPLC-IMER**	**IFU 7a**	**DE-HPLC-IMER**
Grape juice (1)	11.5	5.9	5.7
Grape juice (2)	4.9	6.1	6.4
Grape juice (3)	4.6	6.6	5.8
Grape juice (4)	13.2	8.8	8.8
Grape juice (5)	20.1	9.5	12.1
Grape juice (8)	11.0	7.9	6.1
Grape juice (25)	18.9	14.1	12.2
Grape juice (29)	20.2	13.5	14.4
Grape juice (31)	6.4	2.4	2.0
Grape juice, white (6)	8.5	5.1	4.5
Grape juice, white (26)	7.2	6.5	5.7
Peach nectar (19)	4.1	1.2	0.6
Tomato juice (24)	2.3	0.3	0
Tomato juice (35)	2.6	0	0
Lemon concentrate (32)	1.5	0.5	0

The results displayed in 3.11 are the average results of two analyses. The reproducibility of a method is an important parameter for its reliability. In table 3.12, the standard deviation of the methods is given. Even though two values do not give statistical certainty, a clear tendency is evident: the IFU 7a and in the DE-HPLC-IMER are less reliable and lead to greater variances of the results. The standard deviation of the HPLC-IMER is a lot smaller and it can be concluded that the method therefore leads to more reliable results. Weather or not these results are also closer to the "real" SO_2-content can be discussed critically.

Table 3.12.: Standard deviation of methods, all amounts in [mg/L] SO_2.

	Method		
Juice	**HPLC-IMER**	**IFU 7a**	**DE-HPLC-IMER**
Grape juice (1)	0.2	1.1	0.0
Grape juice (2)	0.1	0.2	0.2
Grape juice (3)	0.0	2.6	0.3
Grape juice (4)	0.0	0.2	0.6
Grape juice (5)	0.0	1.2	0.6
Grape juice (8)	0.1	0.4	0.2
Grape juice (25)	0.0	1.0	0.3
Grape juice (29)	0.1	1.1	1.0
Grape juice (31)	0.1	0.1	0.6
Grape juice, white (6)	0.1	0.1	0.2
Grape juice, white (26)	0.1	0.2	0.2
Peach nectar (19)	0.1	0.4	0.1
Tomato juice (24)	0.0	0.4	-
Tomato juice (35)	0.1	-	-
Lemon concentrate (32)	0.1	0.7	-
Average	0.1	0.5[a]	0.4

[a] After exclusion of outlier (grape juice (3)).

3.7.1.1. IFU 7a versus DE-HPLC-IMER

The two destillation methods are identical in sample preparation and distillation parameters. They differ only in the method of quantification.

While the IFU 7a works with a titrimetric quantification after oxidation of the sulfurous acid to sulfuric acid, the DE-HPLC-IMER applies the more specific detection of sulfite with enzyme reactor and electrochemical detector. The rather unspecific quantification via titration may lead to higher results for sulfites because of volatile acids that may react with the sodium hydroxide solution and be mistaken for sulfites. Therefore it is not surprising that in most cases the DE-HPLC-IMER leads to slightly lower results for sulfite, as with this method false positives are almost impossible.

In a few cases, though, the DE-HPLC-IMER leads to higher results than the IFU 7a. With the very small amount of samples and repetitions these findings are not significant, as the differences are in the range of normal distribution. Yet, with only 3 out of 15 juice samples showing lower values in IFU 7a than in DE-HPLC-IMER, there is a strong indication that the latter leads to results that are closer to the true sufite contents.

The slightly better performance of the DE-HPLC-IMER is counteracted by the considerably higher demand of time, laboratory equipment and financial aspects. In very difficult cases with large amounts of volatile acids it may prevent wrong conclusions, but for most cases, the Monier-Williams based IFU 7a is the method of choice.

3.7.1.2. HPLC-IMER versus IFU 7a

For almost all juices, the detected amounts of sulfites of the HPLC-IMER are higher than those obtained with both of the distillative methods. As the HPLC-IMER is almost immune against false positive results, it may be concluded that during the distillation process there are generally losses of sulfur dioxide that account for too low findings. For example, the oxidation of sulfites during the heating of the flask is possible, as well as losses of small amounts of volatile sulfur dioxides through tiny leaks. Losses may also be caused by the gas stream bubbling too fast through the receiving liquid. Furthermore, it is known, that bound sulfites are not released by the acidic medium during the distillation time.

Despite the more expensive laboratory equipment, this work shows several advantages of the HPLC-IMER over the Monier-Williams distillation:

- time efficiency:
  one HPLC run needs only 8 minutes. The sample preparation can easily be

executed manually, and the standing time of 60 minutes before injection into the HPLC requires no manual action.

- automatic sample preparation:
  the entire sample preparation can be performed by the autosampler, making this method simple to perform even for unexperienced staff. In contrary, the distillation methods require a lot of experience in handling, in order to obtain sufficiently reliable results.
- less dangerous or toxic chemicals:
  the only critical chemical in the HPLC-IMER is the sodium hydroxide solution. If the sample preparation is done by the autosampler, no contact with any chemicals is necessary at all (only for refilling of the reservoirs), whereas the distillation requires a manual refill of caustic acid for every run.
- no falsely positive results:
  the specificity of the enzyme makes false positive results almost impossible, whereas the distillation holds the risk of overestimation of the sulfite content.
- better reproducibility:
  lower possibility of variances and errors leading to a significantly increased reproducibility of the HPLC-IMER compared to the distillation methods.
- much lower detection limit:
  probably the major advantage of the HPLC-IMER is the quantification limit of 0.01 mg/L. The distillation methods are a lot less sensitive, as they do not lead to reliable results under 10 mg/L.

3.8. Comparison of IFU 7a, HPLC-IMER and DE-HPLC-IMER

A comparison of the HPLC-IMER with the distillation method for fruit juices (IFU 7a) and with the newly developed DE-HPLC-IMER was conducted.

The data obtained from all three types of analyses is shown in tables 3.13 and 3.14.

The sulfite amounts found with the different methods vary greatly. Whereas the IFU 7a and the DE-HPLC-IMER, both distillatory methods, lead to almost the same results, the HPLC-IMER yields the highest amounts without exception. In the case of the red grape juice, the mean HPLC-IMER result was almost 90% above that of the IFU 7a. For the white grape juice, the average result of the HPLC-IMER was 70% higher than that of the IFU 7a.

As the chance of false positives with the HPLC-IMER is negligible, one must conclude, that the high results are likely to be much closer to the true amounts of sulfites in those grape juices than the lower results.

Table 3.13.: Red grape juice: data from comparison of three different methods for sulfite analysis.

	HPLC-IMER	**DE-HPLC-IMER**	**IFU 7a**
Arithmetic mean [mg/L]	17.29	9.53	9.15
Variance	0.085	0.442	0.647
Standard deviation	0.291	0.666	0.80
Rel. standard dev.	1.68%	6.98%	8.79%

Table 3.14.: White grape juice: data from comparison of three different methods for sulfite analysis.

	HPLC-IMER	**DE-HPLC-IMER**	**IFU 7a**
Arithmetic mean [mg/L]	10.41	6.47	6.12
Variance	0.061	0.46	0.186
Standard deviation	0.246	0.678	0.431
Rel. standard dev.	2.36%	10.47%	7.04%

DE-HPLC-IMER and IFU 7a both are methods based on a distillation procedure. The similarity of results between them is therefore not surprising. It is most likely, that through acidic treatment and the following distillation, not all sulfites were liberated as sulfur dioxide. Large amounts of sulfites probably remain in their bound state in the sample.

Attempts to liberate those strongly bound sulfites with excess sodium hydroxide solution prior to an HPLC-IMER detection were only partly successful. There was a recovery of some sulfites, yet the amounts did not account for the entire "missing" sulfites. These tests will have to be rerun in the future, with reaction times of more than one hour and more alkaline solution.

With the IFU 7a, both grape juice samples yielded amounts lower than 10 mg/L SO_2. Not so with the HPLC-IMER: whereas the white grape juice was just slightly above the legal limit of 10 mg/L SO_2, the red grape juice would clearly not meat the legal requirements, if the HPLC-IMER was the official method for the analysis of sulfites.

Not only does the method yield the highest results, the HPLC-IMER also shows the best reproducibility of all three methods, when comparing the method parameters variance, standard deviation and relative standard deviation. Especially in the low concentration range of about 10 mg/L SO_2, a good reproducibility of the method is essential for a reliable judgement of the marketability of the products. Both grape juices would be within the legal limits for SO_2, when analyzed with the IFU7a. However, for both juices, the results obtained with the HPLC-IMER are above 10 mg/L SO_2, and especially the red grape juice exceeds the limit clearly.

3.9. Storage Study of Grape Juices

In the production process of grape juices, the amount of previously added sulfites decreases to a certain level. Dietrich and Patz have monitored the changes in the amount of sulfites during all steps of production (see appendix B.5 for details). With the HPLC-IMER, it is possible to reproducibly analyze juice samples for the changes in sulfite contents during storage. It is assumed that sulfite contents in a sample decrease due to oxidation processes and to the formation of more complex addition products. The alkaline sample preparation of the HPLC-IMER is expected to release all forms of bound sulfites. Therefore, only a true loss of sulfites due to oxidation or due to diffusion of sulfur dioxide through the package will lead to decreased amounts of sulfites detected in the juice. In the first of two studies, the changes in sulfite contents were determined for 15 juices after opening, and again after 11 weeks of storage at cool (4°C) temperature. The results are shown in table 3.15.

Table 3.15.: Sulfite contents after 11 weeks of storage of different juice samples.

Sample	**1st analysis** [mg/L] SO_2	**11 weeks later** [mg/L] SO_2	**change** %
Grape juice (1)	14.5	11.5	-21
Grape juice (2)	5.6	4.9	-12
Grape juice (3)	5.6	4.6	-18
Grape juice (4)	14.9	13.2	-11
Grape juice (5)	24.1	20.1	-17
Grape juice (8)	11	11	0
Grape juice (25)	16.9	18.9	12
Grape juice (29)	22	20.3	-8
Grape juice (31)	7.3	6.4	-12
Grape juice, white (6)	9.7	8.5	-12
Grape juice, white (26)	7.5	7.3	-3
Peach nectar (19)	4.7	4.1	-13
Tomato juice (24)	2.5	2.3	-8
Tomato juice (35)	2.4	2.6	9
Lemon concentrate (32)	5.6	1.5	-73

As expected, most of the juices contained less sulfur dioxide after 11 weeks of storage. By far the strongest decrease had appeared in the lemon concentrate. After the end of the storage time, only 27% of the initial sulfite-amount was still

present. Most of the other juices have lost around 10% of their initial values, two juices even showed an increase in measured sulfites. After all, there is no consistent trend in these results. Even though most of the examined juices show the expected results, the variances are too high to draw definite conclusions. This might be caused by the differences between the samples. Neither were all the juices produced at the same time or under similar conditions, nor were they comparable in their matrices or the packaging. There are too many differences for drawing conclusions from this comparison.

A second storage study was carried out with a more homogenous pool of samples. One grape juice was chosen, and packages were all stored under identical conditions (at 20°C). For each analysis, two packages were opened and each was analyzed in duplicate with the standard HPLC-IMER method. The goal was to determine the rate of decrease of the sulfite content in a regular shelf product[3].

Figure 3.27 shows the results of the storage study graphically.

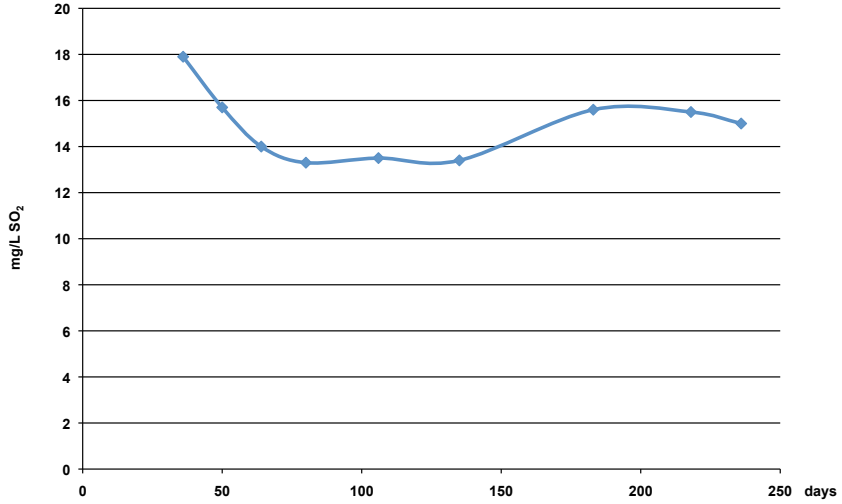


Figure 3.27.: Sulfite contents of a grape juice, stored at room temperature.

The x-axis displays the days after production date, the y-axis the results of the sulfite content in mg/L, calculated as SO_2. The first analysis was performed

[3]rio d'oro Premiumdirektsaft, 100% grape juice, 1L Pure-Pak®

on day 36 after production date. During the next four weeks, the sulfite content dropped almost linearly (approximately 0.16 mg/L/day SO_2), leveling out at about 13.5 mg/L SO_2. Surprisingly, after about 19 weeks there is a rise almost up to 16 mg/L SO_2, followed by a slow decrease. The results of the first weeks were what we expected from this study. A continuous drop of sulfite levels, due to oxidation processes, levelling out at some point.

The further results were rather unexpected, considering the supposition that with the alkaline disintegration it is possible to release all combined forms of sulfite. If this supposition is correct, the increase in sulfites can not be due to an internal release of formerly bound sulfites. The increase is not extremely large, but still it is significantly greater than the standard deviation of this method and it was found in every juice sample analyzed after day 135. A systematical error of the method after day 135 is unlikely to be the reason for this unexpected increase. Every analysis was carried out with freshly prepared standard sulfite solution. Therefore, an error caused by the incorrect concentration of the standard solution is highly unlikely.

Is an internal production of sulfites possible? It is known that some sorts of yeast are able to produce considerable amounts of sulfites. But the juice was stored under normal conditions and analyzed before the expiration date, so microbial effects are not to be expected. However, grape juices undergo several reactions during storage. A degradation of sulfur containing amino acids may lead to the formation of additional sulfites, causing the rise in the sulfite contents of the grape juice.

Considering all of the issues discussed above, it seems necessary to conduct another storage study to either support the findings of this study or to exclude possible errors.

It is noteworthy, that all results of this study showed sulfur dioxide amounts in the juices that are clearly above the legal limit of 10 mg/L. The juice would therefore not be marketable, if the HPLC-IMER was the official method for the analysis of sulfur dioxide in food.

For comparison, the same juice was analyzed with the DE-HPLC-IMER and IFU 7a. When the HPLC-IMER yielded amounts of 17.3 mg/L SO_2, the results of the distillation methods were slightly below 10 mg/L SO_2 (9.5 mg/L SO_2 with the DE-HPLC-IMER and 9.2 mg/L SO_2 with the IFU 7a).

Thus, with the official method for sulfite analysis, the SO_2-content of the red grape juice is within the legal limit of 10 mg/L, even though the true amount of sulfur dioxide within the juice, analyzed with the HPLC-IMER, is considerably higher than 10 mg/L SO_2.

4. Conclusion

4.1. Conclusion and future research

In this work, several parameters of the method were investigated and improved, with a focus on effective sample preparation and suitability of different sulfite oxidizing enzymes.

4.1.1. Sample Preparation

For most of the food matrices, an addition of sodium hydroxide solution, followed by a reaction time of one hour and a dilution with carbonate buffer was the optimal sample preparation. However, some samples, especially those with high amounts of anthocyanins, required longer reaction times for an exhaustive release of bound sulfites.

Experiments with HPLC-UV-MS^n revealed the anthocyanin contents of different grape products. The formation of an anthocyanin-sulfite addition product was shown, however, it was not stable under the acidic HPLC conditions. This leads to the conclusion, that the very strong bonds of sulfites with matrix components are not simply anthocyanin-sulfonates, but rather complex molecules, including polyphenols, sulfites and probably other molecules as well, that are likely to be formed during the production steps of a grape product.

The sample treatment with sodium hydroxide solution leads to a time-dependent release of bound sulfites. The maximum sulfite release is reached within minutes up to about one hour for most samples, whereas some samples require up to four hours of alkaline treatment.

Whether this treatment is able to free all bound sulfites can not be determined. However, the HPLC-IMER always leads to higher sulfite findings than the distillatory methods. Thus, there is a strong possibility that the results obtained with the HPLC-IMER are closer to the true sulfite amounts of a sample, than those obtained with other methods.

Irrespective of the duration of the alkaline sample treatment, the sample preparation in HPLC-IMER is rather simple, and it does not require expensive materials or chemicals nor experienced laboratory staff. All preparation steps, as there are the addition of sodium hydroxide solution to the sample, mixing, waiting and dilution with carbonate buffer, can be executed by a programmable autosampler. Thus, large numbers of samples can be prepared and analyzed without requiring a lot of manual work. Additionally, an autosampler provides great reproducibility.

4.1.2. Enzyme Comparison

The comparison of animal and plant sulfite oxidases for application in the HPLC-IMER was an important task in this work. Two different kinds of sulfite oxidases were applied in the HPLC-IMER and both were equally stressed. The animal sulfite oxidase was, under the chosen conditions, significantly less stable than the plant sulfite oxidase. Furthermore, the linear range of sulfite oxidation of the plant enzyme was much larger than that of the chicken liver sulfite oxidase. Until today, only the sulfite oxidase from chicken liver is commercially available. This enzyme is already successfully applied in different biosensor methods. However, the application of a plant sulfite oxidase from *Arabidopsis thaliana* may in the future become more popular, if this enzyme becomes commercially available.

4.1.3. Comparison with other methods

Compared to other methods for sulfite analysis, the HPLC-IMER shows a lower detection limit, better reproducibility and higher sulfite recoveries for all analyzed samples. Today, the legal limit for sulfites of 10 mg/L is based on the limit of quantification of the Monier-Williams destillation. With the HPLC-IMER, a new legal limit may come into discussion, that will be based on the needs of sulfite sensitive persons rather than on analytical limitations.

With the simple sample preparation, especially by using the features of a programmable autosampler, the HPLC-IMER is after all more reliable and requires less effort than the established methods for sulfite quantification in food.

A round robin test is already planned to be executed by many laboratories within Europe. The results of this test will provide valuable information on the method parameters repeatability and reproducibility.

After a successful interlaboratory comparison of this method, the HPLC-IMER may in the future become an official method for sulfite quantification.

This will be a step in the direction of providing safer food for all consumers.

Appendix A.

A.1. Abbreviations

AiF	Arbeitsgemeinschaft industrieller Forschungs-vereinigungen "Otto von Guericke" e.V.
AiF (engl.)	German Federation of Industrial Research Associations
AU	UV absorption units
BMWi	Bundesministerium für Wirtschaft und Technologie
CEAD	Coulometric electrode array detection
DE-HPLC-IMER	Destillation - High performance liquid chromatography with immobilized enzyme reactor
e. g.	exempli gratia (for example)
EDTA	ethylenediaminetetraacetic acid
ER	enzyme reactor
ESI	electrospray interface
FDA	U.S. Food and Drug Administration
g	gram
HPLC	High performance liquid chromatography
i. d.	inside diameter
i. e.	id est (that is)
IFU	Internationale Fruchtsaft Union
IMER	Immobilized enzyme reactor
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
kDa	kilodalton
L	litre
LC	liquid chromatography
LDPE	low density polyethylene
LFGB	Lebens- und Futtermittelgesetzbuch
LMKV	Lebensmittelkennzeichnungsverordnung
LOD	limit of detection
LOQ	limit of quanitifcation
MAK	Maximale Arbeitsplatz-Konzentration
MAK (engl.)	maximum allowable concentration
mg	milligram

mL	millilitre
mm	millimeter
MS	mass spectrometry
NMR	nuclear magnetic resonance
pH	potentia Hydrogenii
PPO	polyphenol oxidase
SOx	sulfite oxidase
TFA	trifluoroacetic acid
UHQ	ultra high quality
UV	ultraviolet
v	volume
V	volt
Vis	visible light
ZZulV	Zusatzstoffzulassungsverordnung

A.2. Legislation

Regulations (EC), English version

Regulation (EC) No 178/2002
Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety
OJ L 31, 1. 2. 2002, p. 1–24

Regulations (EC), German version

Verordnung (EG) Nr. 178/2002
Verordnung (EG) Nr. 178/2002 des Europäischen Parlaments und des Rates vom 28. Januar 2002 zur Festlegung der allgemeinen Grundsätze und Anforderungen des Lebensmittelrechts, zur Errichtung der Europäischen Behörde für Lebensmittelsicherheit und zur Festlegung von Verfahren zur Lebensmittelsicherheit
Zuletzt geändert durch Art. 1 ÄndVO (EG) 202/2008 vom 4. 3. 2008 (ABl. Nr. L 60 S. 17)
(ABl. Nr. L 31 S. 1)
EU-Dok.-Nr. 3 2002 L 0178

Directives (EC), english version

Directive 2003/89/EC
DIRECTIVE 2003/89/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs
OJ L 308, 25. 11. 2003, p. 15–18

Directive 2000/13/EC
DIRECTIVE 2000/13/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs
OJ L 109, 6. 5. 2000, p. 29–42

Directive 2007/68/EC
COMMISSION DIRECTIVE 2007/68/EC of 27 November 2007 amending Annex

IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients
OJ L 310, 28. 11. 2007, p. 11–14

Directive 2006/142/EC
COMMISSION DIRECTIVE 2006/142/EC of 22 December 2006 amending Annex IIIa of Directive 2000/13/EC of the European Parliament and of the Council listing the ingredients which must under all circumstances appear on the labelling of foodstuffs
OJ L 368, 23. 12. 2006, p. 110–111

Directives (EC), German version

Richtlinie 2000/13/EG
Richtlinie 2000/13/EG des Europäischen Parlaments und des Rates vom 20. März 2000 zur Angleichung der Rechtsvorschriften der Mitgliedstaaten über die Etikettierung und Aufmachung von Lebensmitteln sowie die Werbung hierfür
Zuletzt geändert durch Art. 1 ÄndRL 2007/68/EG vom 27. 11. 2007 (ABl. Nr. L 310 S. 11)
(ABl. Nr. L 109 S. 29)
EU-Dok.-Nr. 3 2000 L 0013

German Regulations

Lebensmittel-Kennzeichnungsverordnung – LMKV
Verordnung über die Kennzeichnung von Lebensmitteln
In der Fassung der Bekanntmachung vom 15. Dezember 1999
Zuletzt geändert durch Art. 1 VO zur Änd. d. Lebensmittel-KennzeichnungsVO und der KosmetikVO vom 18. 12. 2007 (BGBl. I S. 3011)
(BGBl. I S. 2464)

Lebensmittel- und Futtermittelgesetzbuch – LFGB
Lebensmittel-, Bedarfsgegenstände- und Futtermittelgesetzbuch
In der Fassung der Bekanntmachung vom 26. April 2006
Zuletzt geändert durch Art. 12 G zur Änd. des BundespolizeiG und and. G vom 26. 2. 2008 (BGBl. I S. 215)
(BGBl. I S. 945)

Zusatzstoff-Zulassungsverordnung – ZZulV
Verordnung über die Zulassung von Zusatzstoffen zu Lebensmitteln zu technologischen Zwecken
Vom 29. Januar 1998
Zuletzt geändert durch Art. 3 VO zur Änd. lebensmittelrechtl. Vorschriften vom 30. 9. 2008 (BGBl. I S. 1911)
(BGBl.I S. 230)

Appendix B.

B.1. Short Description of the Research Project AiF 14583

This research project was supported by the FEI (Forschungskreis der Ernährungsindustrie e. V., Bonn), the AiF and the Ministry of Economics and Labour. AiF-Project No.: 14583 N. Title: "Bestimmung von Schwefeldioxid in Früchten und Fruchtprodukten durch HPLC-Biosensorkopplung".

The project was realized in cooperation with the Forschungsanstalt Geisenheim, Institut für Oenologie und Getränkeforschung, FG Weinanalytik und Getränkeforschung. The project coordinator, Dr. Sprenger of the Faethe Labor GmbH (Paderborn) provided sulfurized and unsulfurized juices and jams for the experiments.

The plant sulfite oxidase that was successfully employed in the HPLC-IMER, was provided by the Technische Universität Carolo-Wilhelmina zu Braunschweig, Institut für Pflanzenbiologie, Abteilung für Molekular- und Zellbiologie der Pflanzen.

A short description of the project in German and in English will be presented on the following pages.

For further evaluation of the HPLC-IMER method presented in the research project, a round robin test is currently planned to be accomplished with partners throughout Europe. The results of the test may in the future lead to the acceptance of the HPLC-IMER as an official method for the determination of sulfites in foodstuffs.

FORSCHUNGSKREIS DER ERNÄHRUNGSINDUSTRIE E.V.

AiF 14583 N

Bestimmung von Schwefeldioxid in Früchten und Fruchtprodukten durch HPLC-Biosensorkopplung

Koordinierung:	Forschungskreis der Ernährungsindustrie e.V. (FEI), Bonn
Forschungsstelle I:	Forschungsanstalt Geisenheim, Institut für Oenologie und Getränkeforschung, FG Weinanalytik u. Getränkeforschung Prof. Dr. H. Dietrich
Forschungsstelle II:	Universität Bonn Institut für Ernährungs- und Lebensmittelwissenschaften FG Lebensmittelchemie Prof. Dr. R. Galensa
Industriegruppe:	Verband der Deutschen Fruchtsaft-Industrie e.V., Bonn Projektkoordinator: Dr. C. Sprenger, Faethe Labor GmbH, Paderborn
Laufzeit:	2005 – 2008
Zuwendungssumme:	€ 230.200,-- (Förderung durch BMWi via AiF/FEI)

Ausgangsituation:

Schwefeldioxid ist eine Verbindung, die in vielerlei Hinsicht für die Lebensmittelindustrie von großem Interesse und deren Wirkungs- und Einsatzspektrum gut bekannt ist. Die exakte Bestimmung der Substanz, vor allem im unteren Konzentrationsbereich (<10 ppm), ist allerdings mit der offiziellen nasschemischen Analysenmethode (IFU7a) zu unsicher. Dies kann bei der Überprüfung von gesetzlich festgelegten Grenzwerten oder beim Nachweis einer Schwefelung in Produkten sogar zur Rechtsunsicherheit führen, da unterschiedliche Ergebnisse erhalten werden. Die Probleme resultieren vorwiegend aus der großen Reaktivität des Schwefeldioxids, zum anderen bedingt sie wiederum seine vielfältigen technologisch positiven Wirkungen. Unerwünscht ist die Eigenschaft als Pseudoallergen, die bei entsprechend empfindlichen Personen Asthma auslösen kann. Durch die Verabschiedung der EU-Richtlinie 2003/89/EG, die bei der Allergenkennzeichnung auch für SO_2-Gehalte ab 10 mg/kg gilt (seit 25.11.2004), wird der Bedarf an einer zuverlässigen Analysenmethode noch dringender.

Ziel des Forschungsvorhabens war es daher, die analytischen Probleme bei der Erfassung des Schwefeldioxids in unterschiedlichen Produkten durch den Einsatz der HPLC-Biosensorkopplung zu lösen bzw. zu minimieren. Diese Methode bietet neben der Erfassung im Spurenbereich simultan zusätzlich zur Retentionszeit eine qualitative Absicherung durch das spezifische, enzymatische Signal.

Forschungsergebnis:

Die Methode wurde für zahlreiche Fruchtsäfte, fruchtsafthaltige Getränke, Fruchtprodukte und für eine Vielzahl weiterer Probenmaterialien eingesetzt. Während in allen Fällen für die chromatographischen Analysen einheitliche HPLC-Bedingungen gewählt wurden, mussten die Aufarbeitungsbedingungen bei festen und flüssigen Matrices logischerweise variiert werden. Um in sehr komplexen, festen Proben Störungen im Spurenbereich ausschliessen zu können, wurde zusätzlich ein Kombinationsverfahren von Destillation und HPLC-Enzymreaktorkopplung entwickelt und erprobt (DE-HPLC-IMER). Die Schwefeldioxidgesamtgehalte konnten sowohl im Spuren- als auch im Minorkomponenten-Konzentrations-Bereich in allen Proben gemessen werden.

Bei den geforderten Enzymvergleichen erwies sich die gentechnisch hergestellte, pflanzliche Sulfitoxidase der bisher nur zugänglichen tierischen, in einigen Punkten als überlegen, allerdings ist diese ebenfalls für die Methode geeignet. Die (selbst hergestellten) Enzymreaktoren überzeugen durch ihre äußerst hohen Stabilitäts- und Standzeiten. Eine weitere Sulfitoxidase aus einem marinen Organismus konnte nicht erfolgreich immobilisiert werden.

Methodenvergleiche mit den destillativen Verfahren und mit einer FIA-Methode zeigten eindeutig die Vorteile der HPLC-IMER vor allem bei niedrigen Konzentrationen. So kann z.B. als ungeschwefelt deklarierte Bioware auch auf Gehalte von unter 1 ppm überprüft werden. Ein entscheidender Punkt ist hierbei die Identitätsabsicherung. Zumal bei flüssigen Proben ist weiterhin die einfache Probenvorbereitung und die Möglichkeit der schnellen Serienanalytik durch einen programmierbaren Probengeber zu nennen. Dies gilt ebenfalls für die FIA, allerdings nicht im Spurenbereich. Die resultierenden Unterschiede in den gefundenen Gehalten beruhen auf der unterschiedlichen Freisetzung des gebundenen SO_2 im alkalischen bzw. bei den destillativen Methoden im sauren Millieu. Unbekannte Bindungsformen wurden auch nach verschiedenen Schwefelungsversuchen von Traubensäften nicht nachgewiesen, während bereits beschriebene bestätigt werden konnten.

Es zeigte sich, dass durch die HPLC-IMER-Methode mit alkalischer Probenaufarbeitung in allen Fällen mehr Schwefeldioxid ermittelt wurde als bei den destillativen Verfahren mit dem Zusatz von Phosphorsäure. Dies gilt speziell für den unteren, rechtlich relevanten Konzentrationsbereich (10 ppm), wo teilweise bis zu einem Drittel mehr SO_2 gefunden wurde.

Versuche mit dem Einsatz eines coulometrischen Elektrodenarray-Detektors oder mit der DE-HPLC-IMER-Kopplung, die schon niedrige Nachweisgrenze (0,1 ppm) und die qualitative Absicherung noch zu verbessern, führten bisher zu keinem positiveren Ergebnis.

Wirtschaftliche Bedeutung:

2003 betrug der Pro-Kopf-Verbrauch für Fruchtsäfte und Fruchtnektare ca. 42 Liter. Die Herstellerbranche erzielte damit einen Umsatz von 3,6 Mrd. Euro. Alle an der Produktion von Fruchtsäften und Fruchtnektaren beteiligten Unternehmen unterziehen sich einer umfangreichen Qualitätskontrolle bei der Herstellung sämtlicher Erzeugnisse. Dies ist aus Wettbewerbs- und Kostengründen unbedingt notwendig, da sich kein Betrieb fehlerhafte Chargen leisten kann. Die Fruchtsaftbranche selbst umfasst rund 445 Unternehmen. 424 sind im Verband der Fruchtsaft-Industrie organisiert. Der überwiegende Teil der Unternehmen (97 %) sind KMU, die somit in besonderem Maße von den Ergebnissen dieses Vorhabens und seinem Beitrag zur Qualitätssicherung profitieren.

Vor allem bei der Beurteilung von Produkten mit niedrigen SO_2-Gehalten im unteren, rechtlich relevanten Konzentrationsbereich, gibt es oft Probleme, die in kritischen Fällen durch Ablehnung oder Beanstandung der Ware zu finanziellen Verlusten führen können. Nach den bisherigen Messungen ergibt die HPLC-Biosensorkopplung in einer gegebenen Probe immer den höchsten SO_2-Wert. Insofern wäre man damit automatisch auf der rechtlich sicheren Seite, wenn der gefundene Gehalt unter dem Grenzwert liegt. Ebenfalls die Deklarationsüberprüfung von Angaben wie „schwefelfrei" bei Bioware oder bei Herstellern, die bewusst Produkte „garantiert" ohne Zusatzstoffe anbieten wollen, ist so eindeutig möglich.

Die entwickelte Methode ist einfach zu handhaben und durch die langen Standzeiten der Enzymreaktoren und der Detektorelektroden auch preiswert. Die Umrüstung eines entsprechenden vorhandenen HPLC-Systems ist problemlos.

Publikationen (Auswahl):

1. FEI-Schlussbericht 2008.
2. Theisen, S., Kothe, L. und Galensa, R.,: HPLC-IMER as a sensitive method for analysis of SO_2 in fruit juices. 27th Intern. Symp. on Chromatography, Münster, 21.-25.09.2008.
3. Tschoepe M., Herzig B., Sprenger C., Theisen S. und Galensa R.: Bestimmung von Schwefeldioxid in Aprikosen und daraus hergestellten Fruchtprodukten mittels HPLC/ Biosensorkopplung (Enzymreaktorkopplung). Regionaltagung NRW der Lebensmittelchemischen Gesellschaft, Bonn, 8.03.2006, Lebensmittelchem. 60 (6), 138 (2006).

Short description of the research project AiF 14583:
"Analytical determination of sulfur dioxide in fruits and fruit products by HPLC-biosensor-coupling"

Initial situation
Sulfur dioxide is a compound that has in multiple matters a huge meaning for the food-industry and whose wide range of effects and uses are well known.
Using the usual official methods of wet-chemical analysis, an exact determination is not possible, especially not with a concentration level below 10 ppm.Therefore the results differ in a high variety. This fact may lead to uncertainty regarding the law in the case of controlling the law given limits or to problems of control the sulphating of in food samples. These problems in exact determination are caused by the high reactivity of sulfur dioxide, whereas the same quality implies a high amount of technological effects. However the character of being a pseudoallergene to sensitive persons is not desired. The change of the EU-Guideline 2003/89/EG on November 25th has increased the future need of analysis: Passing this guideline set a limit of 10 mg/kg for declaring SO_2-concentrations.

Aim of this research project was to solve or minimize the analytical problems of detecting SO_2 in different products by use of the HPLC-biosensor-coupling.
This method offers not only the possibility of detecting trace amounts of SO_2, it also holds the opportunity of using the specific retention time and the enzymatic signal for validation.

Research results
The HPLC method has been applied to almost all of the products mentioned in the application as well as to many other food products.
The analytical conditions for the chromatography were identical for all products, while the sample preparation method had to be adjusted for the different solid and liquid matrices.
Additionally, in order to avoid mistakes in the trace-analyses of very complex solid samples, a combination of two methods was developed.
This combination, called DE-HPLC-IMER (abbreviation for **De**stillation - **H**igh **P**erformance **L**iquid **C**hromatography - **Im**mobilized **E**nzyme **R**eactor), allows for an accurate trace analysis of sulfur dioxide even in very complex solid samples.

Comparing two different types of enzymes revealed the advantages of the phytogenetic sulfite oxidase over the animal kind, the latter being the only one commercially available until now. After all both enzymes are well suited for this method. The self-produced enzyme reactors impress with their long-term stability in use as well as in storage.
One other type of sulfite oxidase, extracted from a marine organism, has not been immobilized successfully.

The HPLC-IMER method shows several analytical advantages when compared to other methods like the official destillation methods and a FIA method. This becomes evident especially in the lower concentration ranges of sulfur dioxide. The unequalled low detection limit of the HPLC allows for checking e.g. organic food, declared as free of sulfur dioxide, for trace amounts of even less than 2 ppm. One essential aspect in this matter is the proof of authenticity/identity.
Especially for liquid food samples the HPLC-method has considerable advantages because of the simple sample preparation as well as the possibility of a fast series of analyses by using the programmable autosampler.
The same is true for the FIA, yet not for the detection of very small amounts of sulfur dioxide.
There are different ways of extracting SO_2 from the food samples, that is using acid or base, which may be one reason for the differing results of analyses.
It was not possible to detect new bonds between sulfur dioxide and food compounds, yet some of those already known could be affirmed.

The HPLC method with the alkaline release of SO_2 was shown to always yield higher amounts of SO2 than the distillation method with its addition of phosphoric acid. This is especially true for the lower concentration levels which are judicially important. In the range of 10 ppm or lower the HPLC yielded

results up to one third higher than the other methods.

Experiments using a coulometric electrode array detector (and also with the DE-HPLC-IMER) in order to further improve the very low limit of detection and the qualitative validation did not lead to positive results.

Economic value
In food monitoring findings of sulfur dioxide concentrations close to the legal limit have been causing problems in the correct judgement of a product. The (wrongful) rejection or objection of a critical product can lead to significant financial losses for a company.
The research results of this project indicate that the HPLC method always yields higher amounts of the SO_2 content than any other method.
If a product's amount of SO_2 analysed with the HPLC was below the legal limit it could therefore automatically be considered legally not objectionable.
For the first time it is now possible to verify declarations like "no sulfur added" for organic food or with producers offering special products "guaranteed" without sulfur dioxide.

The method is easy to apply and it is comparably cheap due to the long lasting enzyme reactors and electrodes. The adjustment of an existing HPLC into an HPLC-IMER is rather simple.

The actual legal limit of 10 mg/L SO_2 is based on the analytical implications of the official distillation method.

Presuming positive results of an interlaboratory comparison, this method may eventually become part of the list of official methods or even replace the former method. A new debate about the legal limit due to the different results of both methods would then become obligatory.

Publications / Posters

Tschoepe M., Herzig B., Sprenger C., Theisen S., Galensa R.: Bestimmung von Schwefeldioxid in Aprikosen und daraus hergestellten Fruchtprodukten mittels HPLC/Biosensorkopplung (Enzymreaktorkopplung) (Poster, published in Lebensmittelchemie (2006) 138) Regionaltagung NRW der Lebensmittelchemischen Gesellschaft, Bonn, 8. März 2006

Theisen S., Hänsch R., Mendel R., Galensa R.: Sulfit-Bestimmung mittels HPLC-Biosensorkopplung - Vergleich von tierischer und pflanzlicher Sulfitoxidase (Poster, published in Lebensmittelchemie, in print) 36. Deutscher Lebensmittelchemikertag, Nürnberg, 10. - 12. September 2007

Theisen S., Dietrich H., Galensa R., Giehl A., Herzig B., Patz C., Tschoepe M.: DE-HPLC-IMER – eine Möglichkeit zur spezifischen und empfindlichen SO_2-Bestimmung in festen und flüssigen Lebensmitteln (oral presentation, to be published in Lebensmittelchemie, in print) Regionaltagung NRW der Lebensmittelchemischen Gesellschaft, Bonn, 5. März 2008

Theisen, S., Bonn/D, Kothe, L., Bonn/D, Galensa, R., Bonn/D
HPLC-IMER as a sensitive method for analysis of SO_2 in fruit juices (Poster)
27th International Symposium on Chromatography, Münster, 21.-25. September 2009

B.2. Legal Limits for Sulfites in Food (ZZulV, Annex 5, Part B)

Lebensmittel	Höchstmenge (mg/kg bzw. mg/l), berechnet als freie Säure					
	Ss	Bs	PHB	Ss + Bs *1)	Ss + PHB *2)	Ss + Bs + PHB *3)
1	2	3	4	5	6	7
Käse und Käseanaloge (nur Oberflächenbehandlung)	qs					
Gekochte rote Rüben		2 000				
Häute auf Kollagenbasis mit einer Wasseraktivität von mehr als 0,6	qs					
Eiermalfarbe		4 000	4 000	5 000		
Krebstiere und Weichtiere, gekocht		1 000		2 000		
Nahrungsergänzungsmittel in flüssiger Form				2 000		
Aromen				1 500		

Teil B

Liste 1

Zugelassene(s) Schwefeldioxid und Sulfite

E-Nummer	Zusatzstoff
1	2
E 220	Schwefeldioxid
E 221	Natriumsulfit
E 222	Natriumhydrogensulfit
E 223	Natriummetabisulfit
E 224	Kaliummetabisulfit
E 226	Calciumsulfit
E 227	Calciumbisulfit
E 228	Kaliumbisulfit

Liste 2

Zulassungen **)

Lebensmittel	Höchstmenge (mg/kg bzw. mg/l), berechnet als SO_2	
1	2	
Burger meat mit einem Gemüse- und/oder Getreideanteil von mindestens 4 %	450	
Breakfast sausages	450	
Longaniza fresca und Butifarra fresca	450	
Getrocknete und gesalzene Dorschfische (Gadidae)	200	
Krebstiere und Kopffüßer:		
– frisch, gefroren und tiefgefroren	150	
– Krebstiere der Familien *Penaeidae, Solenoceridae, Aristaeidae*:		
– weniger als 80 Einheiten	150	in den essbaren Teilen
– zwischen 80 und 120 Einheiten	200	
– mehr als 120 Einheiten	300	
Krebstiere und Kopffüßer:		
– gekocht	50	

Lebensmittel	Höchstmenge (mg/kg bzw. mg/l), berechnet als SO_2
1	2
– gekochte Krebstiere der Familien *Penaeidae, Solenoceridae, Aristaeidae*:	
– weniger als 80 Einheiten	135
– zwischen 80 und 120 Einheiten	180
– mehr als 120 Einheiten	270
Hartkekse	50
Stärke	50
Sago	30
Graupen	30
Kartoffeltrockenerzeugnisse	400
Knabbererzeugnisse auf Getreide- oder Kartoffelbasis	50
Geschälte Kartoffeln	50
Verarbeitete (einschließlich gefrorene oder tiefgefrorene) Kartoffeln	100
Kartoffelteig	100
Weiße Gemüsesorten, getrocknet	400
Weiße Gemüsesorten, verarbeitet (einschließlich gefrorene oder tiefgefrorene weiße Gemüsesorten)	50
Getrockneter Ingwer	150
Getrocknete Tomaten	200
Meerrettichzubereitung	800
Pulpe von Speisezwiebeln, Knoblauch und Schalotten	300
Gemüse und Obst in Essig, Öl oder Lake (ausgenommen Oliven und gelbe Paprika in Lake)	100
Gelbe Paprika in Lake	500
Verarbeitete Pilze (einschließlich gefrorene Pilze)	50
Trockenpilze	100
Trockenfrüchte	
– Aprikosen, Pfirsiche, Trauben, Pflaumen oder Feigen	2 000
– Bananen	1 000
– Äpfel oder Birnen	600
– Andere (einschließlich Nüsse mit Schale)	500
Getrocknete Kokosnüsse	50
Obst, Gemüse, Angelikawurzel und Zitrusschalen, kandiert, kristallisiert oder glasiert	100
Konfitüren, Gelees und Marmeladen (ausgenommen Konfitüre extra oder Gelee extra) oder ähnliche Fruchtaufstriche, einschließlich brennwertverminderte Erzeugnisse	50
Jams, jellies und marmelades aus geschwefelten Früchten	100
Pastetenfüllungen auf Früchtebasis	100
Würzmittel auf Zitrussaftbasis	200
Traubensaftkonzentrat zur Selbstherstellung von Wein	2 000
Mostarda di frutta	100
Obstgeliersaft und flüssiges Pektin zur Abgabe an den Verbraucher im Sinne des § 2 Nr. 5 Halbsatz 1	800
Weiße Herzkirschen, rehydratisierte Trockenfrüchte und Litschis in Gläsern	100
Zitronenscheiben in Gläsern	250
Zuckerarten im Sinne der Zuckerartenverordnung, ausgenommen Glukosesirup, auch getrocknet	10
Glukosesirup, auch getrocknet	20
Speisesirup oder Melasse	70
Andere Zuckerarten	40
Überzüge (Sirup für Pfannkuchen, aromatisierter Sirup für Milchmischgetränke oder Speiseeis; ähnliche Erzeugnisse)	40

Lebensmittel	Höchstmenge (mg/kg bzw. mg/l), berechnet als SO_2
1	2
Orangen-, Grapefruit-, Apfel- und Ananassaft für die Abgabe aus Großbehältern in der Gastronomie und in Einrichtungen zur Gemeinschaftsverpflegung	50
Limonen- oder Zitronensaft	350
Konzentrate auf der Basis von Fruchtsäften mit mindestens 2,5 % Gerste (barley water)	350
Andere Konzentrate auf der Basis von Fruchtsäften oder zerkleinerten Früchten; capilé groselha	250
Nichtalkoholische, aromatisierte Getränke, die Fruchtsaft enthalten	20 (nur als Gehalt aus dem Konzentrat)
Nichtalkoholische, aromatisierte Getränke mit mindestens 235 g/l Glukosesirup	50
Traubensaft, unvergoren, zur sakramentalen Verwendung	70
Süßwaren auf Glukosesirupbasis	50 (nur als Gehalt aus dem Glukosesirup)
Bier	20
Bier mit Nachgärung im Fass	50
Alkoholfreier Wein	200
Made wine	260
Obst-/Fruchtwein, Obst-/Fruchtschaumwein (jeweils einschließlich alkoholfreie Erzeugnisse)	200
Met	200
Gärungsessig	170
Senf, außer Dijon-Senf	250
Dijon-Senf	500
Gelatine	50
Fleisch-, Fisch- oder Krebstieranaloge auf Proteinbasis	200
Marinierte Nüsse	50
Zuckermais, vakuumverpackt	100
Destillierte alkoholische Getränke mit ganzen Birnen	50
Salsicha fresca	450
Tafeltrauben	10
Frische Litschis	10 (in den essbaren Teilen)

Teil C Andere Konservierungsstoffe

Liste 1 Nitrite und Nitrate

E-Nummer	Bezeichnung	Lebensmittel	Verwendete Höchstmenge (berechnet als $NaNO_2$) mg/kg	Höchstmenge (§ 2 Nr. 2) (berechnet als $NaNO_2$) mg/kg
E 249	Kaliumnitrit *1)	Fleischerzeugnisse	150	
E 250	Natriumnitrit *1)	Sterilisierte Fleischerzeugnisse (Fo> 3,00) *2)	100	
		Traditionelle nassgepökelte Fleischerzeugnisse (1):		

B.3. Methods for Automatic Sample Preparation

Title: 400ul Probe (Vial 10)+NaOH+Puffer_nodelay_MIX+Probe 10x
Datasource: UNI-PQMCQM3TW1B_local Created: 14.11.2007 09:14:10 by biosensor
Location: Timebase-bst\Programme\NaOH_Zeit_mix+Probe 10x NH1
Timebase: Timebase-bst Changed: 14.11.2007 09:14:10 by biosensor

```
           Sampler.AcquireExclusiveAccess
           Switch_Valve.Position =            1
           Pressure.LowerLimit =              2 [bar]
           Pressure.UpperLimit =              50 [bar]
           MaximumFlowRamp =                  1.00 [ml/min²]
           %A.Equate =                        "%A"
           Flush                              Volume = 250
           Wait                               FlushState
           NeedleHeight =                     1 [mm]
           CutSegmentVolume =                 10 [ l]
           SyringeSpeed =                     5
           TrayTemperature =                  20
           CycleTime =                        0 [min]
           WaitForTemperature =               True
           Pump_1_Pressure.Step =             Auto
           Pump_1_Pressure.Average =          On
           echem_chan2.Step =                 Auto
           echem_chan2.Average =              On
           echem.Step =                       Auto
           echem.Average =                    On
           Dispense  Volume = 800.0, SourceReservoir = Reservoir_C,
DestinationVial = 10
           Pipet  Volume = 400.0, SourceVial = 51, DestinationVial = 10
           Mix  SourceVial = 10, NumberOfTimes = 10, Volume = 1200.0
           Dilute  ConcentrateVolume = 100.0, SourceVial = 10,
DiluentVolume = 800.0, SourceReservoir = Reservoir_B, DestinationVial
= CurrentVial
           Mix  SourceVial = CurrentVial, NumberOfTimes = 10, Volume =
900.0
           Wait                               SampleReady
           Flow =                             0.600 [ml/min]
           Curve =                            5

 0.000     Load
           Wait                               CycleTimeState
           Inject
           Wait                               InjectState
           Pump_1_Pressure.AcqOn
           echem_chan2.AcqOn
           echem.AcqOn
           Sampler.ReleaseExclusiveAccess

 0.100     BeginOverlap
```

Title: 400ul Probe (Vial 10)+NaOH+Puffer_nodelay_MIX+Probe 10x
Datasource: UNI-PQMCQM3TW1B_local Created: 14.11.2007 09:14:10 by biosensor
Location: Timebase-bst\Programme\NaOH_Zeit_mix+Probe 10x NH1
Timebase: Timebase-bst Changed: 14.11.2007 09:14:10 by biosensor

```
 8.000     Pump_1_Pressure.AcqOff
           echem_chan2.AcqOff
           echem.AcqOff

           End
```

Title: Probe mit NaOH_10min delay_aus Vial10_MIX 10x
Datasource: UNI-PQMCQM3TW1B_local Created: 14.11.2007 09:18:42 by biosensor
Location: Timebase-bst\Programme\NaOH_Zeit_mix+Probe 10x NH1
Timebase: Timebase-bst Changed: 14.11.2007 09:18:42 by biosensor

```
           Sampler.AcquireExclusiveAccess
           Switch_Valve.Position =            1
           Pressure.LowerLimit =              2 [bar]
           Pressure.UpperLimit =              50 [bar]
           MaximumFlowRamp =                  1.00 [ml/min²]
           %A.Equate =                        "%A"
           Flush                              Volume = 250
           Wait                               FlushState
           NeedleHeight =                     1 [mm]
           CutSegmentVolume =                 10 [ l]
           SyringeSpeed =                     5
           TrayTemperature =                  20
           CycleTime =                        0 [min]
           WaitForTemperature =               True
           Pump_1_Pressure.Step =             Auto
           Pump_1_Pressure.Average =          On
           echem_chan2.Step =                 Auto
           echem_chan2.Average =              On
           echem.Step =                       Auto
           echem.Average =                    On
           DelaySP  Time = 10.0
           Dilute  ConcentrateVolume = 100.0, SourceVial = 10,
DiluentVolume = 800.0, SourceReservoir = Reservoir_B, DestinationVial
= CurrentVial
           Mix  SourceVial = CurrentVial, NumberOfTimes = 10, Volume =
900.0
           Wait                               SampleReady
           Flow =                             0.600 [ml/min]
           Curve =                            5

 0.000     Load
           Wait                               CycleTimeState
           Inject
           Wait                               InjectState
           Pump_1_Pressure.AcqOn
           echem_chan2.AcqOn
           echem.AcqOn
           Sampler.ReleaseExclusiveAccess

 0.100     BeginOverlap

 8.000     Pump_1_Pressure.AcqOff
           echem_chan2.AcqOff
           echem.AcqOff
```

Title: 1:100 Verdünnun aus Vial 11 mit Wasser
Datasource: UNI-PQMCQM3TW1B_local
Location: Timebase-bst\Programme Created: 13.12.2007 15:24:08 by biosensor
Timebase: Timebase-bst Changed: 13.12.2007 15:24:08 by biosensor

```
           Sampler.AcquireExclusiveAccess
           Pressure.LowerLimit =              1 [bar]
           Pressure.UpperLimit =              50 [bar]
           MaximumFlowRamp =                  1.00 [ml/min²]
           %A.Equate =                        "%A"
           Flush                              Volume = 500
           Wait                               FlushState
           NeedleHeight =                     1 [mm]
           CutSegmentVolume =                 0 [ l]
           SyringeSpeed =                     4
           TrayTemperature =                  20
           CycleTime =                        0 [min]
           WaitForTemperature =               False
           Pump_1_Pressure.Step =             Auto
           Pump_1_Pressure.Average =          On
           echem_chan2.Step =                 Auto
           echem_chan2.Average =              On
           echem.Step =                       Auto
           echem.Average =                    On
           Dilute  ConcentrateVolume = 10.0, SourceVial = 11,
DiluentVolume = 990.0, SourceReservoir = Reservoir_A, DestinationVial
= CurrentVial
           Wait                               SampleReady
           Flow =                             0.600 [ml/min]
           Curve =                            5

 0.000     Load
           Wait                               CycleTimeState
           Inject
           Wait                               InjectState
           Pump_1_Pressure.AcqOn
           echem_chan2.AcqOn
           echem.AcqOn
           Sampler.ReleaseExclusiveAccess

 8.000     Pump_1_Pressure.AcqOff
           echem_chan2.AcqOff
           echem.AcqOff

           End
```

B.4. Official Methods for Sulfite Analysis in Food

Amtliche Sammlung von Untersuchungsverfahren, according to Paragraph 64 LFGB (formerly Paragraph 35 LMBG)

L 00.00 46/1
L 00.00 46/2

Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG

L

Untersuchung von Lebensmitteln

00.00

Bestimmung von **Sulfit** in **Lebensmitteln**
Teil 1: Optimiertes Monier-Williams-Verfahren
(Übernahme der gleichnamigen Norm DIN EN 1988 Teil 1, Ausgabe Mai 1998)

46/1

Nationales Vorwort

Diese Europäische Norm ist vom Technischen Komitee CEN/TC 275 „Lebensmittelanalytik – Horizontale Verfahren" (Sekretariat: DIN) unter intensiver deutscher Mitarbeit erarbeitet worden.

Das zuständige deutsche Normungsgremium ist der Arbeitskreis „Sulfite" des Normenausschusses „Lebensmittel und landwirtschaftliche Produkte" (NAL) im DIN Deutsches Institut für Normung e.V.

Die für die Ermittlung der Präzisionsdaten verwendete ISO 5725 : 1986 ist durch ISO 5725-1 : 1994 ersetzt und wird außerdem durch ISO 5725-2 bis ISO 5725-6 ersetzt, die künftig erscheinen werden.

Deutsche Fassung

Einleitung

Sulfit kann als Konservierungsstoff in Lebensmitteln verwendet werden. Um mögliche negative Auswirkungen auf die Gesundheit zu minimieren, haben viele Länder die Verwendung von Sulfit in Lebensmitteln geregelt. Das führte zur Entwicklung unterschiedlicher Analysenverfahren zum Nachweis und zur Bestimmung von Sulfit in einer Vielzahl von Lebensmitteln.

1 Anwendungsbereich

Diese Europäische Norm legt ein Destillationsverfahren zur Bestimmung von Sulfit (ausgedrückt als Schwefeldioxid) in Lebensmitteln, die einen Sulfitgehalt von mindestens 10 mg/kg aufweisen, fest. Das Verfahren ist in Gegenwart anderer flüchtiger Schwefelverbindungen anwendbar. Es ist nicht anwendbar auf Kohl, getrockneten Knoblauch, getrocknete Zwiebeln, Ingwer, Lauch und Sojaproteine [1]. Es hat sich gezeigt, daß die Untersuchung von isolierten Sojaproteinen zu falsch positiven Ergebnissen führt.

Spezielle Lebensmittel, für die Europäische Normen für die Bestimmung von Sulfit gelten, sind vom Anwendungsbereich dieser horizontalen Europäischen Norm ausgenommen.

2 Normative Verweisungen

Diese Norm enthält durch datierte oder undatierte Verweisungen Festlegungen aus anderen Publikationen. Diese normativen Verweisungen sind an den jeweiligen Stellen im Text zitiert, und die Publikationen sind nachstehend aufgeführt. Bei datierten Verweisungen gehören spätere Änderungen oder Überarbeitungen dieser Publikationen nur zu dieser Norm, falls sie durch Änderung oder Überarbeitung eingearbeitet sind. Bei undatierten Verweisungen gilt die letzte Ausgabe der in Bezug genommenen Publikation.

EN ISO 3696
Wasser für analytische Zwecke – Anforderungen und Prüfungen (ISO 3696 : 1987)

3 Prinzip

Das im Lebensmittel befindliche freie Sulfit einschließlich einer reproduzierbaren Menge an gebundenem Sulfit (z. B. Carbonyladditionsprodukte) wird bestimmt. Die Probenmenge wird mit Salzsäure am Rückfluß erhitzt, wobei Sulfit in Schwefeldioxid umgewandelt wird. Ein Stickstoffstrom,

[1] Es hat sich gezeigt, daß die Untersuchung von isolierten Sojaproteinen zu falsch positiven Ergebnissen im Bereich von 20 mg/kg bis 30 mg/kg, ausgedrückt als Schwefeldioxid, führt. Wenn Lebensmittel, die isolierte Sojaproteine enthalten, untersucht werden, kann daher eine proportionale Erhöhung der Ergebnisse beobachtet werden, die in Betracht gezogen wird.

der unter der Oberfläche der am Rückfluß erhitzten Lösung eingeleitet wird, überführt das Schwefeldioxid durch einen Kühler und ein damit verbundenes Gaseinleitungsrohr in eine Wasserstoffperoxid-Lösung, in der das Schwefeldioxid zu Schwefelsäure oxidiert wird. Die gebildete Schwefelsäure wird gegen Natriumhydroxid-Standardlösung titriert. Der Sulfitgehalt steht in direkter Beziehung zur gebildeten Schwefelsäure. Siehe [1], [2].

4 Chemikalien

Wenn nicht anders angegeben, werden bei der Untersuchung ausschließlich analysenreine Chemikalien und nur Wasser verwendet, das mindestens der Qualität 3 nach EN ISO 3696 entspricht.

4.1 Salzsäure, Stoffmengenkonzentration
c (HCl) = 4 mol/l

Für jede Untersuchung werden 90 ml Salzsäure-Lösung durch vorsichtige Zugabe von 30 ml konzentrierter Salzsäure (36 %) zu 60 ml Wasser hergestellt. Die Salzsäure-Lösung wird täglich frisch hergestellt.

4.2 Natriumhydroxid-Standardlösung,
c (NaOH) = 0,010 mol/l

4.3 Methylrotindikator
250 mg Methylrot werden in 100 ml Ethanol gelöst.

4.4 Wasserstoffperoxid-Lösung, Volumenanteil
φ (H_2O_2) = 3 %

Unmittelbar vor der Verwendung werden 3 Tropfen Methylrotindikator zugefügt und mit Natriumhydroxid-Standardlösung (4.2) bis zum Umschlagspunkt nach gelb titriert. Wenn der Umschlagspunkt überschritten wurde, wird die Lösung verworfen.

4.5 Ethanol oder **mit Methanol vergällter Spiritus**

4.6 Ethanol/Wasser-Mischung,
Volumenanteil φ (C_2H_5OH) = 5 %

4.7 Stickstoff, hohe Reinheit, mit einem Druckregler zur Einhaltung eines Durchflusses von 200 ml/min.

Zum Schutz des Stickstoffs vor Sauerstoff dient ein Absorber, wie er in der gaschromatographischen Analyse verwendet wird.

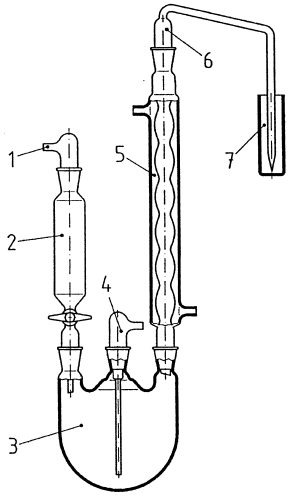
Als andere Möglichkeit kann eine sauerstoffabsorbierende Lösung wie alkalisches 1,2,3-Trihydroxybenzol (Pyrogallol) in einer Gaswaschflasche verwendet werden. Die Lösung wird wie folgt vorbereitet: In die Gaswaschflasche werden 4,5 g 1,2,3-Trihydroxybenzol gegeben. Die Gaswaschflasche wird 2 min bis 3 min mit Stickstoff gespült. Durch Zugabe von 65 g Kaliumhydroxid zu 85 ml Wasser wird eine Kaliumhydroxid-Lösung hergestellt. (Vorsicht: Es entsteht Wärme.) Unter Aufrechterhaltung einer Stickstoffatmosphäre in der Gaswaschflasche wird die Kaliumhydroxid-Lösung in die Gaswaschflasche gegeben.

5 Geräte

5.1 Destillationsapparatur
Die Destillationsapparatur[2)] ist wie folgt zusammengesetzt (siehe Bild 1):

- Einlaßadapter (1) mit Schlauchverbindung und Gummiball, mit dem ein Überdruck über der Lösung erzeugt werden kann. Die Verwendung eines Tropftrichters mit Druckausgleichsrohr wird nicht empfohlen, da sich möglicherweise schwefeldioxidhaltiges Kondensat im Tropftrichter und Druckausgleichsrohr absetzen kann.
- Tropftricher (2), Nennvolumen $\geq$ 100 ml.
- Rundkolben (3), Nennvolumen 1 l, mit drei dichten geeigneten Kegelschliffverbindungen.
- Gaszuführungsrohr (4) in ausreichender Länge, um Stickstoff etwa 25 mm über dem Boden des Rundkolbens einzuleiten.
- Kugelkühler (5), Mantellänge 300 mm.
- Gaseinleitungsrohr (6), aus Glas, mit Maßen nach Bild 2.
- Gefäß (7), Innendurchmesser etwa 25 mm, Länge etwa 180 mm.

ANMERKUNG: Wenn der Gegendruck so niedrig wie möglich gehalten wird, werden Verluste an Schwefeldioxid durch undichte Stellen vermieden. Zum Abdichten aller



1 Einlaßadapter
2 Tropftrichter
3 Rundkolben
4 Gaszuführungsrohr
5 Kugelkühler
6 Gaseinleitungsrohr
7 Gefäß

Bild 1: Apparatur für das optimierte Monier-Williams-Verfahren

[2)] Die Beschreibung der Destillationsapparatur bezieht sich auf [1] und dient lediglich zur Unterrichtung der Anwender dieser Europäischen Norm. Diese Information bedeutet keine Anerkennung des genannten Produktes durch CEN. Gleichwertige Produkte (wie z. B. die Destillationsapparatur wie in ISO 5522 : 1981 [3] beschrieben) dürfen verwendet werden, wenn sie nachweisbar zu den gleichen Ergebnissen führen.

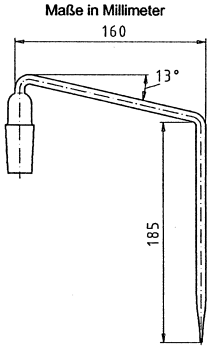


Bild 2: Vergrößerte Darstellung des Gaseinleitungsrohrs für die Monier-Williams-Apparatur

Schliffflächen, außer der Verbindung zwischen dem Tropftrichter und dem Kolben, wird ein dünner Film Hahnfett verwendet. Jede Verbindung wird festgespannt, um die vollständige Abdichtung während der Untersuchung zu sichern.

5.2 Bürette

Nennvolumen 10 ml, mit Überlaufrohr und einer Schlauchverbindung zu einem Rohr, das Siliciumdioxid mit adsorbiertem Natriumhydroxid enthält, oder einem entsprechenden Luftreiniger, damit über der Natriumhydroxid-Standardlösung (4.2) eine kohlenstoffdioxidfreie Atmosphäre aufrechterhalten werden kann.

5.3 Kühlwasserzirkulator

Kühler mit zirkulierender Kühlflüssigkeit, wie einer Mischung aus einem Volumenteil Methanol und 2 Volumenteilen Wasser, oder kontinuierlichem Wasserdurchfluß, bei einer Temperatur von nicht mehr als 15 °C.

5.4 Heizhaube, regelbar auf Temperaturen zwischen 20 °C und 120 °C.

5.5 Labormixgerät oder **Mischer**

5.6 Sauerstoff-Absorber, um den Stickstoff (4.7) gegen Sauerstoff zu schützen.

6 Durchführung

6.1 Allgemeines

Die Probenvorbereitung und die Untersuchung werden so schnell wie möglich durchgeführt, um Verluste an leicht zersetzlichen Sulfiten zu vermeiden.

ANMERKUNG: Um vor der regelmäßigen Durchführung mit dem Verfahren vertraut zu werden und Übung zu bekommen, wird empfohlen, Lebensmittelproben mit bekannten Sulfitgehalten zu untersuchen. Die Untersuchung sollte so durchgeführt werden, daß jeder Sulfitverlust durch Oxidation oder durch Reaktion mit Lebensmittelbestandteilen ausgeschlossen ist. Da Sulfite mit Luft und Lebensmittelmatrices reagieren und instabil sind, werden die Probenmengen mit stabilen Sulfitquellen, nicht mit Natriumsulfit oder ähnlichen Salzen, versetzt. Natriumhydroxymethylsulfonat (HMS), das Bisulfit-Additionsprodukt von Formaldehyd, ist strukturell einigen zusammengesetzten Sulfitformen in Lebensmitteln ähnlich und zur Herstellung von stabilem, mit Sulfit versetztem Untersuchungsmaterial verwendbar.

Für die Untersuchung werden 50 g vorbereitete Probe eines sulfitfreien Lebensmittels in den Rundkolben (Bild 1; Nummer 3) gebracht. Eine aliquote Menge einer wäßrigen Lösung des HMS-Natriumsalzes wird dazugegeben. Die Lösung wird sofort untersucht.

HMS-Wiederfindungsraten von mehr als 80 % aus mit 10 mg/kg versetzten Lebensmittelmatrices erscheinen für die Ermittlung genauer Analysenwerte als ausreichend.

6.2 Probenvorbereitung

6.2.1 Feste Lebensmittel

50 g oder eine andere Probenmenge, die 500 µg bis 1 500 µg Schwefeldioxid enthält, wird in ein Labormixgerät oder einen Mischer gegeben. 100 ml der Ethanol/Wasser-Mischung (4.6) werden zugegeben und mit der Probenmenge kurz vermischt. Das Zerkleinern bzw. Mischen wird nur so lange fortgesetzt, bis die Probenbestandteile soweit zerkleinert sind, daß sie durch die Kegelschliff-Öffnung in den Rundkolben (Bild 1; Nummer 3) gegeben werden können.

6.2.2 Flüssige Lebensmittel

50 g oder eine andere Probenmenge, die 500 µg bis 1 500 µg Schwefeldioxid enthält, wird mit 100 ml Ethanol/Wasser-Mischung (4.6) gemischt.

6.3 Systemvorbereitung

Eine wie in Bild 1 dargestellte Destillationsapparatur (5.1) wird verwendet. Der Rundkolben (Bild 1; Nummer 3) wird in die Heizhaube (5.4) gebracht, und es werden 400 ml Wasser in den Kolben gegeben. Der Hahn des Tropftrichters (Bild 1; Nummer 2) wird geschlossen, und es werden 90 ml Salzsäure (4.1) in den Tropftrichter gefüllt. Der Durchfluß der Stickstoffeinleitung wird auf 200 ml/min ± 10 ml/min eingestellt. Zu dieser Zeit wird auch der Kühlmitteldurchfluß (5.3) im Kühler in Gang gesetzt. In das Gefäß (Bild 1; Nummer 7) werden 30 ml 3%ige Wasserstoffperoxid-Lösung (4.4) gegeben. Nach 15 min sind die Apparatur und das Wasser gründlich von Sauerstoff befreit, und die vorbereitete Einwaage darf in den Rundkolben eingeführt werden.

6.4 Probeneinführung und Destillation

Der Tropftrichter (Bild 1; Nummer 2) wird entfernt und die Probenmenge in dem wäßrigen Ethanol quantitativ in den Kolben (Bild 1; Nummer 3) eingebracht. Die Kegelschliffverbindung wird mit Laborzellstoff gesäubert, der Kegelschliff des Tropftrichters rasch eingefettet und der Tropftrichter wieder auf den Rundkolben aufgesetzt. Jede Schliffverbindung muß auf Dichtigkeit geprüft werden.

Durch einen mit Ventil versehenen Gummiball wird über der Salzsäure-Lösung in dem Tropftrichter Überdruck erzeugt. Der Hahn des Tropftrichters wird geöffnet, damit die Salzsäure-Lösung in den Rundkolben fließen kann. Der Druck wird weiter ausreichend über der Säurelösung aufrechterhalten, um die Lösung in den Kolben zu drücken. Wenn erforderlich, kann hierfür der Hahn kurzzeitig geschlossen werden, um Druck in dem Trichter zu erzeugen. Um zu vermeiden, daß Schwefeldioxid in den Tropftrichter entweicht, wird der Hahn geschlossen, bevor die letzten 2 ml bis 3 ml aus dem Tropftrichter ausgeflossen sind.

Die Heizhaube wird so eingestellt, daß 80 Tropfen/min bis 90 Tropfen/min des Kondensats von dem Kühler in den Kol-

ben zurückgelangen. Der Kolbeninhalt wird 105 min am Sieden gehalten und dann das Gefäß (Bild 1; Nummer 7) entfernt.

6.5 Bestimmung und Berechnung

6.5.1 Titration

Der Inhalt des Gefäßes (Bild 1; Nummer 7) wird sofort mit Natriumhydroxid-Standardlösung (4.2) bis zum Umschlag nach gelb titriert, der länger als 20 s bestehenbleibt. Der Massenanteil an Sulfit, w, ausgedrückt in Milligramm je Kilogramm, wird mit Gleichung (1) berechnet:

$$w = \frac{32{,}03 \cdot V \cdot 1\,000 \cdot N}{m} \qquad (1)$$

Dabei ist:

32,03 das Milliäquivalentgewicht von SO_2, in Gramm je Mol;

N die Stoffmengenkonzentration der Natriumhydroxid-Standardlösung, in Mol je Liter;

V das Volumen an Natriumhydroxid-Standardlösung (4.2), das zum Erreichen des Endpunkts erforderlich war, in Milliliter;

1 000 der Faktor zum Umrechnen von Milliäquivalenten in Mikroäquivalente;

m die in den Rundkolben (Bild 1; Nummer 3) eingeführte Probeneinwaage, in Gramm.

6.5.2 Blindwertbestimmung

Der Blindwert der Chemikalien wird durch Titration bestimmt, und die Ergebnisse werden, wenn notwendig, entsprechend korrigiert.

7 Präzision

7.1 Allgemeines

Einzelheiten über den Ringversuch nach ISO 5725 : 1986 (siehe [4]) zur Ermittlung der Präzisionsdaten sind im Anhang B gegeben. Die Werte, die im Ringversuch ermittelt wurden, sind nicht unbedingt auf andere Konzentrationsbereiche der zu bestimmenden Substanz und andere Matrices anwendbar, als in Anhang B angegeben.

7.2 Wiederholpräzision

Die absolute Differenz zwischen zwei einzelnen Ergebnissen, die ein einzelner Bearbeiter an identischem Untersuchungsmaterial mit denselben Geräten innerhalb der kürzesten möglichen Zeitspanne erhält, wird die Wiederholgrenze r nicht häufiger als in 5 % der Fälle überschreiten.

Die Werte sind:

Maismehl	$\bar{x}$ = 9,2 mg/kg	r = 3,7 mg/kg
Fruchtsaft	$\bar{x}$ = 8,1 mg/l	r = 3,8 mg/l
Meeresfrüchte	$\bar{x}$ = 10,4 mg/kg	r = 4,1 mg/kg

7.3 Vergleichpräzision

Die absolute Differenz zwischen zwei einzelnen Ergebnissen, die von zwei Laboratorien für identisches Untersuchungsmaterial berichtet werden, wird die Vergleichgrenze R nicht häufiger als in 5 % der Fälle überschreiten.

Die Werte sind:

Maismehl	$\bar{x}$ = 9,2 mg/kg	R = 4,0 mg/kg
Fruchtsaft	$\bar{x}$ = 8,1 mg/l	R = 4,5 mg/l
Meeresfrüchte	$\bar{x}$ = 10,4 mg/kg	R = 7,8 mg/kg

8 Untersuchungsbericht

Der Untersuchungsbericht muß die folgenden Angaben enthalten:

- alle notwendigen Informationen zur Identifizierung der Probe;
- eine Verweisung auf diese Europäische Norm oder das verwendete Verfahren;
- die Ergebnisse und Einheiten, in denen sie angegeben werden;
- Datum und Art der Probenahme (wenn bekannt);
- Datum des Probenerhaltes;
- Untersuchungsdatum;
- alle nicht üblichen Einzelheiten, die während der Untersuchung festgestellt wurden;
- alle Arbeitsschritte, die nicht in dieser Europäischen Norm festgelegt sind, wahlweise vorgenommen wurden und das Ergebnis möglicherweise beeinflußt haben.

Anhang A (informativ)

Literaturhinweise

[1] Association of Official Analytical Chemists (AOAC): Official Methods of Analysis (1995), 16. Ausg., Verfahren 990.28, 47.3.43

[2] Hillary et al.: J. Assoc. Off. Chem., Vol 72, Nr 3, 1989, S. 470

[3] ISO 5522 : 1981 Fruits, vegetables and derived products — Determination of total sulfur dioxide content

[4] ISO 5725 : 1986 Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests

Anhang B (informativ)

Präzisionsdaten

Nach ISO 5725 : 1986 [4] ergab der Ringversuch die folgenden Präzisionsdaten. Der Versuch wurde unter Leitung der Food and Drug Administration (FDA) durchgeführt.

Tabelle B.1

Probe	Maismehl	Fruchtsaft	Meeresfrüchte
Jahr des Ringversuchs	1986	1986	1986
Anzahl der Laboratorien	21	21	21
Anzahl der Proben	9	9	9
Anzahl der nach Eliminieren der Ausreißer verbliebenen Laboratorien	18	21	20
Anzahl der Ausreißer (Laboratorien)	3	0	1
Anzahl der anerkannten Ergebnisse	39	42	41
Mittelwert ($\bar{x}$)	9,17 mg/kg	8,05 mg/l	10,41 mg/kg
Wiederhol-Standardabweichung (s_r)	1,33 mg/kg	1,36 mg/l	1,47 mg/kg
Relative Wiederhol-Standardabweichung (RSD_r)	14,49 %	16,90 %	14,13 mg/kg
Wiederholgrenze (r)	3,72 mg/kg	3,81 mg/l	4,12 mg/kg
Vergleich-Standardabweichung (s_R)	1,42 mg/kg	1,62 mg/l	2,77 mg/kg
Relative Vergleich-Standardabweichung (RSD_R)	15,50 %	20,14 %	26,62 %
Vergleichgrenze (R)	3,98 mg/kg	4,54 mg/l	7,76 mg/kg

Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG

L

Untersuchung von Lebensmitteln

00.00

Bestimmung von **Sulfit** in **Lebensmitteln**
Teil 2: Enzymatisches Verfahren
(Übernahme der gleichnamigen Norm DIN EN 1988 Teil 2, Ausgabe Mai 1988)

46/2

Nationales Vorwort

Diese Europäische Norm ist vom Technischen Komitee CEN/TC 275 „Lebensmittelanalytik – Horizontale Verfahren" (Sekretariat: DIN) unter intensiver deutscher Mitarbeit erarbeitet worden.

Das zuständige deutsche Normungsgremium ist der Arbeitskreis „Sulfite" des Normenausschusses „Lebensmittel und landwirtschaftliche Produkte" (NAL) im DIN Deutsches Institut für Normung e.V.

Die für die Ermittlung der Präzisionsdaten verwendete ISO 5725 : 1986 ist durch ISO 5725-1 : 1994 ersetzt und wird außerdem durch ISO 5725-2 bis ISO 5725-6 ersetzt, die künftig erscheinen werden.

Deutsche Fassung

Einleitung

Sulfit kann als Konservierungsstoff in Lebensmitteln verwendet werden. Um mögliche negative Auswirkungen auf die Gesundheit zu minimieren, haben viele Länder die Verwendung von Sulfit in Lebensmitteln geregelt. Das führte zur Entwicklung unterschiedlicher Analysenverfahren zum Nachweis und zur Bestimmung von Sulfit in einer Vielzahl von Lebensmitteln.

1 Anwendungsbereich

Diese Europäische Norm legt ein enzymatisches Verfahren zur Bestimmung von Sulfit (ausgedrückt als Schwefeldioxid) in Lebensmitteln fest. Andere schwefelhaltige Substanzen wie Sulfate, Sulfide oder Thiosulfate stören die Bestimmung nicht. Carbonylsulfitkomplexe reagieren wie freies Sulfit. Isothiocyanate, die z. B. in Senf vorkommen, stören die Bestimmung von Sulfit. Das Verfahren ist nicht anwendbar auf Kohl, getrockneten Knoblauch, getrocknete Zwiebeln, Ingwer, Lauch und Sojaproteine[1]. Es hat sich gezeigt, daß die Untersuchung von isolierten Sojaproteinen zu falsch positiven Ergebnissen führt.

Spezielle Lebensmittel, für die Europäische Normen zur Bestimmung von Sulfit gelten, sind vom Anwendungsbereich dieser horizontalen Europäischen Norm ausgenommen.

2 Normative Verweisungen

Diese Norm enthält durch datierte oder undatierte Verweisungen Festlegungen aus anderen Publikationen. Diese normativen Verweisungen sind an den jeweiligen Stellen im Text zitiert, und die Publikationen sind nachstehend aufgeführt. Bei datierten Verweisungen gehören spätere Änderungen oder Überarbeitungen dieser Publikationen nur zu dieser Norm, falls sie durch Änderung oder Überarbeitung eingearbeitet sind. Bei undatierten Verweisungen gilt die letzte Ausgabe der in Bezug genommenen Publikation.

EN ISO 3696
Wasser für analytische Zwecke – Anforderungen und Prüfungen (ISO 3696 : 1987)

[1] Es hat sich gezeigt, daß die Untersuchung von isolierten Sojaproteinen zu falsch positiven Ergebnissen im Bereich von 20 mg/kg bis 30 mg/kg, ausgedrückt als Schwefeldioxid, führt. Wenn Lebensmittel, die isolierte Sojaproteine enthalten, untersucht werden, kann daher eine proportionale Erhöhung der Ergebnisse beobachtet werden, die in Betracht gezogen wird.

3 Prinzip

Sulfit wird zu Sulfat oxidiert. Die Reaktion wird durch Sulfitoxidase katalysiert, gleichzeitig wird Wasserstoffperoxid gebildet.

$$SO_3^{2-} + O_2 + H_2O \xrightarrow{\text{Sulfitoxidase}} SO_4^{2-} + H_2O_2$$

Das gebildete Wasserstoffperoxid reagiert mit NADH zu NAD^+. Die Reaktion wird durch NADH-Peroxidase katalysiert.

$$H_2O_2 + NADH + H^+ \xrightarrow{\text{NADH-Peroxidase}} 2\,H_2O + NAD^+$$

Die in NAD^+ umgesetzte Menge an NADH, spektralphotometrisch an der Extinktionsabnahme zu messen, ist der Sulfitmenge äquivalent, siehe [1] bis [6].

4 Chemikalien

Wenn nicht anders angegeben, werden bei der Analyse ausschließlich analysenreine Chemikalien und nur Wasser verwendet, das mindestens der Qualität 3 nach EN ISO 3696 entspricht.

4.1 Ammoniumsulfat

4.2 Ethylendiamin-N,N,N',N'-Tetraessigsäure (EDTA)

4.3 Natriumhydrogencarbonat

4.4 Natriumsulfit

4.5 Ammoniumsulfat-Lösung, Stoffmengenkonzentration $c\,[(NH_4)_2SO_4] = 2\ mol/l$

4.6 Natriumhydroxid-Lösung, $c(NaOH) = 0,1\ mol/l$

4.7 Natriumhydroxid-Lösung, $c(NaOH) = 2\ mol/l$

4.8 Triethanolamin-Pufferlösung[2], $c(C_6H_{15}NO_3) = 0,6\ mol/l$, pH 8,0

5,57 g Triethanolamin-Hydrochlorid werden in einem Becherglas in etwa 40 ml Wasser gelöst. Der pH-Wert wird mit Natriumhydroxid-Lösung (4.6) auf pH 8,0 eingestellt. Die Lösung wird in einen 50-ml-Meßkolben überführt und mit Wasser bis zur Marke aufgefüllt. Die Pufferlösung ist bei + 4 °C aufbewahrt ein Jahr haltbar.

4.9 NADH-Lösung[2] (reduziertes Nicotinamid-Adenin-Dinucleotid), $c(NADH) = 7 \cdot 10^{-3}\ mol/l$

25 mg β-Nicotinamid-Adenin-Dinucleotid, Dinatriumsalz (β-NADH-Na_2) und 50 mg Natriumhydrogencarbonat (4.3) werden in 5,0 ml Wasser gelöst. Die Lösung ist bei + 4 °C aufbewahrt mindestens vier Wochen haltbar.

4.10 NADH-Peroxidase-Suspension[2] (EC 1.11.1.1) [7]

Enzymsuspension mit 10 Enzymeinheiten je Milliliter (U/ml)[3] in Ammoniumsulfat-Lösung (4.5) mit einem pH-Wert von etwa 7. Die Suspension ist bei + 4 °C aufbewahrt ein Jahr haltbar.

4.11 Sulfitoxidase-Suspension[2] (EC 1.8.3.1) [7]

Enzymsuspension mit 2,5 Enzymeinheiten je Milliliter in Ammoniumsulfat-Lösung (4.5) mit einem pH-Wert von etwa 7. Die Suspension ist bei + 4 °C aufbewahrt ein Jahr haltbar.

4.12 Vergleichslösung

0,6 g Natriumsulfit (4.4) (entspricht etwa 300 mg Schwefeldioxid) werden auf 0,1 mg eingewogen und mit 37 mg EDTA (4.2) in Wasser gelöst. Die Lösung wird quantitativ in einen 1 000-ml-Meßkolben überführt, mit Wasser bis zur Marke aufgefüllt und gemischt. 100 µl dieser Lösung dienen als Vergleichsprobe, wobei der Sulfitgehalt innerhalb von 30 min bestimmt wird. Der Variationskoeffizient für die Werte dieser Vergleichsproben darf 0,06 nicht überschreiten.

4.13 Polyvinylpyrrolidon, vernetzt (Polyvinylpolypyrrolidon)

4.14 Ascorbatoxidase, z. B. als Ascorbatoxidase-Spatel, mit definierter Aktivität

4.15 Bentonit

5 Geräte

Übliche Laborgeräte und insbesondere die folgenden:

5.1 Wasserbad, auf (60 ± 2) °C regelbar

5.2 Homogenisierer

5.3 Enzymtest-Meßpipetten, 10 µl, 20 µl, 50 µl und 100 µl. Bei der Verwendung von mechanischen Pipetten mit austauschbaren Pipettenspitzen/-kapillaren ist unbedingt darauf zu achten, daß die Pipetten gut kalibriert sind.

5.4 pH-Meter

5.5 Spektralphotometer, für Messungen bei 340 nm geeignet.

5.6 Quarzküvetten mit einer Schichtdicke von 1 cm, auch Einwegküvetten können verwendet werden.

5.7 Zentrifuge, geeignet für eine Zentrifugalbeschleunigung von 2 000 g mit Mischergefäßen oder Zentrifugengläsern geeigneten Volumens.

6 Durchführung

6.1 Herstellung der Probenmeßlösungen

6.1.1 Allgemeines

Hohe Gehalte an Ascorbinsäure von mehr als 100 mg/kg oder 100 mg/l müssen entfernt werden (siehe 6.1.2.3).

Wenn der Sulfitgehalt in der Probenmeßlösung 0,3 g/l überschreitet, muß die Probenmeßlösung vor der Bestimmung verdünnt werden, oder es muß ein kleineres Volumen der Probenmeßlösung zur Bestimmung eingesetzt werden.

6.1.2 Flüssige Lebensmittel

6.1.2.1 Weißweine, Branntweine und Bier

Weißweine und Branntweine werden direkt eingesetzt. Bier sollte zum Entfernen des Kohlenstoffdioxids gefiltert wer-

[2] Es befinden sich Fertigreagenzien im Handel, bei deren Verwendung die Arbeitsvorschrift der Präparate-Hersteller berücksichtigt werden sollte.

[3] Diese Einheit (oft auch internationale Einheit oder Standardeinheit genannt) wird definiert als die Menge Enzym, die die Umsetzung von 1 µmol Substrat je Minute unter Standardbedingungen katalysiert.

den. Manchmal ist eine Entfärbung des Bieres notwendig. Hierfür werden 10 ml des gefilterten Bieres in einem 50-ml-Becherglas mit nicht mehr als etwa 0,7 g Bentonit (4.15) versetzt, 2 min auf dem Magnetrührer gerührt und danach in ein weiteres 50-ml-Becherglas filtriert.

Zur enzymatischen Bestimmung (6.2) werden 100 µl bis 200 µl Wein bzw. 500 µl Branntwein oder Bier eingesetzt.

6.1.2.2 Rotwein

25 ml Rotwein werden in einem Becherglas mit Natriumhydroxid-Lösung (4.7) auf einen pH-Wert von 7,5 bis 8,0 eingestellt, in einen 50-ml-Meßkolben überführt und mit Wasser bis zur Marke aufgefüllt. Bei der Analyse von Rotweinen ist häufig eine Entfärbung notwendig. Diese kann, wie in 6.1.2.3 für Fruchtsäfte beschrieben, durchgeführt werden.

6.1.2.3 Fruchtsäfte

Trübe Fruchtsäfte werden bei etwa 2 000 *g* zentrifugiert. 5 ml Saft werden in ein Becherglas gegeben, und der pH-Wert wird mit Natriumhydroxid-Lösung (4.7) auf 5 bis 6 eingestellt. Ascorbinsäure wird entfernt, indem etwa 40 Einheiten Ascorbatoxidase (4.14) in Lösung zugegeben werden. Die Probe wird 10 min stehengelassen. Die Entfernung von Ascorbinsäure kann auch durch 3minütiges Rühren mit einem Ascorbatoxidase-Spatel (4.14) erfolgen. Der pH-Wert wird mit Natriumhydroxid-Lösung (4.7) auf 7,5 bis 8,0 eingestellt. Bei gefärbten Fruchtsäften wird etwa 0,25 g Polyvinylpolypyrrolidon (4.13) zugesetzt und 1 min gerührt. Diese Mischung wird in einen 10-ml-Meßkolben überführt, mit Wasser bis zur Marke aufgefüllt und filtriert. Zur enzymatischen Bestimmung (6.2) werden 200 µl dieser Lösung eingesetzt.

6.1.3 Feste Lebensmittel

Die Probe wird gründlich homogenisiert und mit Wasser 5 min bei 60 °C unter gelegentlichem Schütteln extrahiert. Vor der Untersuchung wird die Probe auf Raumtemperatur abgekühlt. Die Probenmengen sind je nach dem zu erwartenden Sulfitgehalt zu wählen. Bei Kartoffelchips z. B. werden 5,0 g homogenisierte Probe in einen 50-ml-Meßkolben gegeben und 40 ml Wasser zugefügt. Der Meßkolben wird verschlossen und die Probe in einem Wasserbad (5.1) 5 min bei 60 °C unter gelegentlichem Schütteln extrahiert. Anschließend wird der Meßkolben 15 min stehengelassen oder im Wasserbad bei 20 °C auf Raumtemperatur abgekühlt und mit Wasser bis zur Marke aufgefüllt (V_3 = 50 ml). Falls notwendig, wird die Lösung zentrifugiert.

Für einige andere Lebensmittel werden die folgenden Probenmengen vorgeschlagen:

Trockenfrüchte	1,0 g Probe/50 ml Wasser
Konfitüren	5,0 g Probe/50 ml Wasser
Gewürze	0,1 g Probe/50 ml Wasser
Getrocknete Kartoffelprodukte	2,0 g Probe/50 ml Wasser

Zur enzymatischen Bestimmung werden jeweils 100 µl bis 500 µl eingesetzt (siehe 6.2).

6.2 Bestimmung

Die Bestimmung wird bei 20 °C bis 25 °C nach Tabelle 1 in einer Quarzküvette (5.6) mit einem Probenvolumen von üblicherweise 100 µl durchgeführt. Wenn sich das Probenvolumen von 100 µl unterscheidet, so ist die Menge des zuzusetzenden Wassers so zu variieren, daß das Endvolumen von Wasser und Probenlösung 2,00 ml beträgt.

Falls die Reaktion nicht zum Stillstand gekommen ist, werden die Extinktionen so lange im 2-min-Abstand überprüft, bis die Extinktionsänderung konstant ist. Bei konstanter Extinktionsabnahme muß die gemessene Extinktion A_2 durch Extrapolation der Schleichreaktion auf den Zeitpunkt der Zugabe der Sulfitoxidase-Suspension korrigiert werden.

7 Auswertung

Die Probenextinktionen müssen wie folgt berechnet werden:

$$A_{\text{Probe}} = A_{1\ \text{Probe}} - A_{2\ \text{Probe}} \quad (1)$$

$$A_{\text{Leerwert}} = A_{1\ \text{Leerwert}} - A_{2\ \text{Leerwert}} \quad (2)$$

$$A = A_{\text{Probe}} - A_{\text{Leerwert}} \quad (3)$$

Der Extinktionswert A_{Probe} muß 0,05 überschreiten, anderenfalls muß ein größeres Volumen an Probenmeßlösung eingesetzt werden.

Der Sulfitgehalt (Schwefeldioxidgehalt) als Massenkonzentration, ϱ, in Gramm je Liter, oder als Massenanteil, w, in Gramm je Kilogramm, wird mit Gleichungen (4) bzw. (5) berechnet:

$$\varrho = \frac{V_1 \cdot M \cdot A \cdot F}{\varepsilon \cdot d \cdot V_2 \cdot 1\,000} \quad (4)$$

Tabelle 1

In Küvetten pipettieren	Probenküvette	Leerwertküvette
Triethanolamin-Pufferlösung (4.8)	1,00 ml	1,00 ml
NADH-Lösung (4.9)	0,10 ml	0,10 ml
NADH-Peroxidase-Suspension (4.10)	0,01 ml	0,01 ml
Probenmeßlösung	0,10 ml	—
Wasser	1,90 ml	2,00 ml
Nach Zugabe aller Reagenzien wird vorsichtig gemischt. Die Extinktionen A_1 der Probenlösung und des Leerwertes werden nach 5 min gegen Luft gemessen. Anschließend wird die Reaktion gestartet durch Zugabe von:		
Sulfitoxidase-Suspension (4.11)	0,05 ml	0,05 ml
Der Inhalt wird gemischt, und die Extinktion A_2 wird nach etwa 30 min abgelesen. Nach 5 min ist eine erneute Extinktionsmessung durchzuführen, um zu prüfen, daß keine weitere Extinktionsänderung stattgefunden hat.		

$$w = \frac{V_1 \cdot M \cdot A \cdot V_3}{\varepsilon \cdot d \cdot V_2 \cdot m \cdot 1\,000} \quad (5)$$

Dabei ist:

V_1 das Volumen der Lösungen in den Küvetten, in Millilitern (hier: 3,16 ml);

V_2 das zur enzymatischen Bestimmung eingesetzte Volumen, in Millilitern (hier: 0,1 ml bis 0,5 ml);

V_3 das Gesamtvolumen der Probenmeßlösungen bei festen Proben, in Millilitern (hier: 50 ml);

M die molare Masse von Schwefeldioxid, in Gramm je Mol (64,1 g/mol);

d die Schichtdicke der Küvette, in Zentimetern (hier: 1 cm);

ε der Extinktionskoeffizient von NADH bei 340 nm ($6{,}3\ \mathrm{l \cdot mmol^{1-} \cdot cm^{1-}}$);

m die Probenmenge, in Gramm, bei festen Proben (6.1.3);

F der Verdünnungsfaktor, wenn bei der Probenvorbereitung eine Verdünnung erfolgte (siehe 6.1, 6.1.2.2 oder 6.1.2.3).

8 Präzision

8.1 Allgemeines

Einzelheiten über den Ringversuch zur Ermittlung der Präzisionsdaten nach ISO 5725 : 1986 [8] sind im Anhang B gegeben. Die Werte, die im Ringversuch ermittelt wurden, sind nicht unbedingt auf andere Konzentrationsbereiche der zu bestimmenden Substanz und andere Matrices anwendbar, als in Anhang B angegeben.

8.2 Wiederholpräzision

Die absolute Differenz zwischen zwei einzelnen Ergebnissen, die ein einzelner Bearbeiter an identischem Untersuchungsmaterial mit denselben Geräten innerhalb der kürzesten möglichen Zeitspanne erhält, wird die Wiederholgrenze r nicht häufiger als in 5 % der Fälle überschreiten.

Die Werte sind:

Wein	$\bar{x}$ = 75 mg/l	r = 8 mg/l
Getrocknete Äpfel	$\bar{x}$ = 800 mg/kg	r = 298 mg/kg
Getrocknete Äpfel	$\bar{x}$ = 960 mg/kg	r = 358 mg/kg
Zitronensaft	$\bar{x}$ = 270 mg/l	r = 37 mg/l
Sultaninen	$\bar{x}$ = 260 mg/kg	r = 45 mg/kg
Bier	$\bar{x}$ = 4,9 mg/l	r = 0,8 mg/l

8.3 Vergleichpräzision

Die absolute Differenz zwischen zwei einzelnen Ergebnissen, die von zwei Laboratorien für identisches Untersuchungsmaterial berichtet werden, wird die Vergleichgrenze R nicht häufiger als in 5 % der Fälle überschreiten.

Die Werte sind:

Wein	$\bar{x}$ = 75 mg/l	R = 16 mg/l
Getrocknete Äpfel	$\bar{x}$ = 800 mg/kg	R = 311 mg/kg
Getrocknete Äpfel	$\bar{x}$ = 960 mg/kg	R = 374 mg/kg
Zitronensaft	$\bar{x}$ = 270 mg/l	R = 79 mg/l
Sultaninen	$\bar{x}$ = 260 mg/kg	R = 129 mg/kg
Bier	$\bar{x}$ = 4,9 mg/l	R = 1,6 mg/l

9 Untersuchungsbericht

Im Untersuchungsbericht sind mindestens anzugeben:

- alle notwendigen Informationen zur Identifizierung der Probe;
- eine Verweisung auf diese Europäische Norm oder das verwendete Verfahren;
- die Ergebnisse und Einheiten, in denen sie angegeben werden;
- Datum und Art der Probenahme (wenn bekannt);
- Datum des Probenerhaltes;
- Untersuchungsdatum;
- alle nicht üblichen Einzelheiten, die während der Untersuchung festgestellt wurden;
- alle Arbeitsschritte, die nicht in dieser Europäischen Norm festgelegt sind, wahlweise vorgenommen wurden und das Ergebnis möglicherweise beeinflußt haben.

Anhang A (informativ)

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Anhang B (informativ)

Präzisionsdaten

Die Präzision des Verfahrens wurde aus den Ergebnissen mehrerer Ringversuche ermittelt, die speziell an Proben mit einem niedrigen Sulfitgehalt teilweise gemäß den Anforderungen von ISO 5725 : 1986 [8] durchgeführt wurden (siehe [1], [4] und [5]).

Die Ringversuche wurden mit Kartoffelflocken, Wein, Zitronensaft, getrockneten Äpfeln, Sultaninen und Bier durchgeführt, wobei die Proben einen Sulfitgehalt von zwischen 0 µg/kg und 960 µg/kg aufweisen. In dem vollständigen Ringversuch untersuchten elf Laboratorien zwölf Proben, und in dem ergänzenden Ringversuch untersuchten sechs Laboratorien acht Proben.

Die Ringversuchsergebnisse zeigen, daß das Verfahren zur Bestimmung von Sulfitgehalten unter 100 mg/kg als SO_2 gut geeignet ist. Bei der Bestimmung sehr geringer Sulfitgehalte ist die Probenart von großer Bedeutung. Bei der Untersuchung von festen Proben oder wenn Sulfit an Partikel gebunden ist (z. B. in Säften), muß mit einem hohen Variationskoeffizienten gerechnet werden, insbesondere, wenn das Untersuchungspersonal wenig Erfahrung mit der enzymatischen Analytik hat. Sulfitkonzentrationen zwischen 1 mg/l und 10 mg/l können in Wein mit guter Zuverlässigkeit bestimmt werden.

Die Nachweisgrenze des Verfahrens, in Extinktionseinheiten ausgedrückt, beträgt 0,04. Bei einer Probenmenge von 1 ml beträgt die Nachweisgrenze 1,2 mg SO_2/kg, berechnet als der Mittelwert einer repräsentativen Anzahl von Blindwerten ($n > 20$) plus der dreifache Variationskoeffizient des Mittelwertes (entsprechend den Empfehlungen der Europäischen Kommission).

Nach ISO 5725 : 1986 ergab der Ringversuch die in Tabelle B.1 (Doppelbestimmungen/Doppelblindwerte) und Tabelle B.2 aufgelisteten Präzisionsdaten (siehe [9]). Der Versuch wurde unter Leitung der „National Food Administration" in Schweden durchgeführt.

Tabelle B.1: Ringversuchsdaten zu Wein, getrockneten Äpfeln und Zitronensaft

Probe	Wein	getrocknete Äpfel	getrocknete Äpfel	Zitronensaft
Jahr des Ringversuchs	1991	1990	1990	1990
Anzahl der Laboratorien	6	11	11	11
Anzahl der nach Eliminieren der Ausreißer verbliebenen Laboratorien	6	10	10	10
Anzahl der Ausreißer (Laboratorien)	0	1	1	1
Anzahl der Proben	1	1	1	2
Anzahl der anerkannten Ergebnisse	6	7	7	10
Mittelwert ($\bar{x}$) mg/kg oder mg/l	75	800	960	270
Wiederhol-Standardabweichung (s_r) mg/kg oder mg/l	3	106	128	13
Relative Wiederhol-Standardabweichung (RSD_r) %	4	13	13	5
Wiederholgrenze (r) mg/kg oder mg/l	8	298	358	37
Vergleich-Standardabweichung (s_R) mg/kg oder mg/l	6	111	133	28
Relative Vergleich-Standardabweichung (RSD_R) %	8	14	14	10
Vergleichgrenze (R) mg/kg oder mg/l	16	311	374	79
ANMERKUNG: Die Daten sind für Wein und Zitronensaft in mg/l und für getrocknete Äpfel in mg/kg angegeben.				

Tabelle B.2: Ringversuchsdaten zu Kartoffelflocken

Probe	Kartoffelflocken	Kartoffelflocken
Jahr des Ringversuchs	1990	1990
Anzahl der Laboratorien	11	11
Anzahl der nach Eliminieren der Ausreißer verbliebenen Laboratorien	10	10
Anzahl der Ausreißer (Laboratorien)	1	1
Anzahl der Proben	1	1
Anzahl der anerkannten Ergebnisse	6	7
Mittelwert ($\bar{x}$) mg/kg	28,3	110
Vergleich-Standardabweichung (s_R) mg/kg	13	15
Relative Vergleich-Standardabweichung (RSD_R) %	45	13
Vergleichgrenze (R) mg/kg	36	42

Nach ISO 5725 : 1986 ergab der Ringversuch die in Tabelle B.3 aufgelisteten Präzisionsdaten. Der Versuch wurde unter Leitung der Abteilung Lebensmittelchemie des Max-von-Pettenkofer-Instituts des Bundesgesundheitsamtes, Berlin, Bundesrepublik Deutschland, durchgeführt (siehe [4], [5]).

Tabelle B.3: Ringversuchsdaten zu Sultaninen und Bier

Probe	Sultaninen	Bier
Jahr des Ringversuchs	1986	1986
Anzahl der Laboratorien	14	16
Anzahl der nach Eliminieren der Ausreißer verbliebenen Laboratorien	13	14
Anzahl der Ausreißer (Laboratorien)	1	2
Anzahl der Proben	1	1
Anzahl der anerkannten Ergebnisse	74	70
Mittelwert ($\bar{x}$)	260 mg/kg	4,9 mg/l
Wiederhol-Standardabweichung (s_r)	16 mg/kg	0,3 mg/l
Relative Wiederhol-Standardabweichung (RSD_r)	6 %	5,8 %
Wiederholgrenze (r)	45 mg/kg	0,8 mg/l
Vergleich-Standardabweichung (s_R)	46 mg/kg	0,6 mg/l
Relative Vergleich-Standardabweichung (RSD_R)	18 %	11,6 %
Vergleichgrenze (R)	129 mg/kg	1,6 mg/l

B.5. SO_2 in the Production of Grape Juices

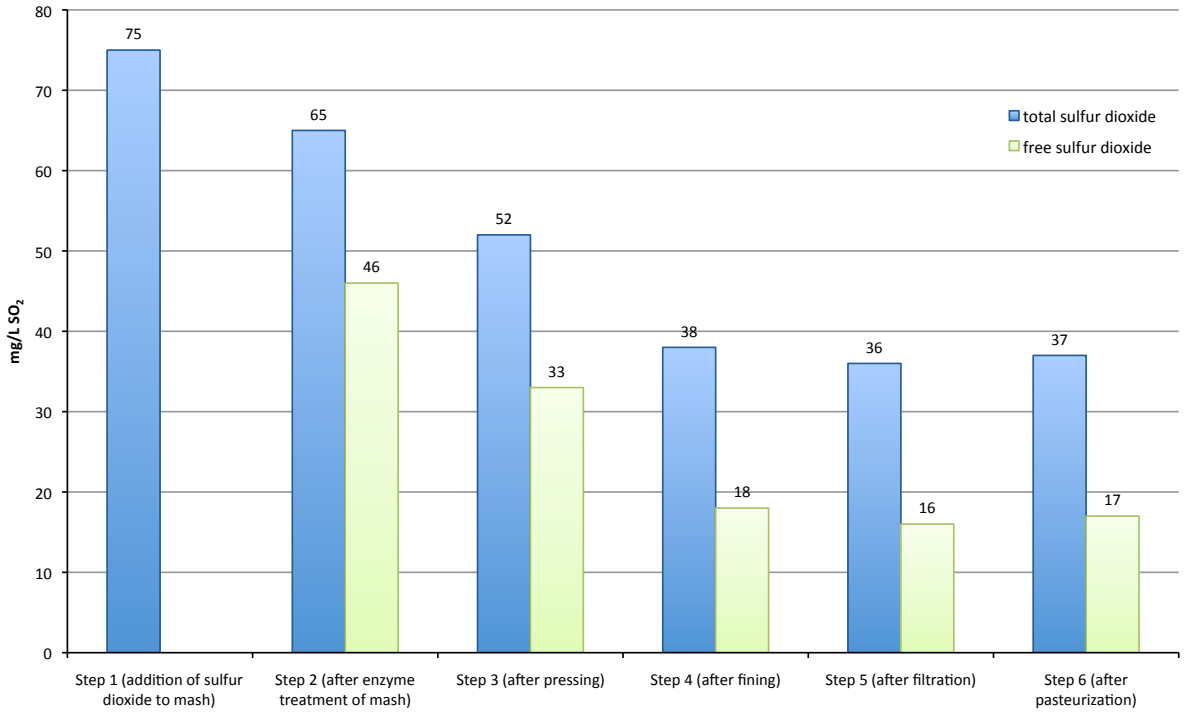

Figure B.1.: Decrease in free and total amounts of sulfur dioxide during the production of grape juice (Blauer Portugieser) with an initial addition of 158 mg/L SO_2.

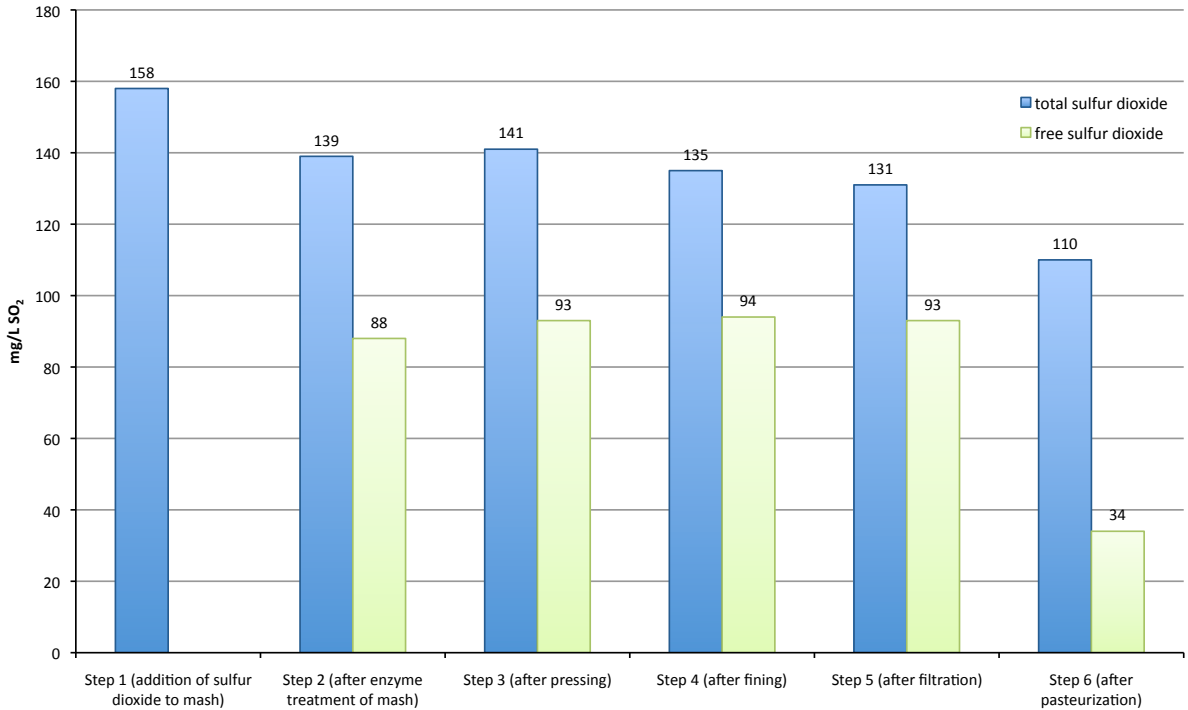

Figure B.2.: Decrease in free and total amounts of sulfur dioxide during the production of grape juice (Blauer Portugieser) with an initial addition of 75 mg/L SO_2.

B.6. Detailed Description of the Smoothies

Chiquita (250 mL)

Erdbeer-Banane:	Apfelsaft, 28% pürierte Erdbeeren, 23% pür. Banane, Orangensaft aus purer Frucht und Fruchtsaft, keine Konzentrate, keine Konservierungsstoffe, keine Zusatzstoffe, kein hinzugefügter Zucker
Himbeer-Granatapfel:	Banane, fr. gepr. Trauben, 17,5% Himbeeren, fr.- gepr. Orange, 7% fr.-gepr. Granatapfel , 1 Tr. Zitrone aus purer Frucht und frischem Fruchtsaft, keine Konservierungsstoffe, keine Zusatzstoffe, kein hinzugefügter Zucker
Kokos-Mango:	Apfelsaft, pür. Banane, weißer Traubensaft, 15% Kokosnußmilch, 4% pür. Mango aus purer Frucht und Fruchtsaft, keine Konzentrate, keine Konservierungsstoffe, keine Zusatzstoffe, kein hinzugefügter Zucker

Granini (2 x 200mL)

Himbeere-Erdbeere-Banane:	35% Apfelsaft aus Konzentrat, 15% Apfelmark, 15% Bananenmark, 14% Himbeermark, 13% Erdbeermark, 4% Apfelstücke, 4% Aroniasaft aus Konzentrat 100% Frucht

Innocent (250 mL)

Brombeere- Himbeere-Boysenben	36% gepr. Äpfel, 18 % pür. Banane, 15% gem. Himbeeren, 10% gem. Boysenbeeren, 8% gem. Brombeeren, 7% gepr. weiße haltbarWeintrauben, 4% Orange, 2% Zitronensaft, kleingemixtes Obst, pure Säfte, keine Konzentrate

Knorr vie (3x 100 mL)

Apfel-Karotte-Erdbeere:	31% Apfelpüree, 29% konz. Karottensaft, 25% konz. Apfelsaft, Orangenfruchtfleisch, 4% Erdbeerpüree, 3% konz. Erdbeersaft, 2,5% konz. Acerolapüree, pflanzl. Ballaststoff, Apfelpektin, konz. Zitronensaft
Traube-Banane-Rhabarber:	37% konz. Traubensaft, 25% konz. Karottensaft, 11% Bananenpüree, 11% Apfelpüree, 5% konz. Rhabarbersaft, Orangenfrucht- fleisch, konz. Acerolapüree, konz. Schwarzer Johannisbeersaft, pflanzl. Ballaststoff, Apfelpektin, konz. Limettensaft

Mövenpick (250 mL)

Blutorange-Apfel	43% Blutorangensaft, 37% Apfelmark, 10% Bananenmark, 6% Apfelsaft, 3% Traubensaft, 1% Aroniasaft aus Fruchtmark bzw. Fruchtkonzentrat, ohne Zusatz von Aromen, ohne Zuckerzusatz, ohne Konservierungsmittel, ohne Farbstoffe

Pro-X (3x100mL)

Erdbeere-Apfel-Karotte	Apfelmark und Apfelsaftkonzentrat (aus 110g Apfelmark und saft), Karottensaftkonzentrat (aus 57 g Karottensaft), Erdbeermark und Erdbeer-saftkonzentrat (aus 16 g Erdbeermark und -saft), 2 g Acerolamark, 0,6 g Orangenzellen, und Zitronensaftkonzentrat (aus 0,4 g Zitronensaft), Stabilisator Pektin, Vitamin C Multifrucht- und Gemüsedrink aus Obst- und Gemüsesaft/-mark (konzentraten) ohne Zuckerzusatz, ohne Konservierungsstoffe

rio d'oro (4 x 100ml) Aldi

Erdbeere-Apfel-Karotte	Apfelmark und Apfelsaftkonzentrat (aus 110g Apfelmark und saft), Karottensaftkonzentrat (aus 57 g Karottensaft), Erdbeermark und Erdbeer-saftkonzentrat (aus 16 g Erdbeermark und -saft), 2 g Acerolamark, 0,6 g Orangenzellen, und Zitronensaftkonzentrat (aus 0,4 g Zitronensaft), Stabilisator Pektin, Vitamin C Multifrucht- und Gemüsedrink aus Obst- und Gemüsesaft/-mark (konzentraten) ohne Zuckerzusatz, ohne Konservierungsstoffe

Schwartau **(Pur Pur)** (250 mL)

Erdbeere-Banane	50% Früchte (25% Erdbeeren, 22% Bananen, Äpfel), 50% Fruchtsaft aus Fruchtsaftkonzentrat (Apfel-, 6% Erdbeer-, Zitronen-, Aronia-) ohne Zuckerzusatz, ohne Zusatz von Aroma- und Konservierungsstoffen

Schwartau **(Fruit 2day)** (2 x 200mL)

Erdbeer-Orange	17% heller Traubensaft, 15% Orangensaft mit Zellen, 13% Apfelsaft, 3% Aronaiasaft, 2% Acerolasaft, 20% Erdbeerpüree, 11% Bananenpüree, 9% Apfelpüree, 5% Ananasstückchen, 5% Ananaspüree, natürliches Aroma Frucht, Fruchtpüree, Fruchtstücke und Fruchtsaft (Säfte aus Konzentrat), ohne Zuckerzusatz

True fruits (250 mL)

purple (versch. Waldbeeren)	36% gepr. Apfel, 17% pür. Banane, 16% gepr. Orange, 10% Himbeeren, 8% gem. Heidelbeeren, 5% gem. Brombeeren, 5% gem. rote Johannisbeeren, 3% gem. schwarze Johannisbeeren 0% Zuckerzusatz, 0% Konservierungsstoffe, 0% Zusatzstoffe, 0% Konzentrate

Valensina (250 mL)

Erdbeere-Banane-Traube	Orangensaft, 23% Erdbeermark, 22% Bananenmark, 12% Traubensaft, kein Konzentrat

VIVA VITAL (200mL) PLUS

Banane-Kirsch	Traubensaft aus Konzentrat, 31% Bananenmark, 30,8% Sauerkirschmark, Aroniasaft aus Konzentrat Mehrfruchtsaft mit Fruchtfleisch, teilweise aus Fruchtsaftkonz. Ohne Zusatz von Zucker, ohne Zusatz von Aromen lt. Gesetz
Himbeer-Pfirsich	29,7% Pfirsichmark, 17,5% Himbeermark, Traubensaft aus Konz., Bananenmark, Apfelsaft aus Konz., Holunderbeersaft aus Konz., 1,9% Orangenfruchtfleisch
Mango-Maracuja	Apfelsaft aus Konz. Bananenmark, 18,3% Mangomark, 8% Maracujasaft aus Konz. , Orangensaft aus Konz., 7,5% Orangenfruchtfleisch
Traube- schw. Johannisbeere	24,9% Traubensaft aus Konz., Apfelsaft aus Konz., 19,5% schw.Johannisbeermark, 16,8% Bananenmark, Apfelmark aus Apfelmarkkonz., Birnenmark aus Birnenmarkkonz., 4,2% Orangenfruchtfleisch, Acerolamark

Schwartau **(Fruit2day)** (4 x 100g) ***zum Löffeln***

Erdbeer-Apfel	77% Apfelpüree, 22% Erdbeerpüree, Aroniasaft aus Konzentrat, Acerolapulpe, natürliches Aroma

B.7. 35 Fruit Juices from Stores for Method Comparison

Juice	Producer	Note
Apple-grape juice (28)	Alete	3
Grape juice (31)	Alnatura	1,2
Apple juice (13)	Amecke	
Multivitamin juice (16)	Amecke	
Grape juice (2)	Amecke (glass bottle)	1
Grape juice (3)	Amecke (Tetrapack)	
Apple-grape juice (27)	babydream	3
Lemon concentrate (32)	Delique	
Grape juice, white (6)	Eckes-Granini	
Grape juice (1)	EUCO GmbH	
Apple juice (15)	Föno	
Grape juice (8)	Fruit du monde	
Lemon juice (33)	Hitchcock	1
Lime juice (34)	Hitchcock	1
Grape juice (5)	Lindauer	1
Peach nectar (19)	Lindauer	
Tomato juice (24)	Lindauer	1
Red currant juice (9)	Lindauer	
Sour cherry juice (10)	Lindauer	
Apple juice (14)	Lindauer	1
Multivitamin juice (17)	Lindauer	
Banana nectar (18)	Lindauer	
Pineapple juice (20)	Lindauer	
Passion fruit nectar (21)	Lindauer	
Grapefruit juice (22)	Lindauer	
Orange juice (23)	Lindauer	
Grape juice, white (26)	Lindavia	
Grape juice (4)	Merziger	1
Grape juice (29)	Niederrhein-Gold	1
Grape juice (30)	Rabenhorst	1,2
Grape juice (25)	REWE	1
Elderflower juice (7)	Van Nahmen	
Plum juice (11)	Van Nahmen	1
Aronia berry juice (12)	Van Nahmen	
Tomato juice (35)	Vitafit	

1: Not from Concentrate, 2: Organic, 3: For Babies

List of Figures

List of Tables

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