INVESTIGATION OF TRITERPENIC ACIDS IN ROSACEAE FRUITS

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The Rose Family

The rose is a rose
And was always a rose;
But the theory now goes
That the apple's a rose,
And the pear is, and so's
The plum, I suppose.
The dear only knows
What will next prove a rose.
You, of course, are a rose,
But were always a rose.

Robert Frost

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CHAPTER 1

1.1 INTRODUCTION AND SCOPE

Terpenes are a large and diverse class of secondary plant metabolites, comprising approximately 30,000 known compounds and occurring predominantly in plants but also in animals and fungi [1-3]. The biosynthesis of these hydrocarbon molecules follows the mevalonate pathway and is based on condensation reactions of isoprene units (Fig. 1) [1, 4]: Here, the carbon backbone of terpenes, in accordance with the so-called isoprene rule [5], is composed of a defined number of linked isoprene units ($(C_5H_8)_n$, n = number of units). Following this rule, terpenes are typically classified in hemiterpenes (n=1), monoterpenes (n=2), sesquiterpenes (n=3), diterpenes (n=4), and triterpenes (n=6) [6]. Biochemical modifications of terpenes, such as oxidation reactions and rearrangements of the carbon backbone, result in the formation of terpenoids, which are categorized in the same manner [1]. Triterpenoids (n=6) are biosynthetically derived from squalene, an acyclic C30 hydrocarbon [6], which is enzymatically converted by squalene epoxidase into 2,3-oxidosqualene [7] (Fig. 1).

Further enzymatic cyclization by (multifunctional) oxidosqualene cyclases (OSCs) and other enzymes, generates more than 100 triterpene scaffolds [4, 7-9], including the intermediate dammarenyl cation, which can be converted into the pentacyclic triterpenic acid (TA) precursors α-amyrin (AM), β-amyrin (BA), and lupeol (Fig. 1) [10]. The common structural skeletons of the ursane, oleanane, and lupane types derived from these precursors are, along with others, typically used to classify triterpenoids [11-12].

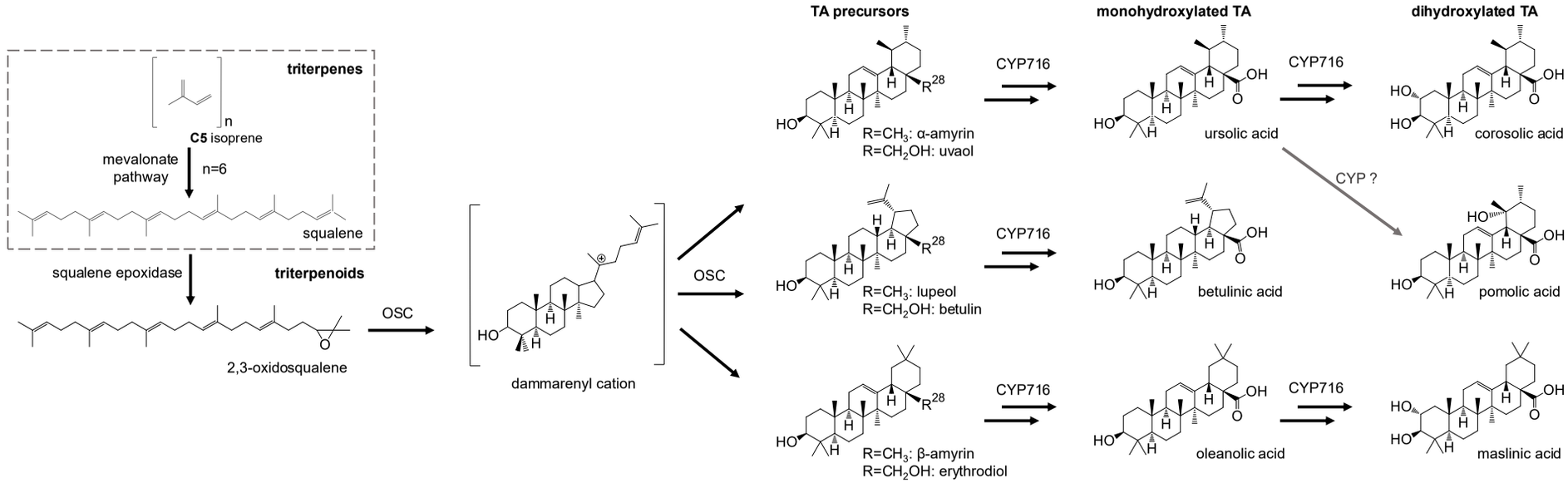

Fig. 1: Biosynthesis of TA: Six isoprene units condense to form squalene, which can be converted into 2,3-oxidosqualene by the squalene epoxidase. The dammarenyl cation is formed by cyclization via oxidosqualene cyclases, which is further cyclized to the TA precursors α-, β-amyrin, or lupeol. CYP P450 monooxygenases (e.g. CYP716) catalyze further modifications, including the addition of hydroxyl groups or formation of carboxylic acids, leading to monohydraxylated and dihydroxylated TA [7, 9, 12].

Cytochrome P450 and transferase-mediated modifications of TA precursors, such as the addition of hydroxy groups and oxidation of primary alcohols to carboxylic acids, result in the great structural diversity of TA [7, 9, 12]: More than 50 different P450 enzymes are known to be involved in the biosynthesis of pentacyclic triterpenoids in plants, particularly members of the CYP716, CYP51, CYP71, CYP72, CYP87, CYP88, and CYP93 families [12]. CYP716s catalyze a three-step oxidation, involving the consecutive formation of hydroxy groups, aldehydes, and carboxy groups on the amyrin or lupeol scaffolds at position C28, leading to the most prominent TA ursolic acid (ursane type), oleanolic acid (oleanane type) and betulinic acid (lupane type) (Fig. 1) [12-13]. The dihydroxylated TA derivatives corosolic acid and maslinic acid are derived from the respective monohydroxylated TA (Fig. 1). For some TA, such as pomolic acid, the CYP families involved in their biosynthesis remain to be identified.

TA are widely distributed in plants and occur as free acids or conjugates such as acylates or glycosides [2, 7, 12]. Although their biological roles remain poorly understood, they are discussed to contribute to plant physiology, e.g. by protecting against dehydration of fruits and herbivores [7, 12]. A comprehensive analysis of TA throughout plant species is still lacking; however, individual studies indicate a high TA content in plants of the *Rosaceae* family (order *Rosales*): Ursolic acid concentrations of up to 2 g/100 g dry weight have been described in apple peel (*Malus domestica* Borkh.) [14-15].

The *Rosaceae* family can be divided into three subfamilies: *Rosoideae*, *Amygdaloideae*, and *Dryadoideae* [16-17], which are in turn classified into more than 90 genera and over 4,000 species, based on fruit morphologies and/or nuclear phylogeny [16-19]. Numerous *Rosaceae* species are cultivated worldwide, and their fruits are an important edible food, particularly of the species from the genera *Malus*, *Prunus*, *Pyrus*, *Fragaria*, *Rubus*, and *Cydonia* [16, 20]. In Europe, production volumes in 2023 were remarkably high for apples (*Malus*, 17 million metric tons), followed by plums and sloes (*Prunus*, 2.7 million metric tons), pears (*Pyrus*, 2.2 million metric tons), strawberries (*Fragaria*, 1.7 million metric tons), cherries (*Prunus*, 760000 metric tons), raspberries (*Rubus*, 590000 metric

tons), and quinces (*Cydonia*, 37000 metric tons) [21]. In Germany, apples were the most widely consumed fruit in 2023/2024, with a per capita intake of 20.1 kg [22].

Around three-quarters of the fruits are consumed fresh, while the remaining portion is processed, for example into juices or jams [23-24]. In particular, during (apple) juice production, up to 30% of the fresh fruit weight, including peel and seeds, remain as by-product, known as pomace [25]. In Germany, up to 250,000 tons of wet apple pomace are produced each year, representing a potentially important natural source of TA [26-27].

To date, TA extraction from plant material mainly relied on organic solvents, such as ethanol, methanol, and ethyl acetate [28]. Both, simple solid-liquid extraction methods [29-30] and more equipment-intensive approaches, such as hot solvent extraction using a Soxhlet apparatus [31], or ultrasonic-assisted extraction, have been described so far [15, 32-33]. The protocols consist of multiple preparation and purification steps and hazardous chemicals, such as petroleum ether [15], to obtain highly pure TA extracts. The use of greener technologies, such as the supercritical fluid extraction, is less common [34-35]. These strategies are time-consuming or technically demanding and therefore mostly confined to well-equipped research laboratories.

Despite the reported high abundance of TA in plants, their occurrence has not yet been comprehensively investigated. Most published analytical methods focused on the determination of individual and selected TA, such as ursolic acid or oleanolic acid [28-29, 36-38]. Here, qualitative and quantitative analyses have been performed using thin-layer chromatography (TLC) [38-39], gas chromatography (GC) [29, 40], or liquid chromatography (LC) [41-42]. As the number of identified triterpenoids in plants increased in recent years [43], research interest has shifted toward more comprehensive analytical procedures, such as the coupling with mass spectrometry (GC-MS, LC-MS), enabling the detection of a broader range of TA derivatives [44-47]. However, only few methods have been described allowing the quantification of both major (e.g.

ursolic acid) as well as minor TA derivatives, including TA precursors [40, 46, 48-50].

Plant-derived TA serve as sustainable raw materials for various applications, including bioderived polymer chemistry [51]. Additionally, TA have a long history of use in traditional herbal medicine, being attributed with hepatoprotective, antifungal, and anti-inflammatory properties [52]. In recent years, growing attention has been directed toward their potential pharmacological applications: Approximately 25% of all medicines worldwide originate from plants [53], representing a sustainable and efficient alternative to the total synthesis of target compounds [1]. Free TA exhibit good solubility in organic solvents [54], but poor solubility in water [55], thereby limiting their bioavailability [56]. The bioavailability of naturally occurring TA derivatives can be enhanced primarily by chemical modifications and complexation [55, 57]: These include, for example, the synthesis of TA esters [58-59] and acylated oximes [60], which have been investigated for their biological activities, including antibacterial [58], antidiabetic [59] and anti-inflammatory [61] properties enabling the investigation of structure–activity relationships [62]. Naturally derived TA therefore represent a significant potential for resource-efficient chemical and pharmacological applications.

To learn more about the occurrence of TA in plants, the development of efficient extraction strategies and comprehensive analytical methods for the characterization of *Rosaceae* fruits is primordial. **Chapter 2** presents the development of a simple and efficient ethyl acetate-based solid-liquid extraction strategy for TA derivatives from plant material, providing a less hazardous approach for their isolation, purification, and concentration according to their chemical properties. The protocol was optimized for implementation in minimally equipped laboratories, including school and educational laboratories. The multistep preparation of the fruits, including boiling in water, enabled the separation of TA from carbohydrates, which represent one of the major components of fruits. Furthermore, cyclohexane was used to remove non-polar matrix compounds. This protocol enabled both high extraction yields and purity, achieving up to 70 g TA/100 g extract. In addition, it highlights the potential for

the sustainable utilization of food (by-products), as substantial quantities of TA can be isolated for subsequent use in synthetic applications and related processes. In **Chapter 3**, a sensitive and comprehensive GC-MS method was developed for the characterization and quantitative analysis of TA in plants. GC-MS analysis enabled the differentiation and quantification of a wide range of both, naturally occurring and modified TA derivatives, based on retention time and specific fragment ions in MS detection. The method was applied on apple peels (*Malus*) of different varieties and validated regarding sensitivity, accuracy and precision. The data revealed apples to be a rich source of TA, with contents exceeding 3.5 g/100 g dry weight. Based on these findings and developed strategies, the occurrence of TA in 17 different genera and 29 species of *Rosaceae* was investigated in detail in **Chapter 4**. The sensitive detection enabled the accurate quantification of a comprehensive set of TA in food-relevant species, with the highest TA levels found in edible fruits of *Amygdaloideae*. Additionally, TA compositions were compared for their specificity towards different genera, species, and cultivars.

Overall, this thesis describes detailed strategies for the extraction and comprehensive analysis of TA from *Rosaceae* fruits, which are a relevant source of TA. This enhances the understanding of TA diversity in plants and provides a basis for their future application in resource-efficient chemical applications and chemotaxonomic studies.

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CHAPTER 2

Efficient and Simple Extraction Protocol for Triterpenic Acids from Apples *

Triterpenic acids (TA), a class of triterpenoids, are widely distributed as secondary metabolites in plants. They have a pentacyclic structure and show high structural diversity. In this work, a simple but efficient method for the extraction and detection of TA derivatives from apple peels is described. The method is technically straightforward and robust and can be implemented in both undergraduate laboratories and science classes or projects in school: Apple peels are (i) extracted with ethyl acetate, (ii) degreased with cyclohexane, and (iii) reconstituted in ethanol. Yields of about 2.1 g of extract/100 g of dry weight apple peel were obtained, which consisted of >70% TA (56% ursolic acid (S-UA), 10% oleanolic acid (OA)). The TA pattern can be evaluated by thin layer chromatography (TLC) using simple detection with a $KMnO_4$ solution. The separation of the different TA derivatives on normal phase TLC plates enables learning how the chemical structure affects the chromatographic separation. The whole procedure requires 3−4 h without the drying steps. The TA extraction represents suitable content for student education since they learn and discuss natural products and secondary plant metabolites. The performance of an extraction, purification of natural products, and observation of chromatographic separation and detection are also learned in this method. Using this simple procedure, up to 1 g/100 g of dry weight S-UA can be generated from apple peels. While only apple peel is used for the experiment, the rest of the apple fruit was eaten by the students. Thus, the experiment itself is a demonstration of how side streams of food production can be used as a source for chemical compounds.

* modified from M. Wiebel, K. Bensberg, L. Wende, R. Grandrath, K. Plitzko, C. Bohrmann-Linde, S.F. Kirsch and N. H. Schebb. "Efficient and Simple Extraction Protocol for Triterpenic Acids from Apples". In: *Journal of Chemical Education*, **2024** *101* (1), 2087-2093. DOI: 10.1021/acs.jchemed.3c01328.

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2.1 INTRODUCTION

Triterpenic acids (TA) belong to the group of triterpenoids. Triterpenoids are one of the major classes of natural products comprising of a large number of secondary metabolites in plants. The triterpenoid biosynthesis begins with C5 isoprene units formed in the mevalonat pathway. Squalene, a C30 triterpene, is synthesized from six of these repeating C5 units. For the synthesis of TA - and many other cyclic terpenes, such as cholesterol - squalene is initially epoxidized to 2,3-oxidosqualene (Fig. 2.1) [1].

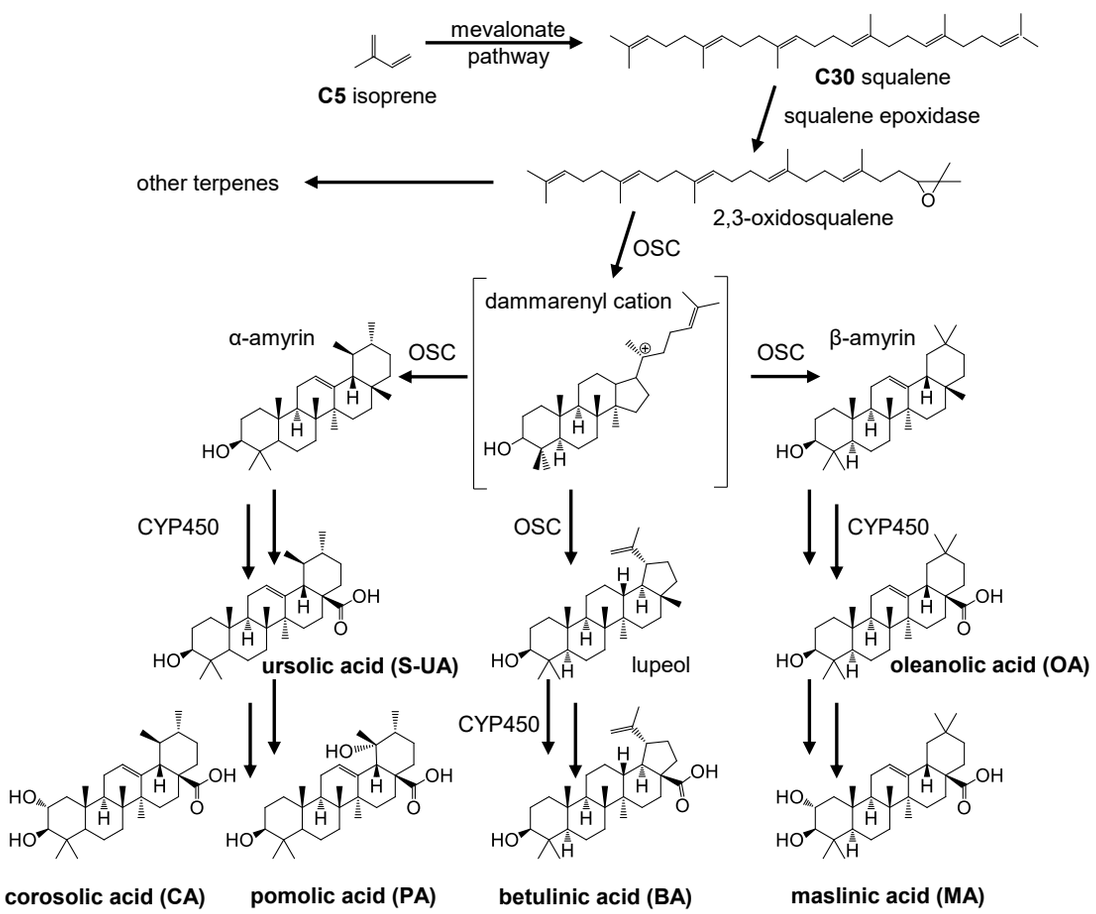

Fig. 2.1: Simplified biosynthetic pathways of triterpenic acids in plants [1-2]. The enzymes OSC (oxidosqualene cyclases) catalyze the conversion of the dammaryl cation to amyrin and lupeol. Further oxidation catalyzed by cytochrome P450 monooxygenases leads to the formation of the triterpenic acids S-UA (ursolic acid), CA (corosolic acid), PA (pomolic acid), OA (oleanolic acid), MA (maslinic acid) and BA (betulinic acid).

This is then cyclized to the dammarenyl cation by oxidosqualene cyclases from where either amyrin or lupeol are formed. Oxygenation by CYP450 monooxygenases leads to the formation of a large variety of TA. In this pathway ursolic acid (S-UA), corosolic acid (CA) and pomolic acid (PA) are derived from α-amyrin, oleanolic acid (OA) and maslinic acid (MA) from β-amyrin and betulinic acid (BA) from lupeol [2-3]. TA are widely distributed in plants. They possess physiological roles in plants due to their ability to protect against dehydration of fruits and against herbivores [4].

Both the monohydroxy TA S-UA, OA and BA and the dihydroxy TA MA, CA and PA are found in apples (*Malus domestica*, *Rosaceae*). High amounts of S-UA in apple peels with up to 2 g/100 g dry weight (DW) have been reported [5]. The content of individual TA in other fruits and plant classes was described in several studies, for example in pears (*Pyrus*, *Rosaceae*), persimmons (*Diospyros kaki*, *Ebenaceae*) and grapes (*Vitis vinifera*, *Vitaceae*) [6]. However, the amount of TA varies between fruits. The experiment described in this paper aims to educate students about secondary plant metabolites as important ingredients of fruits while simultaneously executing a chromatographic separation in the lab using structure-property relationships. As apple peels are often not eaten and typically disposed of (e.g. as component of apple pomace), it is worth pursuing methods of extracting chemically interesting compounds from this food waste.

Several extraction methods for TA from plant material are described. The most commonly used extraction methods are based on hot solvents, Soxhlet extraction, and microwave assisted or ultrasound assisted extraction using sophisticated instrumentation [7-11]. Our goal was to develop an efficient and simple extraction protocol of TA from fresh apple material that can be carried out by students while also achieving high extraction yields and purity. The strategy described is useful for the investigation of this important class of compounds that exist in high amounts of food material but also fosters education about the occurrence and importance of secondary plant metabolites. Additionally, this lab activity could also be linked to addressing education for sustainable development

(ESD) and topics such as food waste or food loss since an apple peel is the largest component of apple pomace, which is a byproduct generated from the industrial extraction of apple juice [12].

2.2 PEDAGOGIC LEARNING GOALS

Via this activity, the students can learn and discuss about natural products and secondary plant metabolites. With regard to the experimental design, the following goals can be set: First, introduce the students to the class of secondary metabolites in plants as interesting compounds for further syntheses, second, strengthen the students‘ laboratory skills in extraction and isolation of natural products and third, learn about structural characterization and identification of compounds by means of chromatography using thin layer chromatography (TLC). If possible, gas chromatography coupled with flame ionization detection (GC-FID) is also studied. The students used structure-property relationships to understand the different steps in the extraction and purification process.

A possible extension of the lab activity could be a link to the framework of ESD or Green Chemistry. With the focus on ESD, it is possible to introduce different perspectives on apples as everyday food, the proper storage of apples, and food waste or new materials from foodwaste in general can be discussed, e.g. apple leather as vegan alternative for traditional leather. Concerning the 12 Principles of Green Chemistry one can refer to selected principles such as «5. Safer Solvents and Auxiliaries» and «7. Use of renewable feedstocks» [13].

2.3 EXPERIMENTAL OVERVIEW

The laboratory experiment comprises the extraction of TA from fresh apple peels, followed by TLC analysis and, if not available, with theory of GC-FID. The material and methods, as well as material which can be used for both demonstration and as a student’s handout in school lessons can be found in the Appendix (Appendix A1-A5, Fig. A1, A2). Different apple varieties, provided by the teacher or brought from home by the students, can be used to compare their TA content and pattern. The efficient and simple extraction strategy allows the students to learn and understand the principles of isolation and enrichment of natural products based

on polarity from natural mixtures (Fig. 2.2). The extraction of different groups of secondary plant metabolites in educational context is already described in several publications, which can be recommended for additional introduction to extraction methods as well as the large group of natural substances present in plants [14-18].

Following the described procedure, the students obtain a white powder consisting of up to >70% of TA. The extraction protocol and the TLC analysis can be performed by students in school or undergraduate classes in higher education. GC-FID analysis can be theoretically demonstrated based on the provided material. If GC analysis can be carried out, only one step - the derivatization procedure - needs to be carried out by the teachers due to safety reasons (s. the Hazards section).

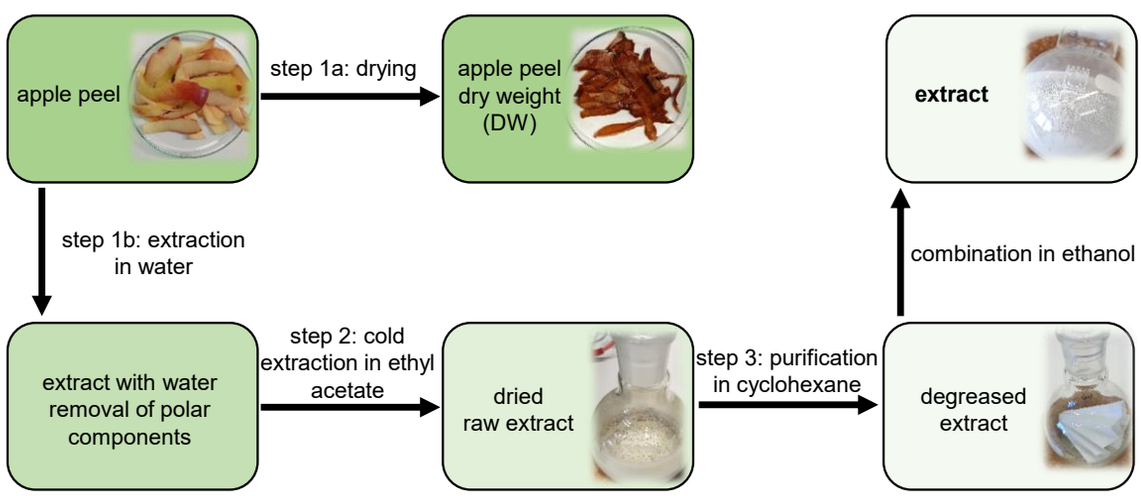

Fig. 2.2: Scheme of the extraction and purification of triterpenic acids from apples. Apple fruits were peeled, dried and extracted with water. The resulting material was extracted with ethyl acetate. For purification, the dried residue was degreased using cyclohexane and reconstituted in ethanol. A more detailed scheme can be found in the SI.

2.3.1 TA EXTRACTION

The detailed protocol for TA extraction as well as an interactive student's handout with detailed information about the experiment is provided in the Appendix A1-A6.

The protocol for TA extraction from the apple peel is carried out in three steps (Fig. 2.2). The apple is first peeled and the dry weight of the peel is determined

(Step 1a). The TA is then extracted: First, the polar peel components are removed by boiling the plant material in hot water for 10 minutes (Step 1b). The dried peels are extracted with ethyl acetate for 30 min (Step 2). Extraction with the medium-polar solvent ethyl acetate efficiently extracts TA from the dried apple peels. The remaining more polar compounds are removed by liquid-liquid extraction with water. In order to learn and train the liquid-liquid procedure, we recommend the school experiment published by McKnelly et al. or Dobberpuhl et al. [19-20]. After evaporation of the organic phase, the dried extract is boiled for 10 min in cyclohexane to remove non-polar compounds (Step 3). In this degreasing step, lipids are removed. The dried residue is dissolved in ethanol and can directly be used for TLC and GC-FID analysis.

Due to the drying steps, the protocol can involve long waiting times. Therefore, a short protocol without drying steps is also provided. This alternative protocol allows the experiment to be completed within 3-4 h, with only slightly lower extraction yields.

2.3.2 Chromatographic characterization

The detailed protocol for the chromatographic analyses of TA extracts by TLC and by GC-FID can be found in the Appendix A1-A2 (Fig. 2.4). In this method, TLC analysis is utilized as a rapid method to separate and detect mono- and dihydroxy-TA derivatives. Application, development and detection of TLC plates demonstrate for the students the basic principles of chromatographic separation. Chromatography is performed on normal phase aluminum sheets where the extract and standard solutions are applied directly onto the TLC plate. The eluents consist of a mixture of ethyl acetate, ethanol, and cyclohexane and the separation is completed in 5-10 minutes. After drying, the plate is dipped in the detection reagent containing an alkaline $KMnO_4$ solution (0.05 M) and compounds are detected as light brown spots on a purple background.

The preparation of the samples and analysis by GC-FID is carried out according to the detailed protocol in the Appendix A.3-A.5. The obtained/provided GC chromatograms show the quantitative TA pattern in the apple peel. (Fig. 2.3).

2.4 HAZARDS

Students should wear safety goggles and lab coats throughout the experiments. Ethyl acetate (H225, H319, H336) is volatile, can irritate the eyes and is flammable. Cyclohexane (H225, H304, H315, H336) is flammable, an irritant, anesthetizing and hazardous to water. Ethanol (H225, H319) is flammable and an irritant. Contact with the skin should be avoided, e.g. by wearing protective gloves. For fire prevention, no sources of ignition should be present and the solvents should be stored and disposed in the appropriate solvent containers.

2.5 STUDENT'S OUTCOMES

The experiment described was developed and applied at an educational institution by a group of three undergraduate students. Based on the results and their feedback to the protocol, the description was optimized, e.g. by including the interactive student handout (Appendix A.6). The yield of extract and TLC analysis from the students were consistent with results described in here.

2.6 RESULTS AND DISCUSSION

2.6.1 PROCEDURE FOR TA EXTRACTION

TA are abundant secondary plant metabolites present in apple peels. However, compared to carbohydrates, minerals and fibers, they are quantitatively minor compounds in plants. Therefore, a TA extraction procedure that isolates and concentrates them based on their chemical properties and removes other major compounds, such as carbohydrates, triacylglycerides and wax in the peel, is essential.

For this purpose, organic solvents of different polarity were used to separate the compounds based on their solubility. TA are medium non-polar compounds. Compounds of low polarity show no solubility in water. In contrast, carbohydrates such as sucrose and several minerals (salts) e.g. potassium choride, are well soluble in water. Extracting the apple material directly with water removes the more polar compounds. This strategy is helpful because medium-polar

compounds would otherwise be coextracted in the future steps. A similar strategy has been used for TA in the past by Fan et al. who treated dried apple pomace with hot water before TA extraction [11].

Ethyl acetate, a medium polar solvent, is used to efficiently extract TA from the dried apple peels. Using liquid-liquid extraction with water, the more polar compounds are transferred to the aqueous phase and then removed.

Aside from the TA, non-polar compounds such as waxes and triglycerides are coextracted with ethyl acetate. These non-polar compounds are well soluble in a non-polar solvent such as cyclohexane, while TA are not. Thus the ethylacetate phase is evaporated to dryness and extracted with cyclohexane to remove nonpolar compounds and to purify the TA (Fig. 2.3). A similar defatting step was described by Geana et al. for the extraction of S-UA and OA using petroleum ether before extraction in hot chloroform [21]. A less hazardous solvent was used, according to principle no.5 of the 12 Principles of Green Chemistry.

In the last step TA are reconstituted in the medium-polar solvent ethanol. Here the TA dissolve very well (experimental solubility of S-UA in EtOH >10 mM; 5 mg/mL). Since the TA are slightly more polar than ethyl acetate, this also leads to a further purification. Ethanol is a solvent frequently used for TA extraction in other protocols [22].

Performing these steps helps the students to understand about the properties of different solvents, their polarity, and their miscibility and their uses for extraction and purification. The use of different solubilities of extracted compounds has already been described in a number of experiments suitable for students and represents an important key learning objective for extraction processes [20, 23-24].

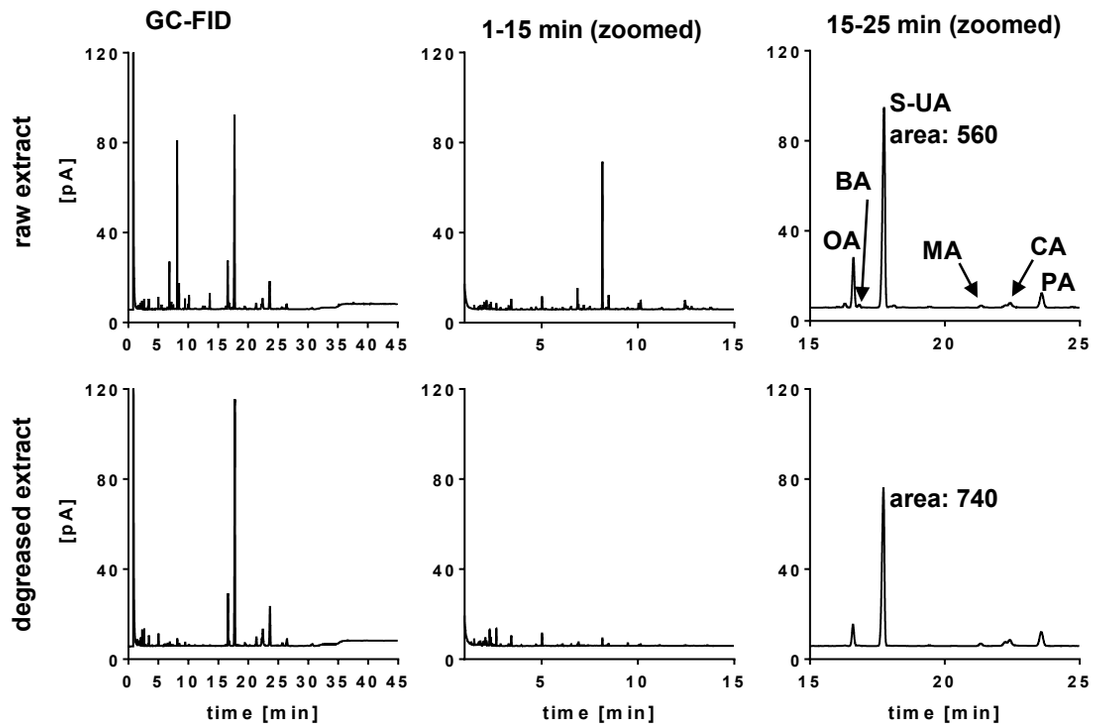

Fig. 2.3: GC-FID analysis of apple extracts. GC-FID chromatograms of the analysis of the raw ethyl acetate extract (top) and degreased extract (3 mg extract/mL) (bottom) of Delbarestivale apple peel. Separation was carried out on a non-polar (DB-5; 30 m, 0.25 mm ID, 0.25 µm film) column separating TA based on their boiling points. OA: Oleanolic acid, S-UA: Ursolic acid, BA: Betulinic acid, MA: Maslinic acid, CA: Corosolic acid, PA: Pomolic acid.

2.6.2 TLC ANALYSIS OF APPLE EXTRACTS

For the TA analysis, GC-FID is the analytical method of choice. It is sensitive and selective using inexpensive and robust instrumentation. However, there is a limited access to these instruments in schools. Therefore, a simple TLC protocol is described here, which is non hazardous and applicable for students (Fig. 2.4). By using this protocol, the mono- and dihydroxy-TA derivatives can quickly be separated and detected (Fig. 2.4). The alkaline $KMnO_4$ solution oxidizes the TA forming light brown MnO_2 making the TA visible as bright spots on a violet ($KMnO_4$) background. The intensity of the light brown spots in the TLC analysis using silica plates (normal phase chromatography) directly demonstrates the difference between peel and pulp samples (Fig. 2.4). No spot is visible when only the solvent is applied (spot 1). When applying the dihydroxy TA derivatives corosolic acid (spot 4) and maslinic acid (spot 5) and the monohydroxy

derivatives ursolic acid (spot 6) and oleanolic acid (spot 7), bright spots are detected. Here, the multiple hydroxylated derivatives show shorter R_f values because of a stronger retention on the normal phase. When applying the apple pulp samples extract (spot 2), no spot is visible. In apple peel extracts (spot 3), spots of high intensity are found for the monohydroxy derivatives while for the dihydroxy derivatives, the intensity is lower indicating a high concentration of monohydroxy-TA and low concentration of dihydroxy-TA in the sample.

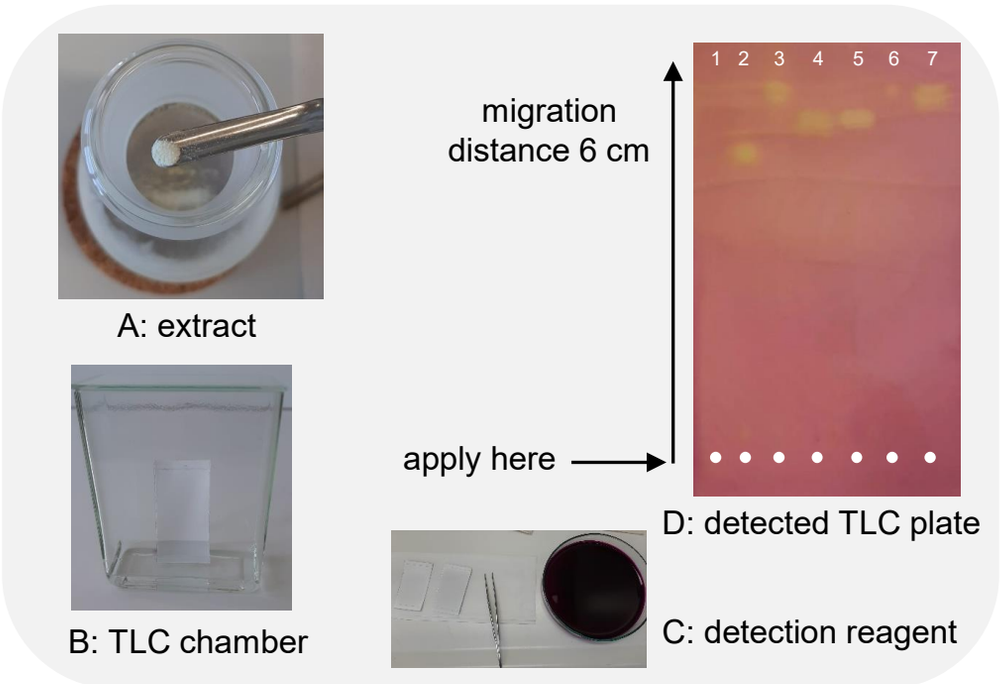

Fig. 2.4: Thin layer chromatography of TA extract. Extracts (3 mg/mL in EA) were separated on a silica gel (normal phase chromatography) TLC plate using cyclohexane/ethanol/ethyl acetate (50/25/25) (*v/v/v*) as the mobile phase. Extract (A) and TA standard solutions were applied on a TLC plate, followed by placement of the TLC into a TLC chamber (B). After drying using a hairdryer the TLC plate was dipped into $KMnO_4$ solution (0.05 M) for detection (C). A result is shown in D: 1: solvent blank, 2: apple pulp extract (3 mg extract/mL EA), 3: apple peel extract (3 mg extract/mL EA, retention factor (R_f) values: 0.70, 0.77), 4: Corosolic acid (0.5 mg/mL, R_f =0.71 ± 0.03, n=3), 5: Maslinic acid (0.5 mg/mL, R_f =0.70 ± 0.03, n=3), 6: Ursolic acid (0.5 mg/mL, R_f =0.77 ± 0.02, n=3), 7: Oleanolic acid (0.5 mg/mL, R_f =0.76 ± 0.02, n=3).

Determining the retardation factors (R_f) values (Fig. 2.4) enables the students to characterize the compounds based on their chromatographic properties. The correlation between the different retention behaviors of TA derivatives depending on the structural properties is another important learning point of the experiment.

2.6.3 GC ANALYSIS OF APPLE EXTRACTS

The results from GC-FID-analysis (including chromatograms for demonstation) demonstrate the efficiency of the extraction strategy (Fig. 2.3). GC is a key analytical technique used to analyze - i.e. separate and detect - compounds in complex mixtures such as food, medical or environmental samples. The compounds are separated based on the distribution between a gas as the mobile phase and a liquid as the stationary phase in a capillary column. The inert gas transports the analytes along a stationary phase and the compounds are mainly separated based on their boiling point/vapor pressure: The flame ionization detector is a robust detector, which can be used for the detection of all organic compounds. The signal area directly depends on the amount of carbon in a peak (mass dependent detector) and it has high linearity [25]. Therefore, peak areas in chromatograms can directly be used to calculate the concentration when calibrating with a similar compound.

By analyzing the extracts by GC-FID or evaluating the provided chromatograms (Fig. 2.3, Appendix A5, Fig. A1) students can directly see the effectiveness of the purification. Samples without prior extraction with water show high abundant signals of polar compounds (Appendix A5 Fig. A1, retention time: 5-15 min) and the degreasing step removes compounds with a low boiling point (Fig. 2.3, Appendix A5 Fig. A2, retention time: 1-15 min).

2.6.4 DETERMINED TA CONCENTRATION IN APPLE PEELS OF DIFFERENT VARIETIES

When using the extraction protocol, the TA content in the apples peel can be calculated with regard to the dry weight determined in step 1a. An interesting application is to compare the apple peel and pulp of different apple varieties. For the Golden Delicious, Boskoop and Delbarestivale apple varieties, significantly higher amounts of TA are found in the peels than in the pulp (Tab. 2.1). Extraction yields of up to 2.1 g/100 g DW were found in the Golden Delicious peel (Tab. 2.1).

The extract consists mainly of TA with up to 50 g/100 g UA and 10 g/100 g OA in Golden Delicious peel samples (Tab. 2.1). This corresponds to a concentration of 1.1 g UA/100 g dry weight resp. 0.2 g OA/100 g dry weight in the Golden Delicious apple peel (Tab. 2.1). These results are consistent with earlier reports.[11] Here, Fan et al. obtained an extraction yield of about 1.3% with a purity of 97% in Fuji apples while using a more complex instrumentation.

Overall the data demonstrates that TA are relevant ingredients and major secondary plant metabolites in apple peel. The analytical strategy outlined here provides a simple and efficient possibility for TA extraction from food by-products.

2.7 KEY LEARNINGS

By using the developed extraction strategy, students experiment with and attain knowledge about an important class of secondary metabolites in plants. They learn how compounds can be extracted and purified based on their polarity using different solvents.

The students acquire skills comprising of the handling of solvents and the gravimetric and chromatographic monitoring of analytical workflows. By analyzing different apple varieties and using the simple extraction protocol, students learn about natural variablities in the composition of food. This is demonstrated based on the TA pattern analyses by GC-FID and TLC. Here, the evaluation of retardation factor values and the different retention help to understand how the chemical structure effects the chromatographic behaviors.

Tab. 2.1: Extraction efficacy and concentration of the main TA in selected apple varieties. Peels and pulp were extracted using the developed protocol and the main TA were quantified by GC-FID by external calibration. OA: Oleanolic acid, S-UA: Ursolic acid, DW: dry weight, LLOQ: 2 µM

	Sample	Yield extract [g/100 g DW]	OA in dry extract [g/100 g]	S-UA in dry extract [g/100 g]	OA in apple [mg/100 g DW]	S-UA in apple [mg/100 g DW]
Hot extraction	Golden Delicious peel	1.3	10.9	55	140	730
Harvest 2022	Boskoop peel	0.8	8.7	33	70	270
Hot extraction	Delbarestivale peel	1.3	9.9	58	130	790
Harvest 2022						
Cold extraction	Delbarestivale peel 1	1.5	9.3	60	140	910
Harvest 2023	Delbarestivale peel 2	1.3	11	70	140	900
	Delbarestivale peel 3	1.4	10.3	62	140	850
	Golden Delicous peel	2.1	9.5	52	200	1100
	Boskoop peel 1	0.9	8.7	48	80	450
	Boskoop peel 2	0.5	4.6	19	20	90
	Boskoop peel 3	1.5	8.7	53	130	790
Hot extraction	Golden Delicious pulp	0.02	0.19	1.2	< 1	< 1
Harvest 2022	Boskoop pulp	0.01	0.19	0.84	< 1	< 1

2.8 CONCLUSION

Herein, an efficient and simple extraction protocol for TA derivatives in apple samples was developed. The protocol can easily be used in student's classes within a 5-session lab activity or even shorter in 3-4 hours if the drying steps are omitted. Monitoring the TA content in all steps demonstrates the efficiency of the extraction strategy. The contents and purities increased from step to step up to a total of 70 g TA/100 g of extract. Overall, the important student's learning subjects are secondary metabolites in food, the extraction and purification of natural products as well as the basics of chromatography. For future demonstrations, the time in between drying periods could be used to focus on the principles of Green Chemistry or on activities to promote education for Sustainable Development.

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CHAPTER 3

Development of a gas chromatography-mass spectrometry (GC-MS) method for the characterization and quantification of triterpenic acids *

Triterpenic acids (TA) are widely distributed secondary metabolites/specialized metabolites in plants. Here a sensitive and comprehensive method for the characterization and quantitative analysis of 20 TA derivatives by means of GC-MS has been developed. The TA were efficiently derivatized using $TMSCHN_2$ and BSTFA before analysis. Chromatographic separation using a DB-5 column (40 m, 0.18 mm ID, 0.25 µm film) enabled the selective detection of naturally occurring and modified TA derivatives. Sample preparation involved the removal of water-soluble matrix compounds and the extraction with ethyl acetate. The electron ionization (EI) led to distinct fragments allowing a structural characterization and specific detection. The method was applied on the analysis of apple peels of different varieties. TA concentrations of up to 3.5 g/100 g dry weight (DW) were found. Ursolic acid (up to 2.9 g/100 g DW) was the main TA, followed by oleanolic acid (up to 500 mg/100 g DW) and pomolic acid (up to 130 mg/100 g DW). Recovery of added TA was good (70-140%) and determined concentrations of naturally occurring TA were in high agreement (75-125%) with those obtained by GC-FID analysis, demonstrating the accuracy of the developed method. The method showed high precision with low intra-day and inter-day variability (≤ 22%). The described method allows for the first time the qualitative and quantitative analysis of a wide range of TA derivatives. This will help to gain more insights into the occurrence of abundant triterpenic acids such as ursolic acid but also low concentrated derivatives such as pomolic acid in plants.

* modified from M. Wiebel, L. Wende, K. Bensberg, T. Zschau, S.F. Kirsch and N. H. Schebb. "Development of a gas chromatography-mass spectrometry (GC-MS) method for the characterization and quantification of triterpenic acids". In: *Food Chemistry*, **2025** *146012*. DOI: 10.1016/j.foodchem.2025.146012.

Author contributions: **MW:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation and Conceptualization. **LW:** Writing – review & editing, Investigation, Formal analysis. **KB:** Writing – review & editing, Methodology, Investigation. **TZ:** Writing – review & editing, Methodology, Investigation. **SFK:** Writing – review & editing. **NHS:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

3.1 INTRODUCTION

Triterpenic acids (TA) are triterpenoids belonging to the large group of secondary/specialized plant metabolites. They are synthesized in plants following the mevalonate pathway. Six C5 isoprene units combine to form a squalene (C30) molecule (Fig. 3.1). Further epoxidation via the squalene epoxidase leads to the formation of 2,3-oxidosqualene, which is folded into a chair-chair conformation and afterwards cyclized. The enzymatically catalyzed cyclization results in a large number of skeletal types (more than 100 triterpene scaffolds are known [1]) as the dammarenyl cation, from which TA derivatives such as α-amyrin (AM) can be formed (Fig. 3.1, 3.2). Further enzymatically catalyzed modifications (via cytochrome P450 monooxygenases and transferases) lead to a great structural diversity such as addition of hydroxyl groups and oxidation of primary alcohols to carboxylic acids, catalyzed by CYP51, CYP71, CYP72, CYP85, CYP87, CYP88 and CYP93 [1-2]. In this pathway, ursolic acid (S-UA), corosolic acid (R-2-OH-UA), and pomolic acid (S-19-OH-UA) are derived from AM, oleanolic acid (OA) and maslinic acid (R-2-OH-OA) from β-amyrin and betulinic acid (BA) from lupeol (Fig. 3.1, 3.2). Triterpenoids are widely distributed in plants and can occur in unmodified forms and as conjugates such as glycosides. In the plant they play physiological roles e.g. protecting against dehydration of fruits and herbivores [1-2].

The TA derivatives S-UA and OA are best described and found in high concentrations in plants of the *Rosaceae* family - most prominently in apples. Here, levels up to 2 g S-UA/100 g DW are found in apple peel [3]. Several extraction protocols have been developed, e.g. for apple (pomace) demonstrating high extraction yields and purity [4-5].

Most studies describing the quantification of TA in plants only focus on S-UA and OA in plants [6-8]. However, the number of characterized triterpenoids increased in recent years [9]. Consequently, comprehensive analytical procedures such as liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) are used covering a large number of TA derivatives [10-12]. However, only few methods have been described including more than 6

TA [13-16]: Here, the major TA (e.g. S-UA, OA, dihydroxylated TA) occurring in plants from the *Oleaceae* family (olive (oil), *Olea europaea* [12]) and from the *Rosaceae* family (apple, *Malus domestica*) were investigated but minor TA derivatives (e.g. S-19-OH-UA, AM) were rarely analyzed [13-16].

The aim of this study was to develop a GC-MS method that enables the quantification of a comprehensive number of TA. For that 20 TA derivatives with varying patterns of functional groups (e.g. hydroxyl groups, ester groups, aldehyde groups) were included (Fig. 3.2, B1). The method was used for the characterization of the TA pattern in different apple varieties.

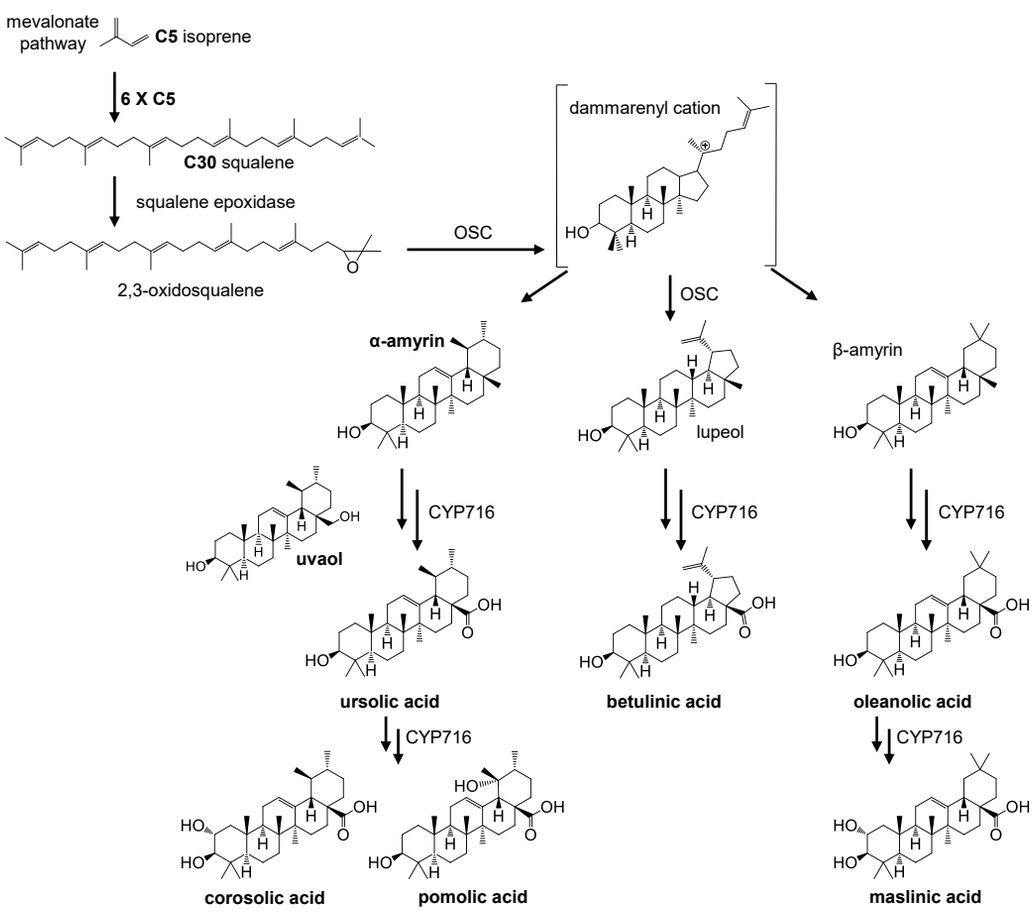


Fig. 3.1: Simplified scheme of biosynthesis of triterpenic acids. Six isoprene units form squalene. From there, the squalene epoxidase catalyzes the formation of 2,3-oxidosqualene. By cyclization via oxidosqualene cyclases, the dammarenyl cation is formed. Further cyclization leads to the formation of α-, β-amyrin, and lupeol. CYP P450 monooxygenases (e.g. CYP716) catalyze further modifications, including the addition of hydroxyl groups or formation of carboxylic acid [17, 27].

3.2 Material and Methods

3.2.1 Chemicals and materials

The TA standards ursolic acid (S-UA) (purity 95%) and oleanolic acid (OA) (purity 95%) were purchased from abcr GmbH (Karlsruhe, Germany), corosolic acid (R-2-OH-UA) (purity ≥98%) and maslinic acid (R-2-OH-OA) (purity ≥98%) from Cayman Chemical (local distributor Biomol, Hamburg Germany), betulinic acid (BA) (purity ≥97%) from TCI Deutschland GmbH (Eschborn, Germany) and pomolic acid (S-19-OH-UA) (purity ≥90%) from Merck (Darmstadt, Germany). All other TA standards were synthesized in house (purity ≥90%, analyzed by NMR). Ethyl acetate (EA), ethanol (EtOH), methanol (MeOH), *n*-hexane (*n*-hex), toluene and pyridine were obtained from Fisher Scientific (Schwerte, Germany), (trimethylsilyl)diazomethane ($TMSCHN_2$, 2 M in *n*-hex) from TCI Deutschland GmbH (Eschborn, Germany) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) from Carl Roth GmbH (Karlsruhe, Germany). Ripe fruits of Golden Delicious (*Malus domestica* Borkh. cv. Golden Delicious, harvest 2023), Fuji (*Malus domestica* Borkh. cv. Fuji, harvest 2024) and Wellant (*Malus domestica* Borkh. cv. Wellant, harvest 2023) were bought from a regional fruit farm in Leichlingen, Germany (51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E) as well as Discovery (*Malus domestica* Borkh. cv. Discovery, harvest 2023) from a regional fruit farm in Wermelskirchen, Germany (51° 6′ 37.6109″ N, 7° 10′ 28.663″ E).

3.2.2 Sample preparation

Fresh apples were peeled and the peels were boiled in water for 10 min in an Erlenmeyer flask. The samples were filtered using standard coffee filters (9×15 cm) and a glass funnel (diameter 10 cm) and the aqueous phase discarded. The residue was dried at 80 °C. The dried peel was ground in a mill (IKA A10, IKA-Werke, Staufen, Germany). In a reaction tube, 2-3 mg of grinded residue was mixed with 1 mL EA following the addition of internal standards (IS) (*R*)-ursolic acid methyl ester (R-UA) and ursenic acid (URA) (2.5 nmol) (Fig. 3.2). Samples were extracted using a thermo shaker (800 rpm, 25 °C, Biometra TSC

Thermoshaker, Analytik Jena, Jena, Germany) for 30 minutes. After mixing with 300 µL of H2O and centrifuging (2 min, 5000 g), the upper EA phase was collected and the aqueous phase again extracted with 500 µL EA. The dried residue was redissolved in 500 µL EtOH, vortexed (Vortex Vortex-Genie 2 G560E, NY, USA) and centrifuged. The EtOH phase was evaporated (Rotary vacuum-concentrator RVC 2-25 CDPlus, Christ, Germany) and the residue dissolved in 200 µL MeOH. For spiking experiments, 2.5 nmol of ursene (UR), rearranged ursolic acid (ar UA), (*R*,*S*)-corosolic acid methyl ester (R,S-2,3-OH-UA), ursonic acid (3-oxo-UA), (*S*,*R*)-corosolic acid methyl ester (S,R-2,3-OH-UA), ursolic acid acetate (UA ac), asiatic acid methyl ester (R-2,23-OH-UA) and 11-oxo- ursolic acid acetate (11-oxo-UA ac) were added to the samples prior extraction (Fig. S1).

For method validation, 8 µL of this solution was used for GC-MS analysis (160 µL for GC-FID analysis). For the analysis of different apple varieties, 8 µL of the solution for high abundant TA (g/100 g DW content) and 50 µL for low abundant TA (mg/100 g DW content) were used. The samples were mixed with 25 µL toluene and 25 µL $TMSCHN_2$ (0.4 M in *n*-hex) and derivatized for 10 minutes at room temperature, evaporated and reconstituted in 25 µL of pyridine and 25 µL BSTFA + 1% TMCS (derivatized S-UA in Fig. 3.3). After derivatization for 30 min at room temperature and evaporation, the residue was dissolved in 40-200 µL *n*-hex for GC analysis.

3.2.3 GC-MS ANALYSIS

GC-MS analysis was carried out using a 5977C GC/MSD (single quadrupole) system (Agilent Technologies, Waldbronn, Germany) operating in electron ionization (70 eV) mode. For chromatographic separation, a DB-5 column (5%-diphenyl/95%-dimethyl polysiloxane), with the dimension of 40 m length, 0.18 mm inner diameter and 0.25 µm film (Agilent Technologies, Waldbronn, Germany) at a flow rate of 1.2 mL/min helium was used. The temperature settings were set to 250 °C (injector), 330 °C (transfer line) and 250 °C (ion source). The separation

was carried out using a linear temperature gradient starting from 200 °C with 10 °C/min to 310 °C and an isothermal step from 11-40 min at 310 °C.

The analyses were carried out in full-scan mode (*m/z* 50-900) as well as in selected ion monitoring (SIM) with a dwell time of 40 ms for quantification. For each TA, one quantifier and two qualifiers were selected (Tab. 3.1, B2).

3.2.4 CALIBRATION AND QUANTIFICATION

The stock solution containing all 20 TA derivatives (1 mM in MeOH) was sequentially diluted in MeOH (4 nM-100 µM for GC-MS and 2-500 µM for GC-FID). The IS concentration was set to 1 µM (GC-MS) and 50 µM (GC-FID).

Quantification was carried out by external calibration using IS: The peak area ratios (analyte/IS) were plotted against the concentration. Calibration functions were calculated by linear regression (weighting: $1/x^2$, Tab. 3.1, B2). The limit of detection (LOD) was determined as concentration with a signal-to-noise ratio of ≥ 3 and the lower limit of quantification (LLOQ) with a signal-to-noise ratio of ≥ 5 and an accuracy of 100 ± 20%.

Tab. 3.1: Parameters of the GC-MS analysis of TA derivatives derivatized with $TMSCHN_2$ and BSTFA. The detected ions in EI (70 eV) are listed and qualifier (underlined) and quantifier (bold) are highlighted. The retention time (mean ± SD, n=5) and peak width at half height (FWHM, mean ± SD, n=5) were determined for standard solutions (10 µM). The limit of detection (LOD, S/N ≥ 3), lower limit of quantification (LLOQ, S/N ≥ 5, accuracy 100 ± 20%) as well as function of the linear calibration and coefficient of determination (R^2) were determined for the quantifier ions in selected ion monitoring mode with a dwell time of 40 ms.

Analyte	Retention time [min]	*m/z ion (*relative intensity above 20%)	FWHM [s]	LOD [nM]	LLOQ [nM]	Regression equation	R^2
URA	19.47 ± 0.01	**262** (50); 203 (85); 191 (50); 189 (15); 133 (60); 119 (30); 95 (20); 81 (20); 69 (25); 55 (20)	3.6 ± 0.1	8	24		
AM	20.77 ± 0.01	**218** (100); 203 (20); 189 (25); 133 (15); 73 (30)	4.2 ± 0.1	8	24	f(x)=1.954x-0.158	0.994
R-UA	22.05 ± 0.01	**262** (100); 203 (80); 190 (50); 189 (15); 133 (45); 129 (20); 75 (20); 73 (40)	4.6 ± 0.1	8	24		
UV	23.68 ± 0.01	496 (55); **216** (50); 203 (60); 201 (20); 189 (30); 188 (30); 133 (30); 129 (40); 119 (30); 109 (20); 107 (20); 105 (20); 95 (30); 75 (45); 73 (100); 69 (20)	5.4 ± 0.1	32	80	f(x)=0.359x+0.007	0.994
OA	24.31 ± 0.01	279 (5); **262** (60); 203 (100); 202 (20); 190 (25); 189 (30); 133 (15); 73 (30)	5.4 ± 0.1	8	24	f(x)=0.830x+0.123	0.989
BA	24.59 ± 0.01	262 (20); 203 (30); 191 (30); 190 (50); **189** (100); 175 (35); 147 (25); 145 (20); 135 (30); 133 (30); 129 (50); 121 (30); 119 (21); 109 (25); 105 (30); 95 (30); 93 (30); 91 (25); 81 (35); 79 (20); 75 (50); 73 (90); 69 (30); 67 (20); 55 (20)	5.6 ± 0.1	160	240	f(x)=0.454x-0.450	0.998
S-UA	25.88 ± 0.01	279 (5); **262** (100); 203 (80); 190 (30); 189 (35); 133 (50); 129 (20); 119 (20); 75 (25); 73 (40)	5.8 ± 0.1	8	24	f(x)=1.09x-0.929	0.986
R-2-OH-OA	29.43 ± 0.01	278 (10); **262** (80); 203 (100); 202 (20); 189 (20); 147 (60); 133 (25); 73 (55)	7.5 ± 0.1	24	32	f(x)=0.746x-0.683	0.998
R-2-OH-UA	30.79 ± 0.01	278 (5); **262** (100); 203 (65); 189 (15); 147 (65); 133 (40); 119 (25); 73 (55)	8.2 ± 0.2	24	32	f(x)=1.004x-1.474	0.990
S-19-OH-UA	33.38 ± 0.01	209 (25); 193 (45); 190 (40); 189 (20); **179** (100); 147 (50); 146 (40); 133 (100); 129 (25); 119 (45); 117 (20); 107 (20); 75 (30); 73 (45)	8.8 ± 0.5	600	700	f(x)=0.184x-0.679	0.987

3.3 RESULTS AND DISCUSSION

A sensitive and selective quantification method for TA analysis using GC-MS was developed. The dwell time was optimized for mass spectrometric detection (Fig. B2). The method was characterized by determining precision and accuracy, and the results were compared with those obtained using GC-FID. Finally, the method was applied to the analysis of TA in peel of different apple varieties.

3.3.1 CHROMATOGRAPHIC SEPARATION

Triterpenic acids are specialized plant metabolites, with a large number of isomers [1, 17]. For their selective analysis an effective chromatographic separation is required.

The chromatographic separation of TA derivatives was performed by an isothermal separation step at 310 °C on a DB-5 column (40 m, 0.18 mm ID, 0.25 µm film) following derivatization to the methyl ester and trimethylsilyl ether (Fig. 3.2, B1). The chromatographic separation showed sufficient resolution (R > 1.5) for all TA, which have been described in *Rosaceae* fruits so far. This comprises ursolic acid (S-UA), oleanolic acid (OA), betulinic acid (BA), corosolic acid (R-2-OH-UA), maslinic acid (R-2-OH-OA), pomolic acid (S-19-OH-UA), alpha-amyrine (AM), uvaol (UV) and ursonic acid (3-oxo-UA) (Fig. 3.2, B1) [18-19]. The dwell time was set to 40 ms, allowing to measure 23 data points per peak when monitoring 12 SIM transitions in parallel (Fig. B2). The elution window of the TA in the GC-MS method using the isothermal step was as long as 40 min, enabling the separation and detection of further TA derivatives. This was demonstrated analyzing 12 synthetic derivatives with different functional groups (Fig. 3.2, B2) reflecting the diversity of TA described in literature. Owing that performance, this is the most comprehensive quantitative analysis method for TA described so far, covering more than 20 TA derivatives:

Earlier GC studies, only analyzed few TA such as OA, S-UA and BA and the derivatives AM and UV [13-16]. Zhan et al. were able to separate S-UA and OA in 15 min [20]. Gu et al. showed baseline separation for S-UA, OA, and BA in a total run time of 40 min [14]. Caligiani et al. additionally included the

dihydroxylated TA maslinic acid (R-2-OH-OA) and corosolic acid (R-2-OH-UA) [10]. Dashbaldan et al. developed a method that covers 19 TA derivatives in a total run time of 75 min but were unable to achieve baseline separation for OA and BA [18]. Guo et al. analyzed 11 TA, within a total run time of more than 45 min [21]. A recently published LC-MS method comprised 14 TA derivatives in a total run time of 60 min [22].

Of note the separation of the TA derivatives by GC is better than by LC: Reversed phase (RP)-liquid chromatography using standard RP18 phases does not allow the baseline separation of the isobaric compounds BA, S-UA, and OA [20, 23] (Fig. B3, B4).

The method described here efficiently separates a wide range of TA in a total run time of 40 min, including 20 TA and achieving baseline separation for BA and OA. Chromatographic separation is crucial for the quantification of TA because most derivatives have a similar fragmentation behavior as described in the following section.

3.3.2 Mass Spectrometric Detection

Mass spectrometric detection following EI (70 eV) enables the structural characterization and sensitive quantification of TA derivatives. Using the large number of covered TA derivatives, we could deduce a specific fragmentation behavior of the TA leading to prominent fragment ions. The EI-MS spectra showed no $[M]^+$ ion, except for ar UA and UV (Tab. 1, Tab. S2), while several fragments were detected in the scan spectra between 50-400 Da (Fig. 3, Fig. B6).

By comparing the spectra of the TA standards, structural suggestions for the fragments could be made (Fig. 3.3, B5, B6, Tab. B1). The fragments were numbered according to the suggested structures (CDE1-CDE4). Applying the fragmentation pathway on the 20 TA, all the expected fragments were found (Fig. B5, Tab. B1). As shown exemplary for S-UA, fragments showing the C-D-E ring were formed by a retro-Diels-Alder reaction (Fig. 3.3). The fragment at *m/z* 262 (CDE1) includes the methyl ester of the carboxylic acid of the TA and could only be detected when using $TMSCHN_2$ for the derivatization.

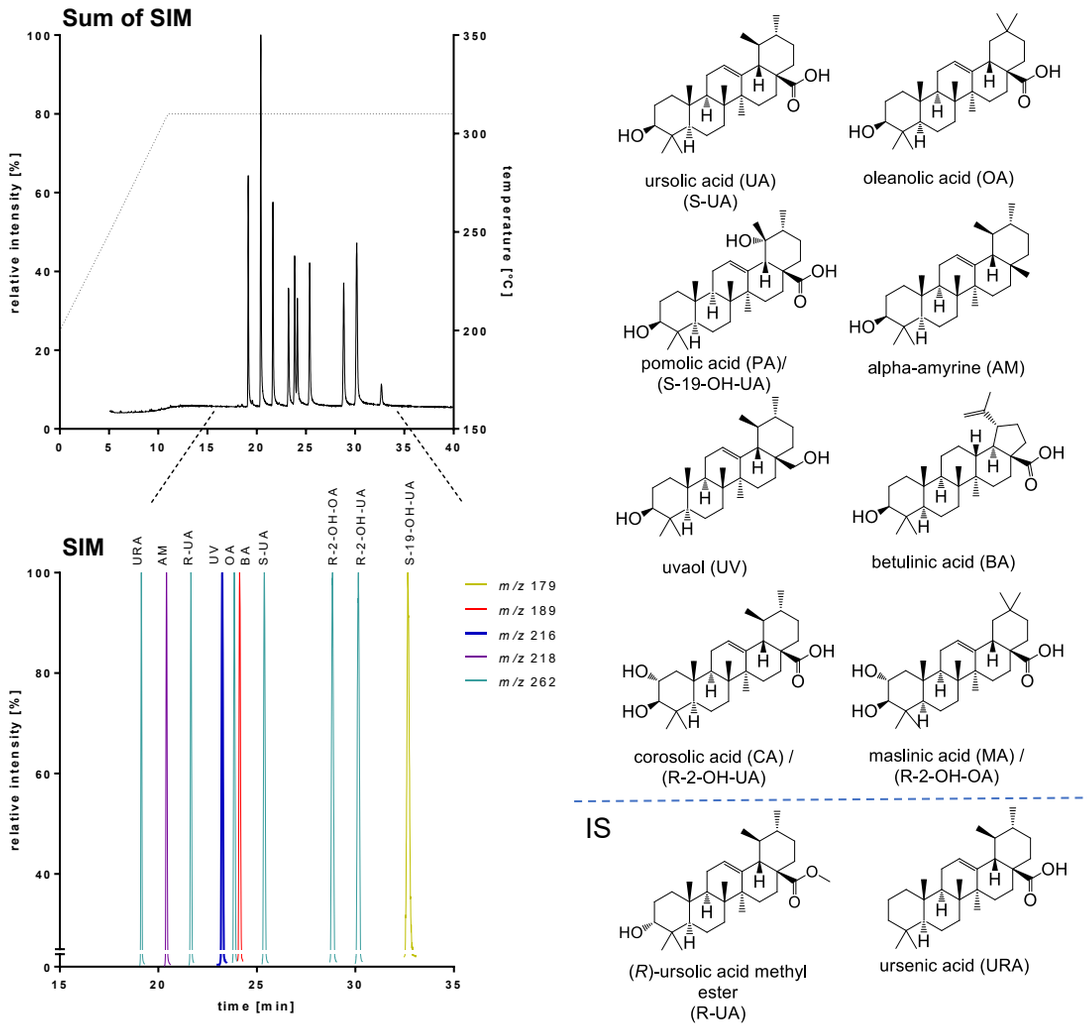


Fig. 3.2: Chromatographic separation of triterpenic acid (TA) derivatives. Left: Separation of a TA mixture of 10 TA (20-100 µM) on a 5%-phenyl/95%-dimethyl polysiloxane column (40 m, 0.18 mm ID, 0.25 µm film). Shown is the sum of SIM (TOP) and the individual SIM traces used for quantification (BOTTOM). Right: Structures of the TA derivatives.

This supports the conversion of the carboxylic acid to its corresponding methyl ester. Consistently, BA, OA, S-UA, R-2-OH-UA, and R-2-OH-OA show this fragment, while AM and UR do not (Figure B5). Instead, the fragment at *m/z* 218 (CDE1) was detected for AM and UR, because these molecules do not possess a carboxy group in ring E but a methyl group at the same position (Fig. B5). When BSTFA was used as derivatization agent alone, an ion at *m/z* 320 was detected for OA, S-UA, BA, R-2-OH-UA, R-2-OH-OA, 3-oxo-UA, URA and UA ac,

indicating the conversion of the carboxylic ester to the trimethylsilyl ether derivative (Fig. 3.3; B6).

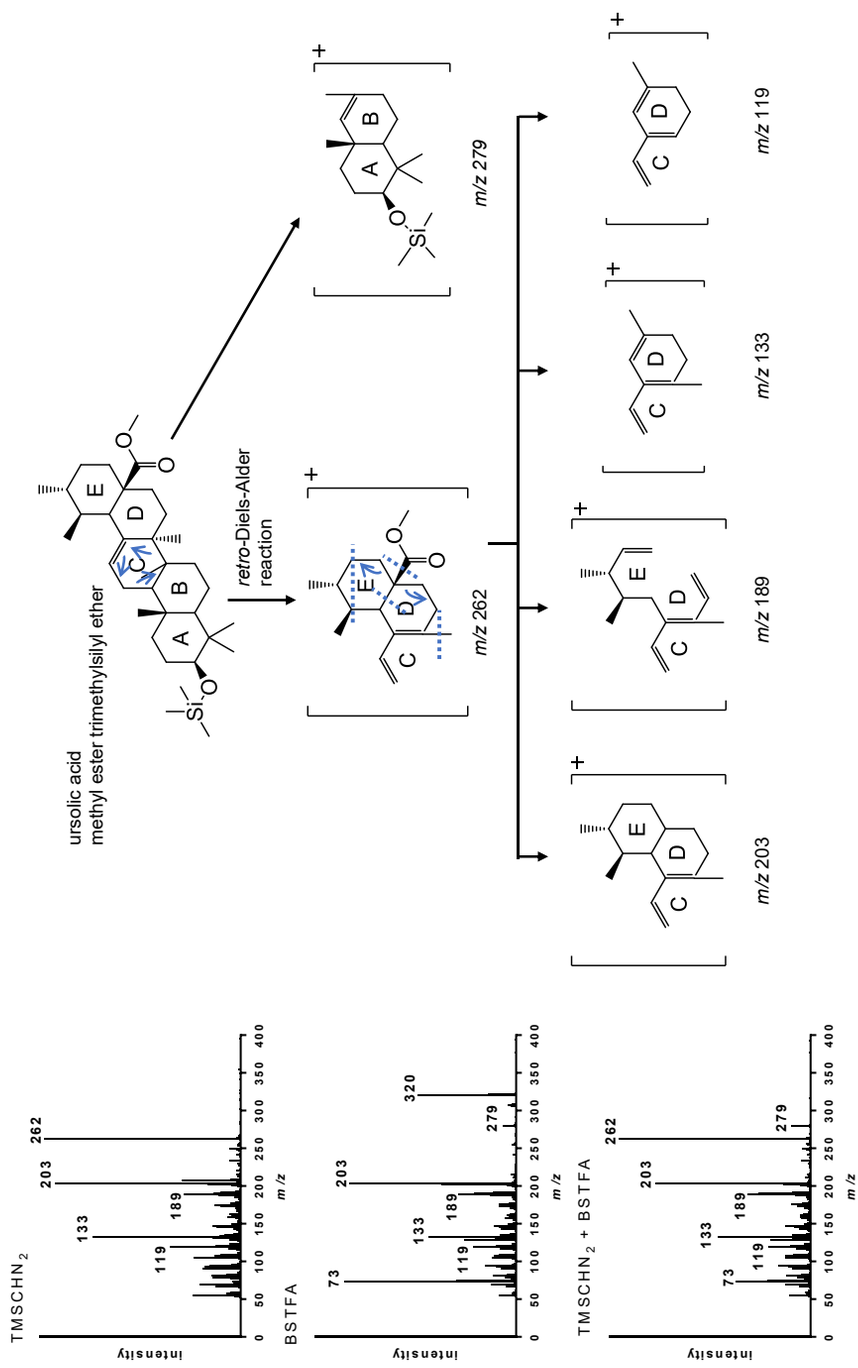


Fig. 3.3: Left: GC-EI-MS spectra (*m/z* 50-400) of S-UA (50 µM) derivatized with $TMSCHN_2$ (TOP), BSTFA (MIDDLE) and both (BOTTOM). Right: Suggested fragmentation pathway giving rise to the most prominent fragment ions.

A further loss of the carboxylic ester group leads to a fragment with *m/z* 203 (CDE2) detected following derivatization with BSTFA as well as $TMSCHN_2$. The fragment at *m/z* 189 (CDE3) is suggested to be formed by an opening of the D-E ring and *m/z* 133 (CDE4) and *m/z* 119 by loss of the E ring.

Retro-Diels-Alder fragmentation is well established for TA. Razborsek et al. also described the fragmentation following retro-Diels-Alder of trimethylsilylated TA S-UA, OA and BA suggesting the same structures of the ions at *m/z* 320, *m/z* 203 (CDE2), *m/z* 189 (CDE3) and *m/z* 133 (CDE4) [24]. Other studies found the same ions at *m/z* 262 (CDE1) for methylesters [14] or ions at *m/z* 320 and *m/z* 203 (CDE2) for the TA trimethylsilylesters [10, 25], but did not use them to deduce the structure of the molecules.

Due to the common fragmentation routes, most TA derivatives give rise to the same ions, thus the *m/z* of ions is not specific. Therefore, non-specific fragments with sufficient intensity were selected for the qualifiers and quantifiers (Tab. 3.1, B2).

3.3.3 SENSITIVITY

The sensitivity was determined based on the signal-to-noise (S/N) ratio of the individual peaks, S/N ≥ 3 for the LOD and S/N ≥ 5 for the LLOQ (accuracy of the calibration 100 ± 20%) were chosen (Fig. 3.4). The concentrations of all TA standards were checked using GC-FID.

The sensitivity for the detection of the TA seems to depend on the used quantifier ions (Tab. 3.1, B2): High sensitivities could be observed for the TA derivates ursenic acid (URA), (*R*)-ursolic acid methyl ester (R-UA) as well as S-UA and OA, using the ion at *m/z* 262. LLOQs of 24 nM (11 pg on column) could be found for these TA. A similar sensitivity was found for the dihydroxylated TA R-2-OH-UA and R-2-OH-OA (32 nM, 15 pg on column). When comparing the performance for S-UA, the LLOQ is better compared to earlier published GC methods with an LLOQ of 550 nM (250 pg on column) [11] and 650 nM (300 pg on column) [25] and in the same range as LC-MS methods with an LLOQ of 43 nM (200 pg on column) [7] to 6 nM (15 pg on column) [22].

Similarly, an LLOQ of 6 nM (15 pg on column) was reported for OA [22]. The published LLOQ of 6 nM (15 pg on column) for 2-R-OH-OA and 2-R-OH-UA [22] were comparable to our method.

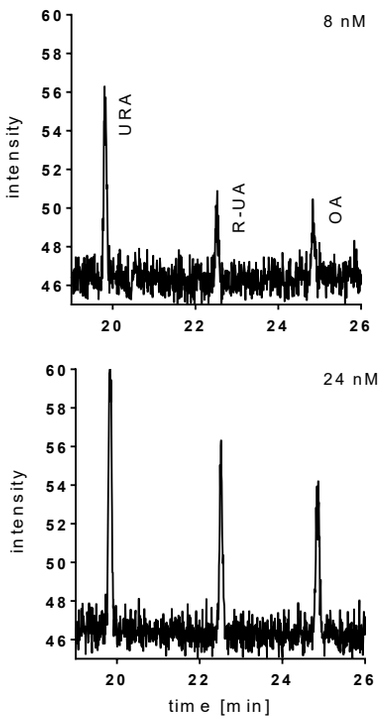


Fig. 3.4: Sensitivity of the developed method: Shown is the SIM signal at *m/z* 262 (dwell time 40 ms) used for the quantification of URA, R-UA and OA at the limit of detection (LOD, S/N ≥ 3) and at the lower limit of quantification (LLOQ, S/N ≥ 5).

Use of the quantifier ion at *m/z* 218 for AM and UR leads to the same sensitivity with LLOQs of 24 nM (10 pg on column). For UV, a higher LLOQ of 80 nM (35 pg on column) results using the quantifier ion at *m/z* 216. Yabukawa et al. also observed a slightly higher LLOQ for UV of 110 nM (244 pg on column) compared to S-UA and OA at 6 nM (15 pg on column) [22].

Interestingly, a tenfold lower sensitivity was found for BA using the quantifier ion at *m/z* 189 (240 nM, 110 pg on column). The fragment ion at *m/z* 189 (CDE 3) was selected due to its high relative abundance (100% rel. intensity, Tab. 3.1) and the better signal-to-noise ratio of the peaks compared to *m/z* 262 and *m/z* 129. Moreover, *m/z* 189 allows to quantify BA in the presence of high concentrations of OA (quantifier at *m/z* 262) as they occur in *Rosaceae* [3, 18]. Also, the analysis of pomolic acid (S-19-OH-UA) was not as sensitive as the other TA with an LLOQ of 700 nM (330 pg on column) using the quantifier ion at

m/z 179 (Tab. 3.1). Again, alternative ions at *m/z* 133, 147 or 189 did not lead to a better sensitivity. This may be explained by the different substitution patterns in the E ring leading to a reduced formation of ions used for quantification.

The effect of the quantifier ion on the sensitivity was also observed for synthetic derivatives of TA (Tab. B1): The LLOQ for the quantifier at *m/z* 262 was 80 nM for 3-oxo-UA (36 pg on column) and UA ac (40 pg on column) and comparable to natural TA derivatives. Compared to 3-oxo-UA, the exchange of the carboxyl group with an aldehyde group in ring E resulting in 3-oxo ursolic acid aldehyde (3-oxo-UA al) (fragment ion at *m/z* 133) led to a tenfold higher LLOQ (900 nM, 390 pg on column). Again, the substituents at the ring E could cause a reduced formation of abundant ions.

Interestingly, the synthetic compound R,S-2,3-OH-UA showed a lower sensitivity with an LLOQ of 500 nM (243 pg on column) using the quantifier ion at *m/z* 262. Compared to R-2-OH-UA, which is detected more sensitively, (*R,S*)-corosolic acid methyl ester (R,S-2,3-OH-UA) only differs in configuration of the hydroxyl group in the A ring. For the trihydroxylated TA asiatic acid methyl etser (R-2,23-OH-UA) an LLOQ of 600 nM (301 pg on column) was observed. This suggests that configuration as well as the number of hydroxyl groups in ring A influences the formation of the CDE ring fragments.

Overall, our GC MS method was more sensitive than previous GC-MS approaches, covering more TA [11, 25]. The enhanced sensitivity may be explained in addition to the good GC separation leading to narrow peaks (s. above) by the two-step derivatization strategy using $TMSCHN_2$ and BSTFA. As shown for the TA OA, S-19-OH-UA, BA, R-UA, and R-2-OH-UA the peak height increased dramatically compared to a derivatization with $TMSCHN_2$ or BSTFA alone (Fig. B6) as used in previous GC-MS methods [10-11].

3.3.4 Accuracy and precision

Accuracy of the method was analyzed in Wellant apple peel samples spiked with TA (1 nmol/1 mg sample) on three different days before extraction with ethyl acetate and subsequent GC-MS analysis (Tab. 3.2). The accuracy was

calculated as recovery, determined by means of GC-MS and was with 70-140% good (Tab. 3.2).

In addition, the concentrations of the naturally occurring TA OA, S-UA, R-2-OH-UA, R-2-OH-OA and S-19-OH-UA (50-900 mg/100 g DW) were determined and compared to those obtained with GC-FID (Tab. 3.2). The results using both detectors were consistent with the determined TA concentrations from 75-125% (comparison of mean and standard deviation). Of note, R-2-OH-UA could not be determined by GC-FID due to coelution with matrix compounds.

The accuracy for measurements in biological matrix was validated by spiking (standard addition procedure) of S-UA and R-2-OH-UA to apple peel extracts prior to the derivatization procedure resulting in a recovery of 93-112% of the added amount. This indicates an appropriate extraction strategy and sufficient excess of derivatization reagents in the presence of matrix (Tab. B3).

The intra-/interday precision was assessed by comparing the TA concentration in Wellant apple peel determined in triplicates on three days (n=3) by means of GC-MS as well as GC-FID and by calculating the relative standard deviation (RSD) (Tab. 3.2). Both parameters were lower 22% demonstrating a good precision for GC-MS and GC-FID analysis.

Tab. 3.2 Analysis of TA in Wellant apple peel. A: The concentration of the detected TA determined by GC-MS (left) as well as GC-FID (right). Intra- and interday precision were calculated as relative SD for repeated (n=3) measurements on three different days. B: Those TA which were not present were spiked to the samples (20-23 mg/100 g dry weight (DW)) and accuracy was calculated:

$$accuracy\ [\%] = \frac{TA\ concentration\ calculated\ [\frac{mg}{100\ g\ DW}]}{TA\ concentration\ spiked\ [\frac{mg}{100\ g\ DW}]} * 100[\%]$$ (each n=3, ± SD, RSD [%]).

GC-MS					GC-FID			
A Concentration ± SD [mg/100 g DW] (RSD [%])					Concentration ± SD [mg/100 g DW] (RSD [%])			
Analyte	Day 1	Day 2	Day 3	Interday	Day 1	Day 2	Day 3	Interday
OA	180 ± 20 (12)	210 ± 50 (20)	210 ± 20 (10)	200 ± 30 (15)	210 ± 40 (18)	230 ± 10 (4)	230 ± 40 (17)	220 ± 30 (13)
S-UA	850 ± 150 (18)	900 ± 200 (22)	1000 ± 120 (12)	900 ± 150 (16)	880 ± 140 (16)	810 ± 120 (14)	910 ± 50 (6)	860 ± 100 (12)
R-2-OH-OA	44 ± 6 (14)	50 ± 8 (16)	53 ± 6 (11)	48 ± 7 (15)	77 ± 5 (6)	73 ± 13 (19)	47 ± 2 (4)	65 ± 15 (23)
R-2-OH-UA	53 ± 9 (16)	57 ± 10 (18)	58 ± 1 (2)	56 ± 7 (12)	coelution			
S-19-OH-UA	220 ± 10 (3)	230 ± 20 (10)	240 ± 30 (20)	230 ± 20 (9)	150 ± 30 (19)	140 ± 30 (21)	170 ± 10 (6)	150 ± 20 (15)
B Mean recovery ± SD [%] (RSD [%])								
UR	150 ± 20 (14)	117 ± 6 (6)	146 ± 6 (4)	140 ± 20 (14)				
ar UA	77 ± 6 (7)	70 ± 25 (35)	91 ± 7 (7)	80 ± 15 (20)				
R,S-2,3-OH-UA	150 ± 10 (8)	126 ± 6 (5)	140 ± 15 (11)	140 ± 15 (11)				
3-oxo-UA	74 ± 7 (10)	70 ± 10 (19)	70 ± 15 (21)	70 ± 10 (16)				
S,R-2,3-OH-UA	70 ± 15 (19)	59 ± 8 (13)	75 ± 1 (21)	70 ± 15 (20)				
UA ac	80 ± 15 (21)	70 ± 2 (3)	80 ± 15 (17)	80 ± 10 (15)				
R-2,23-OH-UA	100 ± 14 (14)	85 ± 10 (12)	110 ± 20 (14)	100 ± 20 (15)				
11-oxo-UA ac	79 ± 9 (12)	60 ± 15 (23)	80 ± 20 (22)	70 ± 15 (20)				

3.3.5 Determining the TA concentration in apple peels

TA concentrations were determined in peels of the apple varieties Fuji, Discovery and Golden Delicious using the described GC-MS method (Fig. 3.5). The TA derivatives S-UA, OA, UV, AM, R-2-OH-UA, R-2-OH-OA and S-19-OH-UA were found in apple peels (Fig. 3.5-A, -B). S UA was by far the most abundant TA, with concentrations between 1.2 ± 0.3 g/100 g DW in Discovery and 2.9 ± 0.5 g/100 g DW in Golden Delicious. OA was the second most concentrated TA in the peels, with 220 ± 50 mg to 550 ± 90 mg/100 g DW. The results for S-UA and OA were consistent with other studies [3] and our previous work on preparative TA extraction [5]. Jäger et al. also found S-UA occurring in highest concentration (0.2-2.1 g/100 g DW), followed by OA (average of 280 mg/100 g DW) in apple peels. The determined concentrations were strongly depending on the analyzed apple variety [3]. Yabukawa et al. found 1.1 g/100 g DW S-UA and 140-190 mg/100 g DW OA in Fuji resp. Golden Delicious peels [22]. The same group found up to 140 mg S-19-OH-UA/100 g DW in Golden Delicious peels [22] which is comparable to our results (130 ± 5 mg/100 g DW in Discovery and 280 ± 20 mg/100 g DW in Golden Delicious). In our study, the dihydroxylated TA R-2-OH-UA/-OA were found in concentrations ranging from 50-150 mg/100 g DW. Additionally, small amounts of UV (10-70 mg/100 g DW) and AM (3-5 mg/100 g DW) were determined in the apple peels. Again, these results are in good agreement with Yabukawa et al. who found 30-80 mg R-2-OH-UA and -OA/100 g DW resp. 20 mg UV/100 g DW in Fuji and Golden Delicious peels [22].

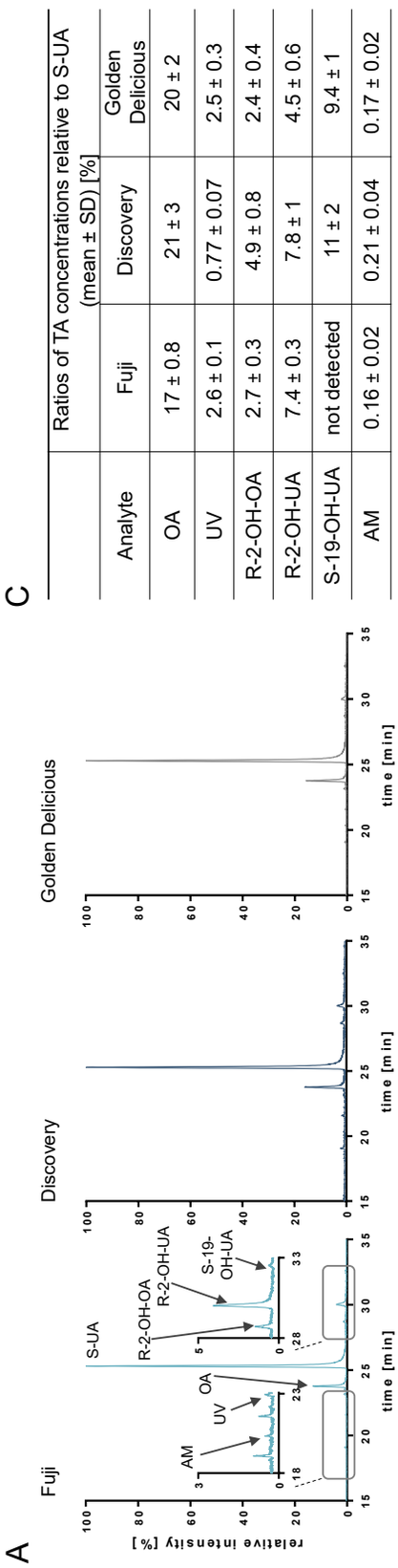
Hence, the TA pattern in the peels is characterized by large concentration differences between the derivatives, and the absolute concentrations vary depending on the apple variety (Fig. 3.5-B). However, when calculating the relative concentration ratio between the different TA to S-UA, the ratios are comparable across the analyzed varieties (Fig. 3.5-C). Only the ratio for UV to S-UA is about 3 times lower in Discovery apples (0.8) compared to the Fuji and Golden Delicious varieties (2.5) (Fig. 3.5-C). It could be assumed that there are characteristic concentration ratios of TA valid for all varieties of the genus *Malus*

domestica. Analyses of further genera of the *Rosaceae* family would be necessary to support this hypothesis.

To summarize, TA were found in high concentrations in apple peels (up to 3.5 g/100 g DW). Due to the high sensitivity of the GC-MS method, even low-concentration derivatives such as AM could be detected, which was often not detected or quantified, even though it is one of the biosynthetic precursors of the main TA S-UA (Fig. 3.1). Interestingly, many studies discuss the plants of the *Oleaceae* family to be a rich source for TA and focus on the quantitative TA analysis in herbs and olives [12, 15]. However, our results show that at least S-UA occurs in similarly high concentrations in *Rosaceae* fruits and could be of interest for extraction from food side streams such as apple pomace, which is produced in thousands of tons every year esp. during apple juice production [26].

3.4 CONCLUSION

A comprehensive and sensitive GC-MS method for the quantification of triterpenic acids (TA) has been developed. TA were methylated and silylated by a two-step derivatization procedure. 20 TA derivatives, including well-known TA derivatives as ursolic acid and oleanolic acid and less concentrated derivatives as pomolic acid and alpha-amyrine were efficiently separated in a total run time of 40 minutes. The method demonstrated good accuracy and precision. The application on the investigation of TA in apple peels in different varieties revealed differences in TA concentration, with ursolic acid being the most abundant compound in all apples, with concentrations between 1.2 ± 0.3 g/100 g DW in Discovery and 2.9 ± 0.5 g/100 g DW in Golden Delicious peel. Oleanolic acid was the second most abundant TA, with concentrations between 220-550 mg/100 g DW. Other detected TA included pomolic acid (up to 280 ± 20 mg/100 g DW), maslinic acid and corosolic acid (50–150 mg/100 g DW), uvaol (10–70 mg/100 g DW), and the low-abundance precursor alpha-amyrine (3–5 mg/100 g DW). The results suggest that characteristic TA concentration ratios may be consistent across *Malus domestica* varieties, which should be further investigated for other *Rosaceae* species.



C

Analyte	Ratios of TA concentrations relative to S-UA (mean ± SD) [%]		
	Fuji	Discovery	Golden Delicious
OA	17 ± 0.8	21 ± 3	20 ± 2
UV	2.6 ± 0.1	0.77 ± 0.07	2.5 ± 0.3
R-2-OH-OA	2.7 ± 0.3	4.9 ± 0.8	2.4 ± 0.4
R-2-OH-UA	7.4 ± 0.3	7.8 ± 1	4.5 ± 0.6
S-19-OH-UA	not detected	11 ± 2	9.4 ± 1
AM	0.16 ± 0.02	0.21 ± 0.04	0.17 ± 0.02

Fig. 5: A: GC-EI-MS chromatograms (sum of SIM) and B: concentration (mean ± SD) of TA in apple peels of the varieties Fuji, Discovery and Golden Delicious. C: Ratios of TA concentrations relative to S-UA content.

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CHAPTER 4

Genera specific occurrence of triterpenic acids in edible fruits of the *Rosaceae* family*

Triterpenic acids (TA) are triterpenoids belonging to the large group of secondary plant metabolites. However, only little information is available about the occurrence and concentration of TA in different fruits of the *Rosaceae* family. In this study, quantitative TA patterns in fruits of 29 species from 17 genera of the *Rosaceae* family were analyzed. TA were detected in all fruits. The concentrations were lower in genera of *Rosoideae* (<1-1200 mg TA/100 g DW) than in those of *Amygdaloideae* (100-7000 mg TA/100 g DW). TA patterns were dominated by mono- and dihydroxylated derivatives. In edible fruits, both the amount of detected TA derivatives and their concentrations were low in *Rubus* (< 10 mg TA/100 g DW), and highest in *Malus*, reaching >3 g TA/100 g DW. *Rosaceae* genera did not show genus-specific TA patterns, as the concentrations and ratios of individual TA to the dominating ursolic acid varied strongly between species. However, cultivar-specific TA patterns were found for *Malus domestica*, independent of harvest year or growing location. This study is the first report about a comprehensive set of more than 20 TA in food-relevant species. These data support future investigations on the nutritional relevance of TA and may improve the chemotaxonomic differentiation of *Rosaceae* cultivars.

* modified from M. Wiebel, L.M. Wende, K. Bensberg, T. Zschau, S.F. Kirsch and N. H. Schebb. "Genera specific occurrence of triterpenic acids in edible fruits of the *Rosaceae* family". (Manuscript submitted for publication.)

Author contributions: **MW:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation and Conceptualization. **LMW:** Writing – review & editing, Investigation, Formal analysis. **KB:** Writing – review & editing, Methodology, Investigation. **TZ:** Writing – review & editing, Methodology, Investigation. **SFK:** Writing – review & editing. **NHS:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

4.1 INTRODUCTION

Triterpenic acids (TA) are triterpenoids belonging to the large group of secondary plant metabolites. They are synthesized in plants in the mevalonate pathway (Fig. 4.1): Six C5 isoprene units combine to form a squalene (C30) molecule which is converted by squalene epoxidase into 2,3-oxidosqualene. Further enzymatic cyclization yields over 100 triterpene scaffolds, including intermediates such as the dammarenyl cation and precursors such as alpha-amyrin (AM) and beta-amyrin. Cytochrome P450 and transferase-mediated modifications such as addition of hydroxy groups and oxidation of primary alcohols to carboxylic acids lead to a great structural diversity of TA [1-2]. TA are widely distributed in plants and can occur in unmodified forms and as conjugates such as glycosides. In plants they play physiological roles e.g. protecting against dehydration of fruits and herbivores [1-2].

Rosaceae is a large plant family belonging to the order *Rosales* [3]. This plant family is distributed worldwide, with a primary occurrence in the temperate zone [4-5]. The family can be further divided into three subfamilies: *Rosoideae*, *Amygdaloideae*, and *Dryadoideae* [4, 6] (Fig. 4.2). The subfamilies are further classified into genera and species, based on fruit morphologies and/or nuclear phylogeny [4, 6]. In total, the *Rosaceae* family comprises more than 90 genera and over 4,000 species [3]. Fruits of the subfamilies *Rosoideae* and *Amygdaloideae* are an important edible food, particularly of the species from the genera *Prunus*, *Rubus*, *Aronia*, *Cydonia*, *Malus*, *Pyrus*, *Mespilus* and *Fragaria* [4-5]. A total world production of edible fruits of *Rosaceae* of about 110 million metric tons was reported in 2005 [5]. These fruits are consumed both fresh and as processed products such as juices and jams [7]. Moreover, *Rosaceae* are widely cultivated as ornamental plants, including species from the genera *Sorbus* and *Pyracantha* [5]. Apple peels (genus *Malus*) are known for their high TA content [8-10]. During food processing (e.g. juice production), large portions of the edible fruits, including peels and seeds, remain as by-products which may represent a relevant source of TA [11]. However, TA concentrations in the *Rosaceae* family have been poorly investigated so far.

TA have a long history of use in traditional herbal medicine, being attributed with hepatoprotective, antifungal, and anti-inflammatory properties [12].

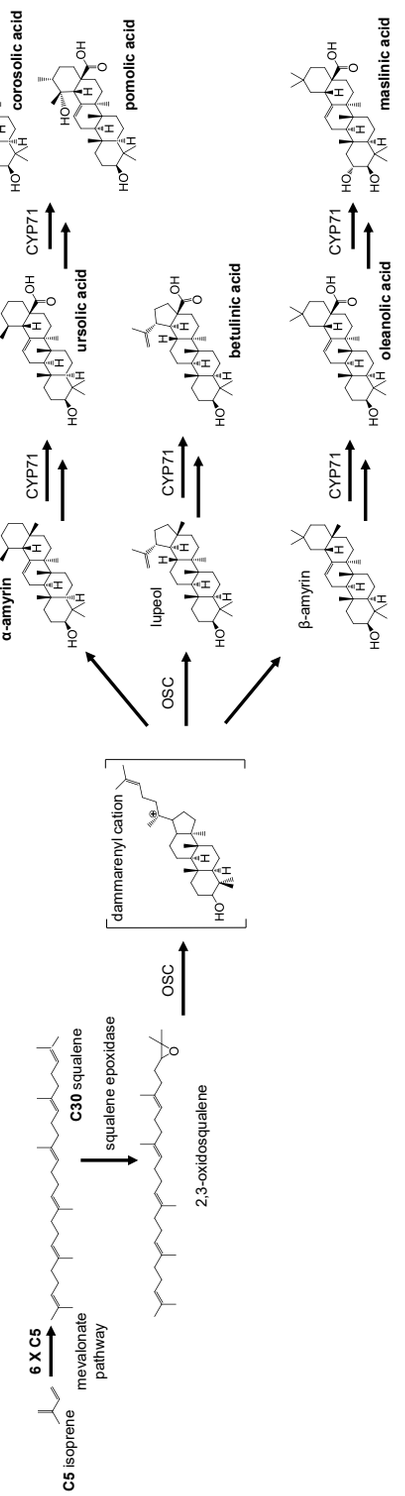


Fig. 4.1: Simplified scheme of biosynthesis of triterpenic acids: Six isoprene units combine to form squalene which is converted into 2,3-oxidosqualene by squalene epoxidase. Cyclization via oxidosqualene cyclases yields the dammarenyl cation, further leading to α-, β-amyrin, and lupeol. Modifications by CYP P450 monooxygenases (e.g. CYP71) introduce hydroxy groups which can be further oxidized to carboxy groups [31, 39].

Although the low water solubility limits their bioavailability [13-14], TA are investigated in pharmacological research, aiming to enhance biological activity by chemical modifications of the structures [15]. This study aims to characterize, quantify and compare the TA pattern in edible fruits of *Rosaceae*. For that, regionally grown fruits of 29 different species from 17 *Rosaceae* genera were analyzed using a comprehensive GC-MS method covering 20 TA derivatives (Fig. 4.2, C1, C2).

4.2 Material and method

4.2.1 Chemicals and materials

The TA standards ursolic acid (S-UA) and oleanolic acid (OA) were bought from abcr GmbH (Karlsruhe, Germany), corosolic acid (R-2-OH-UA) and maslinic acid (R-2-OH-OA) from Cayman Chemical (local distributor Biomol, Hamburg Germany), betulinic acid (BA) from TCI Deutschland GmbH (Eschborn, Germany) and pomolic acid (S-19-OH-UA) from Merck (Darmstadt, Germany). All other TA standards were synthesized in house, as described [8]. Methanol, *n*-hexane, ethyl acetate, ethanol, toluene and pyridine were purchased from Fisher Scientific (Schwerte, Germany), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) from Carl Roth GmbH (Karlsruhe, Germany) and (trimethylsilyl)diazomethane ($TMSCHN_2$, 2 M in *n*-hex) from TCI Deutschland GmbH (Eschborn, Germany). *Rosaceae* samples used in this study were either collected or purchased from regional fruit farms (App.C, Tab. C1 for sample details and locations). Identification of collected fruits was supported by the use of the Android application Flora Incognita (version 3.12.6, provider: TU Ilmenau, Germany, and: Max-Planck-Institut of Biogeochemistry, Jena, Germany).

4.2.2 Quantification of TA (derivatives) in *Rosaceae*

TA in *Rosaceae* samples were analyzed as previously described [8]. In brief, for extraction, 5-50 g of fresh material (peeled or whole fruits with diameters <1 cm, Tab. C1) were used. After aqueous extraction and drying, the residue was mixed

with ethyl acetate and internal standards (Fig. C1). Samples with dry infructescence were directly extracted with ethyl acetate. Liquid-liquid extraction with water was performed, followed by evaporation and dissolution in ethanol. After centrifugation, the organic phase was evaporated and the residue dissolved in 200 µL methanol. 50 µL of this solution was used for derivatization. After derivatization and evaporation, the residue was dissolved in exactly 20-100 µL *n*-hexane for GC-MS analysis. In each preparation batch, apple peel with a defined TA content was included as quality control (Tab. C4). GC-MS analysis was performed using a 5977C GC/MSD (single quadrupole) system (Agilent Technologies, Waldbronn, Germany) operating in electron ionization (70 eV) mode [8]. Quantification was carried out by external calibration with internal standards (Fig. C1). The method was validated and showed good accuracy and precision for the analysis of plant materials [8].

4.3 RESULTS AND DISCUSSION

Triterpenic acids (TA) are secondary plant metabolites found in *Rosaceae* plants, occurring in particularly high concentrations in the peels of apple fruits (>3 g/100 g dry weight (DW), *Malus domestica* Borkh.) [8-9]. Despite the nutritional importance of Rosaceae fruits, only limited information is available about the occurrence of TA in other genera and species in this plant family. Here, a comprehensive GC-MS method covering 20 different TA derivatives (Fig. 2, S1, S2) [8] was used to quantitatively analyze TA in 29 species from 17 *Rosaceae* genera, focusing on common fruits occurring in Northern Europe and evaluating the phylogenetic relationships and the impact of vegetation period and region.

4.3.1 TA PATTERN IN DIFFERENT *ROSACEAE* GENERA

The TA patterns of 17 genera from the subfamilies *Rosoideae* and *Amygdaloideae* were analyzed and compared: TA were detected in fruits of all analyzed *Rosaceae* samples (Tab. 4.1). However, the TA pattern and concentrations differed between genera and species (Tab. 4.1, Fig. 4.2). Representative chromatograms illustrating the TA pattern of selected species

from the fruits of the genera *Rubus*, *Prunus*, *Cydonia*, *Mespilus*, and *Aronia* are shown in Fig. 4.2.

When comparing the TA pattern across *Rosaceae* genera, those belonging to the subfamily *Rosoideae* showed lower TA concentrations (<1 mg to >1 g/100 g DW) compared to the subfamily *Amygdaloideae* (100 mg to 7 g/100 g DW) (Tab. 4.1, Fig. 4.2):

In detail, within the subfamily *Rosoideae*, TA concentrations of only <10 mg/100 g DW were found in whole fruits of *Rubus* (Tab. 4.1). This is consistent with earlier reports, describing TA concentrations between 0.1 and 120 mg/100 g DW of betulinic acid (BA), corosolic acid (R-2-OH-UA), and ursolic acid (S-UA) in 37 varieties of *Rubus idaeus* L. (raspberry) [16].

Notably even lower TA concentrations (<1 mg/100 g DW) were found in whole fruits of *Fragaria* (strawberry) (Tab. 4.1).

Remarkably high TA levels exceeding 1 g/100 g DW were found in the genus *Rosa*, for example in the whole fruits of *Rosa rugosa* (rose hip) (Tab. 4.1). Dashbaldan et al. investigated the TA content in the cuticular waxes of *Rosa rugosa* and reported similarly high concentrations of up to 420 mg/100 g wax extract [17].

In contrast, all analyzed genera of *Amygdaloideae* showed TA concentrations of more than 100 mg/100 g DW, with the highest levels observed in the genus *Malus* (apple) and concentrations of >3 g TA/100 g DW in fruit peel and 7 g TA/100 g DW in apple pomace with S-UA as main (monohydroxylated) TA (Tab. 4.1, Fig. 4.2). Equally high levels have been reported in the literature: Wozniak et al. found a total TA content of up to 1 g/100 g in apple pomace, also identifying S-UA as main derivative [18]. Besides apple, S-UA was the main TA in the genera *Crataegus* (hawthorn), *Cydonia* (quince), *Pyrus* (pear), *Cotoneaster* and *Aronia* (chokeberries) (Tab. 4.1, Fig. 4.2). This dominating occurrence of S-UA is consistent with previous studies describing the TA content in genera of *Amygdaloideae* [9, 17, 19-24].

Fig. 4.2: A: Phylogenetic tree of *Rosaceae*, based on fruit morphologies and nuclear phylogeny, modified and simplified from [4, 6]. Bold: Genera of food-related *Rosaceae* analyzed in this study. **B:** Quantitative pattern of TA in the whole fruit or fruit peel.

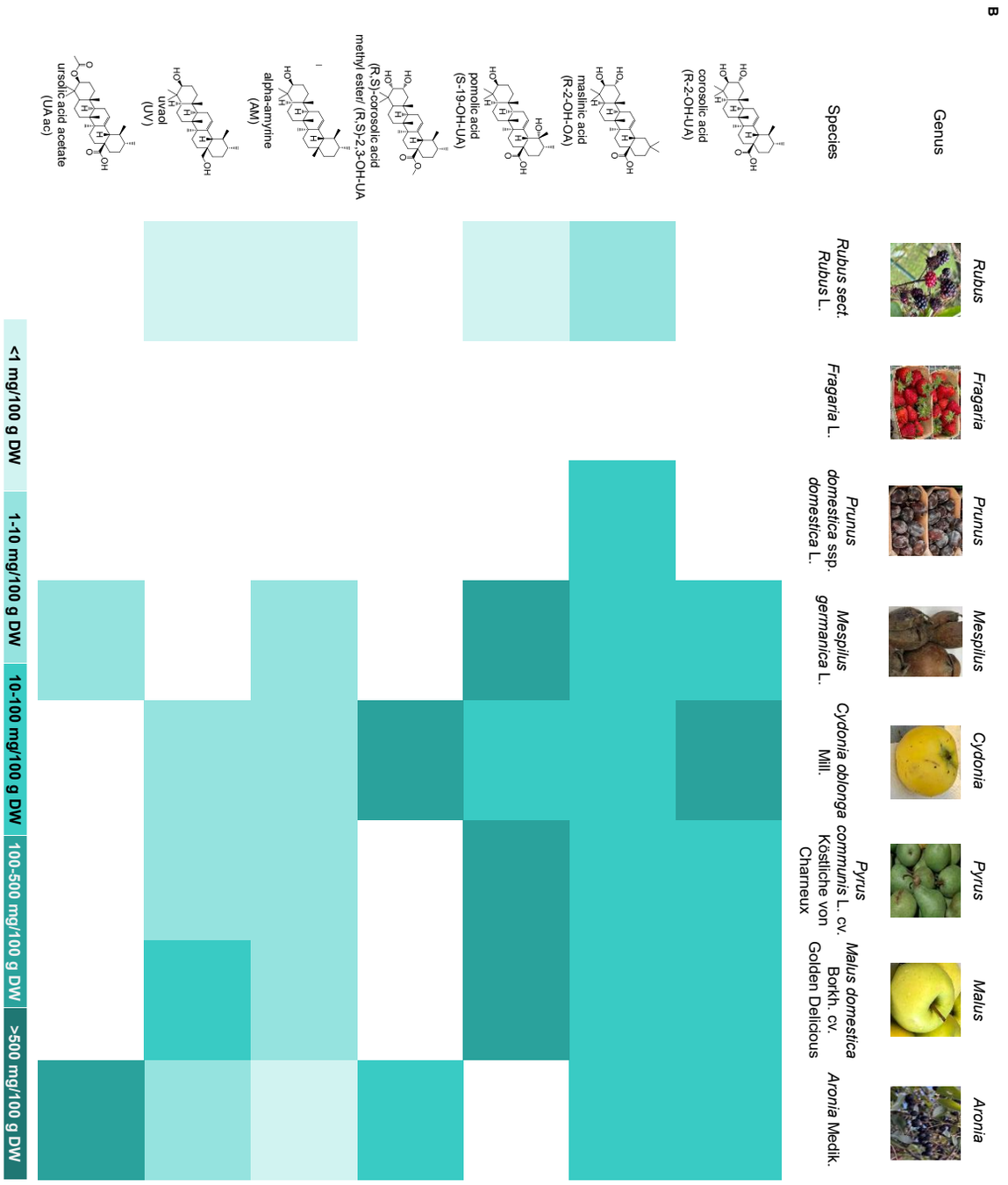


Fig. 4.2 (continued)

Interestingly, dihydroxylated TA were predominant in *Mespilus* (medlar) peel (370 mg S-19-OH-UA/100 g DW) and in whole fruits of *Pyracantha* (firethorn) (up to 260 mg R-2-OH-UA/100 g DW) belonging to the same subfamily (Tab. 1, S3).

Ursolic acid acetate (UA ac) was exclusively found in *Aronia*, *Rosa*, *Mespilus* and *Sorbus* with high concentrations in *Aronia* (110 mg/100 g DW) and *Rosa multiflora* (140 mg/100 g DW) (Tab. 4.1). Here, the relative ratio of UA ac to S-UA was about 40%, consistent with reports about the cuticular wax in *Aronia melanocarpa* [17]. This relatively high content of UA ac seems to be characteristic for *Aronia*. Moreover, (R,S)-2,3-dihydroxy-UA (an isomer of R-2-OH-UA), was exclusively detected in *Cydonia*, *Cotoneaster* and *Aronia* (Tab. 4.1, Fig. 4.3), a TA derivative which has not yet been quantified in *Rosaceae* so far.

The TA precursors alpha-amyrine (AM) and uvaol (UV) were found in all analyzed genera and species at lower concentrations compared to carboxylic acid-containing derivatives (Tab. 4.1) which is consistent with reports about fruits of *Rosa*, *Aronia* and *Malus* [17].

Based on the characteristic fragment ions (Tab. C3, Fig. C3), the vast majority of occurring TA are covered by the comprehensive GC–MS method used in this study covering 20 TA derivatives (Fig. 4.2, C1, C2) [8]. In addition to the analyzed TA, a few minor peaks were detected in the chromatograms, which are likely further TA derivatives based on the fragment ions (Fig. 4.3, C3).

In summary, TA are present in all *Rosaceae* genera. In all genera investigated, the TA pattern was dominated by monohydroxylated or dihydroxylated TA. Notably high TA concentrations of more than 100 mg/100 g DW were observed in the subfamily *Amygdaloideae*, with the highest levels found in edible fruits (>3 g TA/100 g DW in apple peel). Therefore, we focus in the following on *Rosaceae* fruits used as food.

4.3.2 TA CONCENTRATION IN EDIBLE FRUITS OF THE GENERA *RUBUS*, *PRUNUS*, *PYRUS* AND *MALUS*

Fruits from species of *Rubus*, *Prunus*, *Pyrus*, and *Malus* such as apples, pears, cherries and raspberries are globally cultivated and consumed in the US and Europe [25-26]. To investigate a genus-specific pattern, different species of these food-related genera were analyzed (Fig. 4.4): The number of detected TA derivatives as well as their concentrations were lowest in *Rubus*, followed by *Prunus* and *Pyrus*. The highest content was found in *Malus*, reaching up to 3 g/100 g DW.

In detail, within *Rubus*, both the pattern and TA concentrations determined in the whole fruits varied considerably among species, with concentrations being approximately 1000-fold lower than in *Malus* peel (Tab. 4.1, Fig. 4.4). For example, in *Rubus sect. Rubus* L. (blackberries), both mono- and dihydroxylated TA were detected, whereas in *Rubus phoenicolasius* (Japanese wineberry), only AM and S-UA were found, with total TA concentrations below 10 mg/100 g DW. In a previous study, Wang et al. quantified TA in *Rubus idaeus* L. and found similar TA concentrations. Additionally, they were able to demonstrate a high TA content variation among different varieties of this species [16].

Up to 300 mg TA/100 g DW were determined in the peel of *Prunus* (Tab. 4.1, Fig. 4.4). In individual species such as *Prunus spinosa* L. (blackthorn) and *Prunus domestica* subsp. *syriaca* (Borkh.) Janch. ex Mansf. (mirabelle), BA was found. Overall, *Prunus* showed highly diverse TA patterns, with the highest concentrations found in *Prunus spinosa* L. Berni et al. analyzed S-UA and OA in *Prunus avium* L. (sweet cherry), extracting both mesocarp and exocarp (whereas we analyzed only the exocarp, i.e. the peel), and found up to 10 mg S-UA/100 g fresh weight and 2.5 mg OA/100 g fresh weight [27].

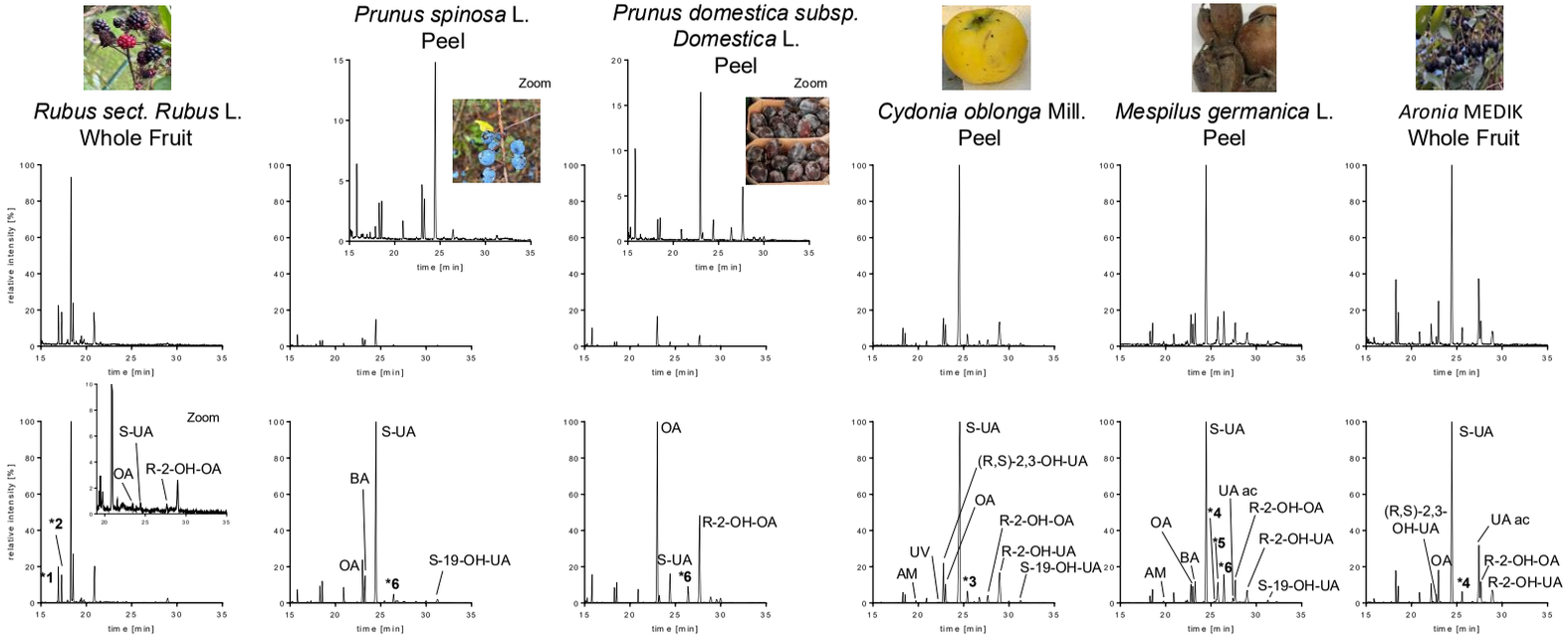


Fig. 4.3: GC-EI-MS chromatograms and mass spectra of edible fruits of the *Rosaceae* family (blackberries, blackthorn, plums, quince, medlar, chokeberries). Top: The TIC (50-900 amu) and (bottom) sum of SIM chromatograms of typical ions of TA (Tab. C2) are shown. Peaks of compounds which were not covered by the method are numbered *1-*6. S-UA: ursolic acid, OA: oleanolic acid, BA: betulinic acid, UV: uvaol, AM: alpha-amyrin, R-2-OH-OA: maslinic acid, R-2-OH-UA: corosolic acid, S-19-OH-UA: pomolic acid, (R,S)-2,3-OH-UA: (*R,S*)-corosolic acid methyl ester, UA ac: ursolic acid acetate

Interestingly, similar high TA concentrations were found in the peel of species of *Pyrus* (pear, up to 1.4 g TA/100 g DW) and *Malus* (apple) (Tab. 4.1, Fig. 4.4). However, the concentration of individual TA differed strongly between species within a genus (e.g., *Pyrus pyraster* L. (Du Roi) and *Pyrus communis* L.) and between the cultivars investigated (e.g. *Pyrus communis* L. cv. Köstliche von Charneux and Doyenné du Comice or *Malus domestica* Borkh. cv. Gala, Rubinette, Boskoop). These findings are consistent with previous studies: Andre et al. investigated 109 apple varieties and demonstrated a strong variation in TA levels [28]. Sun et al. showed that the absolute TA content in pear fruit and peel varied greatly across ten different pear varieties, with S-UA concentrations ranging from 2.5 to 7 mg/100 g fresh weight which is supporting our findings [21].

Previous studies suggest that differences in TA patterns and concentrations may be linked to the expression of key enzymes of TA biosynthesis, such as oxidosqualene cyclases (OSCs) and cytochrome P450 monooxygenases (CYP450s) [29-31]. In *Malus domestica*, OSC expression appeared to correlate positively with the concentration of S-UA and OA [31]. However, the abundance of these enzymes has only been investigated for few plants and the correlation of TA pattern with the expression of the enzymes of their synthesis needs to be evaluated in future studies.

In conclusion, when comparing species within one genus, the TA pattern was species- and cultivar-/variety-dependent. In addition, the calculated relative ratios of individual TA to S-UA differed greatly among species, indicating that *Rosaceae* genera do not show genus-specific TA patterns (Tab. S2, Fig. 4).

4.3.3 Impact of harvest locations and years on the TA concentration in cultivars of the same species

To investigate a cultivar-specific TA pattern, the TA content in cultivars of the same *Malus* species from different locations and harvest years was determined (Fig. 4.5). *Malus domestica* Borkh. cv. Elstar (Elstar apples) from three different farms showed comparable concentrations and relative ratios of individual TA to S-UA (Fig. 4.5-A,-D).

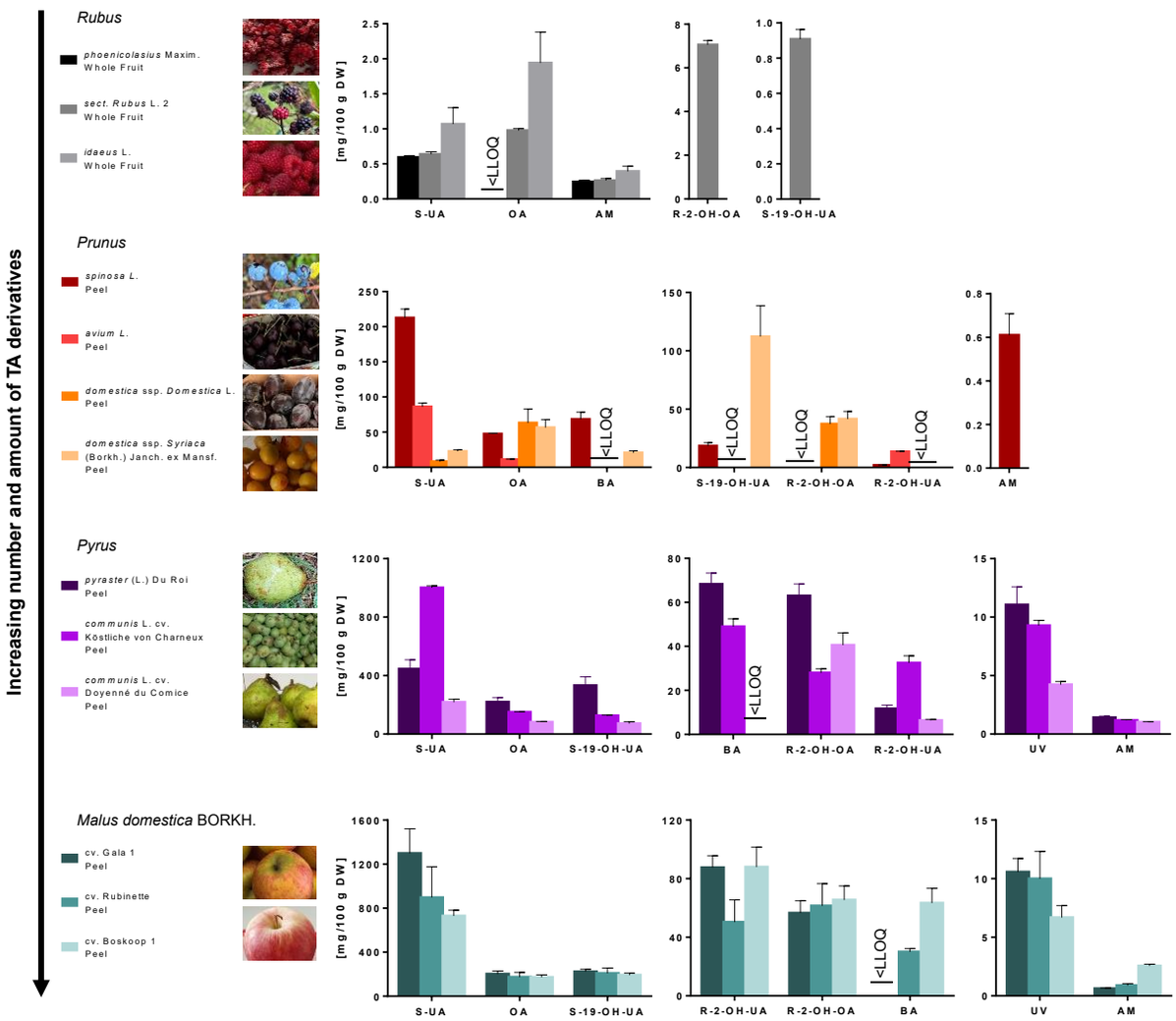
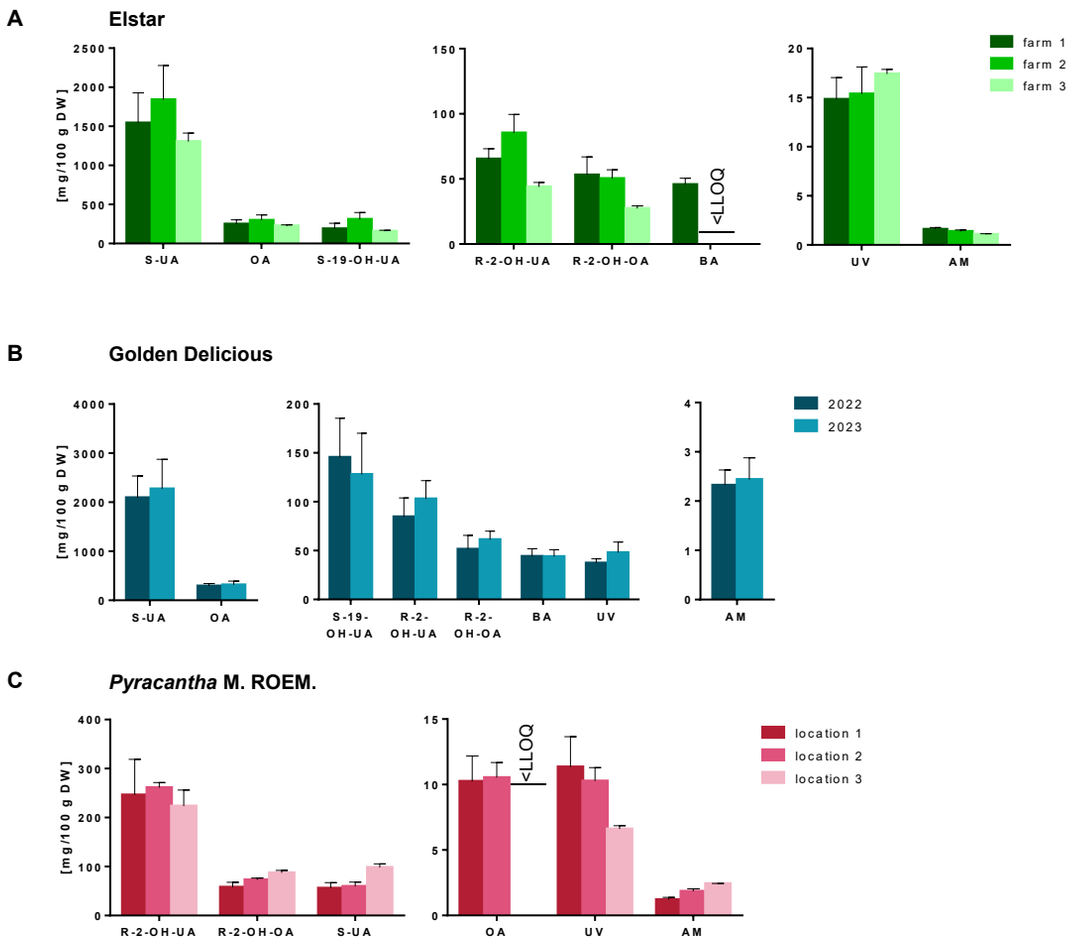


Fig. 4.4: TA concentrations in exemplary edible fruits of the genera *Rubus*, *Prunus*, *Pyrus* and *Malus*. 3-4 different varieties of the genera were analyzed and the amount of detected TA compared (mean ± SD, n=3). S-UA: ursolic acid, OA: oleanolic acid, BA: betulinic acid, UV: uvaol, AM: alpha-amyrin, R-2-OH-OA: maslinic acid, R-2-OH-UA: corosolic acid, S-19-OH-UA: pomolic acid.



D

TA	Relative Ratio TA/S-UA (mean ± SD) [%]				
	Elstar farm 1	Elstar farm 2	Elstar farm 3	Golden Delicious 2022	Golden Delicious 2023
OA	16.3 ± 1.6	16.1 ± 0.4	17.4 ± 0.6	14.1 ± 1.3	13.9 ± 0.2
BA	3.1 ± 0.9	<LLOQ	<LLOQ	2.2 ± 0.6	2.0 ± 0.2
R-2-OH-OA	3.6 ± 1.1	2.8 ± 0.3	2.1 ± 0.2	2.5 ± 0.3	2.7 ± 0.3
R-2-OH-UA	4.4 ± 0.7	4.7 ± 0.8	3.4 ± 0.3	4.1 ± 0.5	4.6 ± 0.3
AM	0.11 ± 0.04	0.08 ± 0.01	0.08 ± 0.01	0.11 ± 0.02	0.11 ± 0.02
UV	1.0 ± 0.2	0.85 ± 0.06	1.3 ± 0.1	1.8 ± 0.3	2.1 ± 0.1
S-19-OH-UA	12 ± 4	17 ± 2	12.0 ± 0.1	7 ±1	6 ± 1

Fig. 4.5: Top: TA concentrations (mean ± SD, n=3) in the peel of *Malus domestica* cv. Elstar from three different farms (A), in the peel of *Malus domestica* cv. Golden Delicious from two different harvest periods (B), in three different plants of *Pyracantha* (C). Bottom: The percentage of TA relative to S-UA content (mean ± SD) in *Malus domestica* (D). S-UA: ursolic acid, OA: oleanolic acid, BA: betulinic acid, UV: uvaol, AM: alpha-amyrin, R-2-OH-OA: maslinic acid, R-2-OH-UA: corosolic acid, S-19-OH-UA: pomolic acid.

Viškelis et al. also analyzed the effect of apple-tree location across different countries on the TA content and found that the cultivar Auksis showed a significant difference in TA content, whereas the cultivar Ligol did not [32]. However, this study compared samples from different countries, while the samples in our study originated from a single region.

The analysis of cv. Golden Delicious apples from two harvest years (2022 and 2023), originating from the same farm, also revealed comparable TA concentrations and consistent relative ratios (Fig. 4.5-B,-D). Similar TA concentrations and relative ratios were also found in the cultivars Gala 1-2. An earlier study reported variations in S-UA content between harvest years in the apple cultivars Discovery and Aroma, while it remained constant in Gloster [33].

Interestingly, the fruits of *Pyracantha* (firethorn), which were collected at three locations and which is mainly cultivated as an ornamental plant, showed similar high TA concentrations (exceeding 400 mg/100 g DW) (Fig. 4.5-C). The dihydroxylated TA R-2-OH-UA was identified as the main TA (220–250 mg/100 g DW), which was not described so far.

For the same cultivars of *Malus domestica* and for *Pyracantha* the TA pattern, concentrations, and relative ratios were comparable and characteristic regardless of the harvest year or location (from one region). Unlike previous findings describing pronounced variability in TA concentrations [32-33], our study showed stable TA levels for the apple cultivars Elstar, Golden Delicious, and Gala.

The high content and structural diversity of TA in *Rosaceae* fruits demonstrates that they are relevant food ingredients. Despite their low bioavailability [13-14], TA have been described to exhibit anti-inflammatory and antioxidant activities. In a mouse model it has been reported that TA protect against carbon tetrachloride–induced liver injury [34] and reduce oxidative stress and inflammation by modulating MAPK signaling [35]. Regarding anti-inflammatory activities, S-UA has been shown to induce apoptosis by activating TNF-α-induced, caspase-3 mediated pro-apoptotic pathways, while inhibiting NF-κB-induced anti-apoptotic pathways in the human B16F-10 melanoma cell line [36]. In addition, studies in

human T-cell leukemia cell lines have demonstrated that TA lead to an inhibition of NF-κB activation suppressing cyclooxygenase-2 expression, thereby modulating inflammatory signaling pathways [37]. Furthermore, S-UA was shown to reduce the expression of matrix metalloproteinase-9 in the rat C6 glioma cell line, inhibiting tumor cell migration and invasion [38].

These biological activities highlight the importance of a comprehensive analysis of the TA content in food, as described for a large number of *Rosaceae* here.

Species	*Filipendula ulmaria* (L.) Maxim.	*Rubus phoenicolasius* Maxim.	*Rubus sect. Rubus* L. 1	*Rubus sect. Rubus* L. 2	*Rubus idaeus* L.	*Rosa canina* L. 1	*Rosa canina* L. 2	*Rosa rugosa* Thunb.	*Rosa multiflora* Thunb.	*Fragaria* L.
Analyzed	Dry infructescence	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit	Peel	Whole Fruit	Peel	Whole Fruit	Whole Fruit
S-UA	33.6 ± 0.9	0.59 ± 0.01	1.5 ± 0.1	0.64 ± 0.04	1.1 ± 0.2	12 ± 3	250 ± 50	630 ± 60	90 ± 15	0.55 ± 0.09
OA	<LLOQ	<LLOQ	2.2 ± 0.1	0.98 ± 0.03	1.9 ± 0.4	9.4 ± 2.8	890 ± 210	110 ±15	90 ± 4	<LLOQ
BA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	28 ± 2	104 ± 8	<LLOQ
R-2-OH-UA	53 ± 10	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	47 ± 2	20 ± 4	14 ± 3	<LLOQ
R-2-OH-OA	35 ± 2	<LLOQ	<LLOQ	7.1 ± 0.2	<LLOQ	<LLOQ	56 ± 4	13 ± 5	81 ± 12	<LLOQ
S-19-OH-UA	<LLOQ	<LLOQ	2.3 ± 0.2	0.91 ± 0.05	<LLOQ	20 ± 2	<LLOQ	48 ±10	<LLOQ	<LLOQ
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	<LLOQ	0.24 ± 0.01	0.60 ± 0.09	0.26 ± 0.03	0.39 ± 0.07	0.63 ± 0.13	1.9 ± 0.3	0.48 ± 0.08	1.7 ± 0.2	<LLOQ
UV	2.2 ± 0.1	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	4.5 ± 0.6	<LLOQ	<LLOQ
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	140 ± 80	<LLOQ

Tab. 4.1 (continued)

Species	*Sorbus aucuparia* L.	*Sorbus aria* (L.) Crantz	*Pyrus communis* L. cv. Williams Christ	*Pyrus pyraster* (L.) Du Roi	*Pyrus communis* L. cv. Köstliche von Charneux	*Pyrus communis* L. cv. Doyenné du Comice	*Malus domestica* Borkh. (Apple pomace)	*Malus domestica* Borkh. cv. Galmac	*Malus domestica* Borkh. cv. Santana	*Malus domestica* Borkh. cv. Elstar 1
Analyzed	Whole Fruit	Whole Fruit	Peel	Peel	Peel	Peel	Pomace	Peel	Peel	Peel
S-UA	410 ± 40	22 ± 1	197 ± 7	450 ± 60	1000 ± 10	220 ± 20	5200 ± 700	870 ± 150	1900 ± 200	1500 ± 400
OA	130 ± 20	13 ± 1	46 ± 6	220 ± 30	150 ± 3	82 ± 4	710 ±100	250 ± 40	280 ± 30	250 ± 50
BA	<LLOQ	97 ± 14	44 ± 8	68 ± 5	49 ± 4	<LLOQ	<LLOQ	37 ± 4	<LLOQ	46 ± 5
R-2-OH-UA	13 ± 2	<LLOQ	<LLOQ	12 ± 2	32 ± 3	6.3 ± 0.5	620 ± 80	53 ± 9	56 ± 7	65 ± 8
R-2-OH-OA	50 ± 15	<LLOQ	18 ± 3	63 ± 5	28 ± 2	41 ± 6	280 ± 30	60 ± 9	24 ± 3	53 ± 14
S-19-OH-UA	190 ± 10	<LLOQ	63 ± 4	330 ± 60	126 ± 4	70 ± 10	620 ± 80	<LLOQ	150 ± 20	190 ± 70
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	4.8 ± 0.4	<LLOQ	0.98 ± 0.50	1.4 ± 0.1	1.18 ± 0.04	1.03 ± 0.03	3.9 ±0.4	0.63 ± 0.03	1.1 ± 0.4	1.6 ± 0.2
UV	18 ± 2	<LLOQ	4.0 ± 0.6	11 ± 2	9.3 ± 0.4	4.2 ± 0.3	49 ± 5	5.4 ± 0.6	17 ± 3	15 ± 2
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

Tab. 4.1 (continued)

Species	*Malus domestica* Borkh. cv. Elstar 2	*Malus domestica* Borkh. cv. Elstar 3	*Malus domestica* Borkh. cv. Gala 1	*Malus domestica* Borkh. cv. Gala 2	*Malus domestica* Borkh. cv. Rubinette	*Malus domestica* Borkh. cv. Golden Delicious 1	*Malus domestica* Borkh. cv. Golden Delicious 2	*Malus domestica* Borkh. cv. Topaz	*Malus domestica* Borkh. cv. Delbarestivale	*Malus domestica* Borkh. cv. Wellant 1
Analyzed	Peel	Peel	Peel	Peel	Peel	Peel	Peel	Peel	Peel	Peel
S-UA	1800 ± 400	1300 ± 100	1300 ± 200	1300 ± 200	900 ± 300	2300 ± 600	2100 ± 400	1180 ± 70	1400 ± 300	810 ± 30
OA	300 ± 70	230 ± 10	200 ± 30	200 ± 20	170 ± 40	310 ± 80	300 ± 50	168 ± 3	210 ± 10	170 ± 20
BA	<LLOQ	<LLOQ	<LLOQ	33 ± 1	30 ± 2	44 ± 7	44 ± 8	27 ± 1	31 ± 3	36 ± 7
R-2-OH-UA	85 ± 15	44 ± 3	88 ± 8	55 ± 5	50 ± 15	100 ± 20	84 ± 20	76 ± 4	30 ± 2	62 ± 5
R-2-OH-OA	50 ± 7	27 ± 2	57 ± 8	38 ± 7	61 ± 15	61 ± 9	51 ± 15	40 ± 4	28 ± 3	67 ± 1
S-19-OH-UA	310 ± 90	160 ± 10	220 ± 20	170 ± 20	210 ± 50	130 ± 40	150 ± 40	81 ± 5	150 ± 10	220 ± 30
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	1.4 ± 0.2	1.1 ± 0.1	0.62 ± 0.04	0.79 ± 0.05	0.88 ± 0.16	2.4 ± 0.4	2.3 ± 0.3	0.64 ± 0.03	1.0 ± 0.1	0.86 ± 0.20
UV	15 ± 3	17 ± 1	10.6 ± 1.2	11 ± 1	10 ± 2	48 ± 10	37 ± 4	12 ± 1	12 ± 1	5.8 ± 0.7
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

Tab. 4.1 (continued)

Species	*Malus domestica* Borkh. cv. Wellant 2	*Malus domestica* Borkh. cv. Wellant 3	*Malus domestica* Borkh. cv. Fuji	*Malus domestica* Borkh. cv. Boskoop 1	*Malus domestica* Borkh. cv. Boskoop 2	*Malus domestica* Borkh. cv. Boskoop 3	*Malus domestica* Borkh. cv. Falstaf	*Malus domestica* Borkh. (cv. unknown)	*Malus domestica* Borkh. cv. Mairac	*Malus domestica* Borkh. cv. Berlepsch
Analyzed	Peel	Peel	Peel	Peel	Peel	Peel	Peel	Peel	Peel	Peel
S-UA	970 ± 50	980 ± 40	1600 ± 500	730 ± 50	93 ± 7	880 ± 30	1700 ± 100	1500 ± 300	530 ± 60	780 ± 50
OA	330 ± 10	210 ± 20	210 ± 40	170 ± 20	27 ± 3	160 ± 6	250 ± 20	290 ± 40	119 ± 4	170 ± 4
BA	50 ± 6	37 ± 2	32 ± 4	63 ± 10	53 ± 2	39 ± 2	39 ± 4	52 ± 4	28 ± 4	40 ± 9
R-2-OH-UA	95 ± 9	102 ± 6	110 ± 20	90 ± 15	4.9 ± 0.6	78 ± 5	99 ± 9	55 ± 8	95 ± 22	35 ± 3
R-2-OH-OA	84 ± 7	81 ± 6	60 ± 9	65 ± 10	26 ± 3	58 ± 5	51 ± 5	43 ± 3	51 ± 3	59 ± 9
S-19-OH-UA	270 ± 20	330 ± 60	43 ± 10	193 ± 17	74 ± 10	150 ± 20	107 ± 12	515 ± 2	180 ± 10	210 ± 20
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	1.2 ± 0.1	0.99 ± 0.13	1.4 ± 0.3	2.6 ± 0.1	2.9 ± 0.6	1.4 ± 0.3	1.1 ± 0.1	1.1 ± 0.1	0.45 ± 0.05	0.77 ± 0.18
UV	7.7 ± 0.5	6.3 ± 0.6	40 ± 8	7.0 ± 1.0	3.0 ± 0.2	5.8 ± 0.2	15 ± 1	14 ± 2	3.8 ± 0.1	5.6 ± 0.4
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

Tab. 4.1 (continued)

Species	*Malus domestica* Borkh. cv. James Grieve		*Cotoneaster horizontalis* Decne.	*Cotoneaster dammeri* C.K.Schneid.	*Cotoneaster bullatus* Bois	*Cotoneaster dielsianus* E.Pritz.	*Aronia* Medik.	*Pyracantha* M.Roem.	*Pyracantha* M.Roem.	*Pyracantha* M.Roem.
Analyzed	Peel		Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit
S-UA	1500 ± 400		1100 ± 100	85 ± 9	600 ± 70	320 ± 40	270 ± 10	56 ± 11	59 ± 9	98 ± 8
OA	210 ± 40		120 ± 10	27 ± 4	73 ± 8	74 ± 11	50 ± 2	10 ± 2	11 ± 1	<LLOQ
BA	37 ± 6		<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
R-2-OH-UA	90 ± 20		112 ± 5	52 ± 7	70 ± 12	20 ± 3	41 ± 3	250 ± 70	260 ± 10	220 ± 30
R-2-OH-OA	46 ± 6		48 ± 2	46 ± 6	44 ± 11	49 ± 7	58 ± 2	58 ± 10	73 ± 4	87 ± 5
S-19-OH-UA	74 ± 17		<LLOQ	87 ± 16	<LLOQ	17 ± 1	<LLOQ	<LLOQ	<LLOQ	<LLOQ
R,S-2,3-OH-UA	<LLOQ		<LLOQ	<LLOQ	7.7 ± 1.3	<LLOQ	12.0 ± 0.4	<LLOQ	<LLOQ	<LLOQ
AM	0.68 ± 0.11		3.0 ± 0.3	1.9 ± 0.1	3.8 ± 0.6	2.1 ± 0.1	0.97 ± 0.06	1.2 ± 0.2	1.9 ± 0.2	2.4 ± 0.1
UV	6.0 ± 1.0		17 ± 1	9.0 ± 1.0	19 ± 1	10 ± 1	5.8 ± 0.5	11 ± 2	10 ± 1	6.6 ± 0.2
UA ac	<LLOQ		<LLOQ	<LLOQ	<LLOQ	<LLOQ	110 ± 4	<LLOQ	<LLOQ	<LLOQ

4.4 Conclusion

This is the first report providing comprehensive analyses and comparison of TA patterns of 17 different *Rosaceae* genera. High TA levels were found in edible fruits of the *Amygdaloideae*, underlining their relevance as important (dietary) sources of these compounds. The highest levels (>3 g/100 g DW) were found in apple peels (*Malus domestica*), while TA concentrations were low in *Rubus* (< 10 mg TA/100 g DW). While TA patterns were dominated by mono- and dihydroxylated derivatives, no clear genus- or species-specific TA patterns were identified; however, TA composition appeared to be stable within individual cultivars or varieties. Overall, these findings expand the knowledge of TA diversity in *Rosaceae* and highlight the importance of further research, as the fruits are widely consumed and a relevant source of TA, while their TA patterns are of interest for chemotaxonomic investigations as well as for pharmacological applications.

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CHAPTER 5

5.1 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

TA are a highly diverse class of secondary plant metabolites that can occur as free acids or as conjugates, such as acylates and glycosides [1-3]. Although TA are primarily associated with protective properties against herbivors and dehydration in plants [2-3], they represent an important class of compounds in traditional herbal medicine and modern pharmacological research. Both TA and their modified derivatives have been reported to exhibit hepatoprotective [4], antibacterial [5], antidiabetic [6] and anti-inflammatory [7] properties. Consequently, there is a growing interest in developing efficient extraction strategies from natural sources, as well as in elucidating TA patterns and their distribution across the plant kingdom, which requires comprehensive analytical approaches and methodologies.

Within this thesis, a comprehensive analytical strategy was established enabling the detailed analysis of TA in Rosaceae fruits. An extraction strategy was developed that enables the isolation of TA from plant material in high purity and yield. Furthermore, the protocol was adapted for the quantitative analysis of TA patterns in *Rosaceae* fruits, and its reliability and reproducibility were thoroughly assessed. A GC-MS method was developed for the simultaneous qualitative and quantitative analysis of a wide range of TA derivatives and allowed the characterization of TA patterns in plants.

In summary, the developed strategy provides both qualitative and quantitative analytical access to plant-derived TA and enables their extraction in high yields and purities, highlighting plants as valuable natural sources of TA.

Chapter 2 describes the extraction strategy developed for isolating TA from *Rosaceae* fruits. For the extraction of TA from *Rosaceae* fruits, the selection of suitable solvents was crucial. These secondary plant metabolites are present as

minor compounds within the plant matrix. The solvents were selected based on the solubility of TA, which is low in water [8] but high in organic solvents. They were selected to faciliate the removal of additional matrix components during extraction, thereby improving extract purity and minimizing natrix effects during instrumental analysis. The initial extraction step, involving the removal of water-soluble matrix components, was essential to separate major compounds such as carbohydrates. While TA, particularly S-UA, exhibit high solubility in methanol and ethanol, and these solvents have been widely applied as extraction solvents [9-10], their miscibility with water made them unsuitable for the developed protocol. Therefore, the medium-polar solvent ethyl acetate, which also provides good solubility for TA but is immiscible with water, was selected. It efficiently extracted TA, whereas more polar compounds were subsequently removed by liquid–liquid extraction with water.

For obtaining extracts of high purity, a degreasing step using cyclohexane was required to remove lipids, which are primarily present in the wax layer. During the different extraction and purification steps, as well as through the use of solvents with varying polarity, a partial loss of TA could not be completely avoided. This was caused due to multiple transfer steps and the relatively high solubility of TA in organic solvents [11-12]. Nevertheless, each purification step increased extract purity and (up to 70 g TA/100 g extract) and yield (>2 g TA/100 g dry apple peel) within 3–4 hours, demonstrating the effectiveness of the strategy. In contrast, only trace amounts of TA were detected in the apple pulp. Simple detection strategies, including TLC and GC-FID, allowed for the rapid detection and monitoring of extraction efficiency through comparison with TA standards. Unlike many previously reported approaches, the described extraction strategy can be implemented in minimally equipped laboratories, as it is less time-consuming and technically demanding [9, 13-15]. The described protocol can also be used to isolate high quantities of TA from food by-products such as apple pomace. The extraction results highlight the potential of food (by-products), as significant amounts of TA can be isolated for subsequent use in chemical and pharmacological applications.

In Chapter 3, the extraction protocol was adapted and scaled down for analytical purposes to enable comprehensive qualitative and quantitative characterization of TA in *Rosaceae* fruits. The procedure was further optimized to minimize TA losses during analysis; accordingly, the cyclohexane purification step was omitted, simplifying the workflow and improving time efficiency. Suitable internal standards were added to determine the extraction efficiency. Of note, deuterated internal standards were not available; therefore, structurally similar compounds not being present in the fruit matrix were chosen. The addition of TA standards prior to extraction with ethyl acetate showed good recovery for both naturally occurring and modified TA derivatives. Intra- and interday precision were evaluated by comparing the TA concentrations of apple samples analyzed by GC-MS and GC-FID, showing excellent agreement. Furthermore, the sufficient excess of derivatization agents during the derivatization of biological samples was confirmed. Previously developed instrumental-analytical methods for TA were limited to a few target compounds, such as S-UA and OA, or did not allow quantitative investigations in untargeted approaches [11, 16-19].

Based on authentic TA standards, GC-FID and GC-MS methods were established in this thesis, enabling both the characterization of *Rosaceae* samples and the quantitative determination of more than 20 TA derivatives in plant extracts, many of which were isobaric compounds. Consequently, most TA derivatives exhibited similar fragmentation behavior in MS detection, so that a sufficient chromatographic separation was crucial. This observation is consistent with previously reported GC–MS and LC–MS methods [20-21].

Evaluation of the fragmentation behavior of TA and the selection of suitable fragments as quantifier and qualifier ions, in combination with the retention times, enabled a sensitive and robust analytical approach. Application of the developed method to apple samples allowed the determination of both low- and high-abundant TA and revealed S-UA to be the most abundant TA in *Malus*. The dominance of S-UA in *Malus* is consistent with previous findings [9, 22]. However, the presence of TA precursors at detectable levels has rarely been addressed,

but has increasingly come into focus in recent years [20], highlighting the need for more comprehensive analytical studies such as the present work.

This thesis aimed to characterize the TA pattern in a broad range of *Rosaceae* fruits (chapter 4). GC-MS analyses of quantitative TA patterns in fruits from 17 different genera and 29 species of the *Rosaceae* family focused on common fruits occurring in Northern Europe, while also evaluating phylogenetic relationships and the influence of vegetation period and region. TA were detected in the fruits of all analyzed *Rosaceae* samples, with higher concentrations found in the subfamily *Amygdaloideae* compared to *Rosoideae*. To date, no comprehensive studies have compared TA patterns across different subfamilies and genera. Most studies have focused on single plant species or on comparisons of several species within a single genus.

Of note, TA contents were determined in the peels of fruits with removable skins, whereas in small berries, the whole fruits were analyzed. TA patterns were dominated by mono- and dihydroxylated derivatives. A broad concentration range of TA was observed among the investigated fruits, with particularly low concentrations (<1 mg/100 g DW) in whole fruits of strawberries (*Fragaria*) and high concentrations in apple peels (*Malus*), reaching >3 g TA/100 g DW, where S-UA represented the main derivative. Differences in TA content have been reported between genera and species of the *Rosaceae* family [22-24], with *Malus* being described as a particularly TA-rich genus [25]. However, systematic investigations are still completely lacking, and some genera, such as *Mespilus*, have so far received little to no attention and were described for the first time within this thesis. TA precursors were found in all analyzed genera and species, but at lower concentrations compared to the corresponding carboxylic acid-containing derivatives. Cultivar-specific TA patterns were found in *Malus*, independent of harvest year or growing location. However, *Rosaceae* genera did not exhibit genus-specific TA patterns, as the concentrations and ratios of individual TA to the dominating ursolic acid varied strongly between species. This work provides an overview of the TA patterns in the most food-relevant genera. The developed analysis could be extended in future studies to other genera and

growth conditions, potentially contributing to their chemotaxonomic differentiation or authenticity evaluation of food.

In total, this thesis represents the first comprehensive report including more than 20 derivatives for the TA analysis in food-relevant *Rosaceae* species and expand the current knowledge of TA diversity within this plant family. The development of a comprehensive analytical approach not only enabled the detailed characterization of TA in food-relevant *Rosaceae* species but also provided insights into their potential as rich natural sources of TA. However, systematic investigations of TA patterns across different genera are still lacking and would be valuable for advancing chemotaxonomic studies in the future.

The developed extraction protocol could, be applied to the large-scale isolation, purification, and utilization of TA from *Rosaceae* fruits for chemical and pharmacological applications. Future studies could employ the developed analytical methods to extend the characterization to a broader range of plant families and food-relevant matrices. This would contribute to a deeper understanding of TA structural diversity and help to identify particularly TA-rich sources. In addition to the analyzed TA, a few minor peaks were detected in the chromatograms of species such as *Prunus*, *Aronia*, and *Mespilus*, which are likely further TA derivatives based on their fragment ions. These detected compounds could be further structurally elucidated using complementary analytical techniques, such as LC-MS/MS or NMR spectroscopy.

All in all, this thesis expands the knowledge of the occurrence of TA in food-relevant *Rosaceae* fruits. The combination of novel extraction strategies and a comprehensive GC-MS method enabled the characterization and quantification of a broad range of TA derivatives in plants.

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SUMMARY

TA are a diverse class of triterpenoids found in plants. These secondary plant metabolites are discussed to contribute to plant physiology, for example by protecting fruits against dehydration and defending against herbivores. Their biological role, however, remains poorly understood, although (research) interest for plant-derived raw materials increased in recent years, as they can be used in fields such as polymer chemistry and pharmacology. TA are traditionally used in herbal medicine and are attributed with various biological activities, including antibacterial, antidiabetic, and anti-inflammatory properties. Consequently, research interest for the quantitative characterization, extraction, and usage of TA is increasing. Although individual plant species are known for their high and/or specific TA content, strategic and comprehensive investigations across plant families have not been performed so far.

The aim in **Chapter 2** was to develop a simple and efficient strategy for the isolation, purification, and concentration of TA from plant material (apples, *Malus*), enabling a rapid and low-hazardous extraction. The solvents for the solid–liquid extraction were carefully chosen to allow the removal of other matrix compounds, such as carbohydrates and lipids, and to permit the implementation in educational institutions. The described protocol first involved the removal of polar matrix compounds by boiling the plant material in hot water. Subsequent extraction with the medium-polar solvent ethyl acetate efficiently isolated the TA from the plant material. Co-extracted lipids were removed in a degreasing step by boiling in cyclohexane. The residue was dissolved in ethanol and then further analyzed by TLC or FID. For chromatographic characterization, a simple and rapid detection method (within 10 minutes) was developed using TLC, allowing the detection and differentiation of mono- and dihydroxylated TA. This method could be of particular advantage in educational contexts. To determine the quantitative TA pattern of plant extracts, a GC-FID method was developed,

including a derivatization step during sample preparation. Although more time-consuming, this approach offers the advantage of determining both, concentration yields and purity, and can be used to monitor the extraction process by comparing the peak areas and heights of extracts from the individual protocol steps.

Investigation of different apple cultivars and fruit parts showed that the majority of TA are located in the peels, with only trace amounts found in the pulp, and that TA yields depend on the cultivar analyzed. The strategy achieved high extraction yields of up to >2 g TA/100 g dry apple peel and high purities of up to 70 g TA/100 g extract in a total extraction time of 3-4 hours. The developed method showed results comparable to extraction approaches using hot solvents, such as Soxhlet extraction. Consequently, the protocol represents an efficient strategy to isolate substantial amounts of TA from natural sources, including food by-products such as apple pomace which is generated during juice production and consists of pulp, peel, and seeds.

In order to comprehensively investigate the TA patterns of *Rosaceae* fruits, a GC-MS method was developed in **Chapter 3** for the simultaneous characterization and quantification of TA derivatives occurring at low and high concentrations. Sample preparation was based on the extraction protocol developed in Chapter 2, however, the degreasing step was excluded. For method optimization, 20 different naturally occurring and modified TA derivatives were used to enable broad structural characterization of the plant extracts.

Chromatographic separation using a DB-5 column (40 m, 0.18 mm ID, 0.25 µm film) within a total run time of 40 minutes allowed the selective detection of naturally occurring and modified TA derivatives. A sufficient chromatographic separation was crucial for the quantification of TA because most derivatives exhibited similar fragmentation behavior. Mass spectrometric detection following EI (70 eV) enabled structural characterization and sensitive quantification of the TA derivatives. Quantification was performed by external calibration using internal standards. Analysis of TA in apple peels of different varieties revealed

differences in TA concentrations among the cultivars Fuji, Discovery, and Golden Delicious, with levels of up to 3.5 g TA/100 g DW. Ursolic acid (up to 2.9 g/100 g DW) was the predominant TA, followed by oleanolic acid (up to 500 mg/100 g DW) and pomolic acid (up to 130 mg/100 g DW). The recovery of added TA was good (70–140%), and the measured concentrations of naturally occurring TA agreed well (75–125%) with those obtained by GC-FID analysis, demonstrating the accuracy of the developed method. The method also showed high precision, with low intra-day and inter-day variability (≤ 22%).

The use of the developed analytical approach enabled, for the first time, the characterization of a large set of 17 *Rosaceae* genera and 29 species in **Chapter 4**. TA were detected in all investigated genera, but patterns and concentrations varied depending on the species: The patterns were dominated by mono- or dihydroxylated derivatives. Notably high TA concentrations of more than 100 mg/100 g DW were observed in edible fruits of the subfamily *Amygdaloideae*, with the highest levels found in apple peels (>3 g TA/100 g DW), whereas very low TA concentrations (<1 mg/100 g DW) were found in fruits of the subfamily *Rosoideae*, such as strawberries (*Fragaria*). In food-relevant species, the number of detected TA derivatives and their concentrations were lowest in *Rubus*, followed by *Prunus* and *Pyrus*. The highest content was found in *Malus*, reaching up to 3 g TA/100 g DW. No clear genus- or species-specific TA patterns were identified. For the same cultivars of *Malus domestica*, TA patterns and concentrations were comparable and characteristic, regardless of harvest year or location within a region.

Overall, this thesis provides an efficient approach for the extraction, characterization, and quantification of TA in food-relevant *Rosaceae* species. The developed extraction protocol revealed *Rosaceae* fruits to be a relevant source of TA. The detailed analytical approach enhanced the understanding of TA diversity, patterns, and concentrations in several *Rosaceae* species, which highlights the potential for their future use in chemotaxonomic studies or resource-efficient chemical and pharmacological applications.

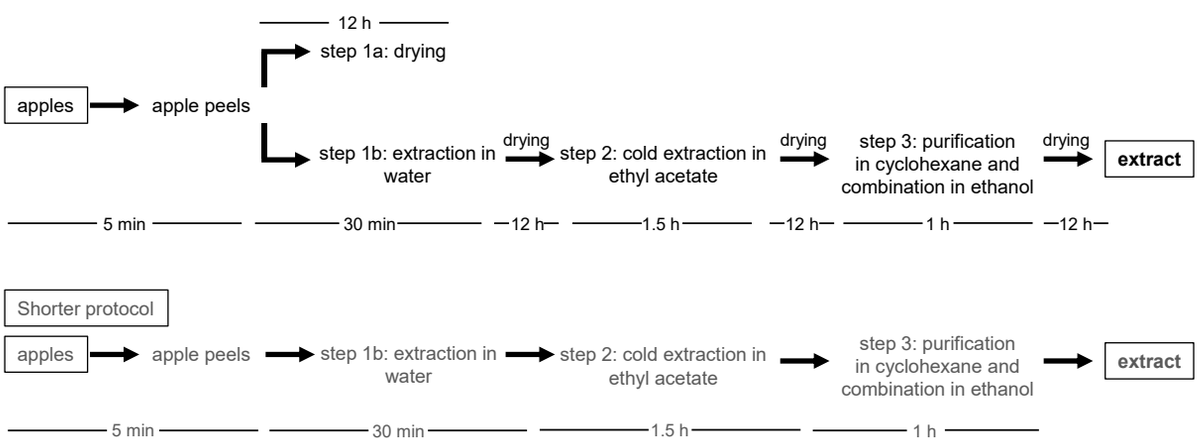
APPENDIX

A APPENDIX FOR CHAPTER 2

Appendix A.1

Detailed protocol of the TA extraction procedure – instructions for students

The following detailed protocol should be used for TA extraction. An interactive student's handout is recommended to be used for a more illustrative presentation of TA extraction in the classroom. Important: The steps marked with an asterisk (*) can be skipped if only little time is available. The following timeline shows the time required for extraction. The shorter protocol allows the extraction to be performed in 3 hours, with slightly lower extraction yields.



Fruit preparation and removal of polar compounds

- Peel 2-3 fresh apple fruits.

a.) (*) Determination of dry weight (DW). The dry weight is needed as a reference for information on TA content in [g/100g DW]:

- Weigh out a dry glass dish.
- Weigh exactly an amount of ~20 g (± 1 g) peel on a weighed glass dish and dry it in an oven at 80 °C (± 5 °C) over night (~12 hours or longer).
- Weigh out the glass dish including the dried material and calculate the dry weight (formula 1):

$$DW\ \left[\frac{g}{100\ g}\right] = \frac{weight\ (dish + dried\ peels)[g] - weight\ (dish)[g]}{weight\ (fresh\ apple\ peels)[g]} * 100 \left[\frac{g}{100\ g}\right] \quad (1)$$

b.) Preparation of plant material for extraction. With this step the water-soluble components are removed.

This step is carried out simultaneously with 1a:

- Weigh exactly an amount of 20 g peel (± 1 g) in a (Erlenmeyer) flask and add 100 mL water.
- Boil the solution for 10 min (± 2 min), using an electrical plate or gas burner.
- Let the suspension cool down.
- Place a coffee filter (e.g. boat shaped 9×15 cm corresponding to size 2 for European coffee machines, or a basket-typ filter of similar size) in a funnel (diameter about 10 cm) and filter the suspension.
- Discard the aqueous solution.

TA extraction

In this step the components which are soluble in organic solvents are extracted from the peels from 1b.

- (*) Place the peels from 1b on a glass dish and dry them in an oven at 80 °C (± 5 °C) over night (~12 hours or longer).
- Transfer the peels into an (Erlenmeyer) flask.
- Add 100 mL ethyl acetate and a magnetic stirrer.
- Place the flask on a magnetic stir plate and let it stir for ~30 (±5) min.
- Put a funnel (diameter about 10 cm) on top of a separation funnel (capacity volume 250 mL).
- Place a small filter paper (e.g. diameter 18.5 cm) in the funnel and filter the organic liquid extract into the separation funnel.
- Add 30 mL H_2O.
- Shake the two-phase mixture (1. liquid liquid extraction).
- Leave the separation funnel until phase separation.
- Discard the lower aqueous phase.
- Add again 30 mL H_2O (2. liquid liquid extraction) to the upper organic phase and shake.

- Leave the separation funnel until phase separation.
- Discard the lower phase.
- Weigh out a dry round bottom flask (capacity volume 250 mL).
- Transfer the upper phase into the round bottom flask.
- Use a rotary evaporator for the removal of organic solvent (evaporation under reduced pressure, 40 °C, 240 mbar), if not available: heat and evaporate it gently till almost no solvent remains.
- (*) Dry the flask with the residue in an oven at 80 °C (±5 °C) over night (12 hours or longer).
- (*) Calculate the dried raw extract (formula 2).

$$yield\left[\frac{g\ raw\ extract}{100\ g\ dried\ peel}\right] = \frac{weight\ (flask + raw\ extract\ )[g] - weight\ (flask)[g]}{weight\ (fresh\ apple\ peels)[g] * DW\left[\frac{g\ dried\ peels}{100\ g\ fresh\ peels}\right]} * 100\left[\frac{g}{100\ g}\right] \quad (2)$$

Purification of TA extract: Removal of non polar compounds

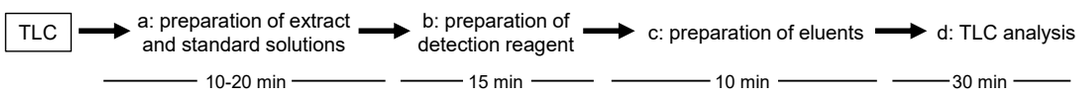
In this step the TA extract is purified, using the non-polar solvent cyclohexane in order to remove non-polar components. The more polar TA remain as solids in the flask. Finally they are dissolved in the solvent ethanol and can be analyzed after the evaporation of the medium-polar solvent.

- Add 30 mL cyclohexane and 2-3 boiling stones into the flask and boil the suspension for 10 min (± 2 min) under reflux, using an electrical plate.
- Let the suspension cool down.
- Place a small filter paper (e.g. diameter 18.5 cm) in the funnel and filter the organic liquid extract. Keep the flask with the remaining solid!
- Discard the liquid phase.
- (*) Dry the filter paper and flask in a dry oven at 80 °C (± 5 °C) over night (~12 hours or longer).
- Put the filter paper into the flask and add 30 mL ethanol.
- Shake vigorously for 5 min (may use a ultrasonic bath if available).
- Weigh out a dry round bottom flask (capacity volume 100 mL).
- Place a new filter paper (e.g. diameter 18.5 cm) in a funnel and filter the organic phase in the 100 mL flask.

- Use a rotary evaporator for the removal of organic solvent (evaporation under reduced pressure, 40 °C, 180 mbar), if not available: heat and evaporate gently till almost no solvent remains.
- (*) Place the flask in a dry oven at 105 °C (± 5 °C) over night (~12 hours or longer).
- (*) Weigh out the dried material and calculate the yield of extract in analogy to step 2 (formula 2).

Appendix A.2

Detailed description of TLC analysis



The following detailed protocol should be used for TLC analysis of the TA extracts:

a) Preparation of extract and standard solutions:

- Weigh exactly an amount of ~3 mg (± 1 mg) extract in a test tube.
- Add 1 mL ethyl acetate and vortex (or shake well).
- If TA standards are available (this step needs to be prepared by teachers):
  - Prepare a 1-2 mM solution of TA (~ 1 mg/mL).

b) Preparation of detection reagent $KMNO_4$ 0.05 M

- Weigh in 1.5 g $KMnO_4$, 10 g K_2CO_3 and 0.125 g NaOH in a flask and dissolve the solids in 200 mL water.
- Fill the detection reagent in a flat glass dish.

c) Preparation of eluents

- Mix 5 mL cyclohexane, 2.5 mL EtOH and 2.5 mL EA in a 50 mL tube and vortex or shake.
- Fill (10 mL) of the solvent mixture in a TLC chamber (20 × 20 cm) (Fig. 1.4B).

d) TLC Analysis

- Cut silica gel TLC plates into a piece of about 8 × 6 cm and use a pencil to mark a starting line at a distance of 10 mm from the bottom of the plate.
- Apply the extract and standard solutions (1-2 µL) as small spots on the line using thin glass capillaries (example in Fig. 4). Keep a distance of ~5 mm between the spots and let the spots dry at room temperature.
- Put the TLC plate in the chamber with eluents and observe the chromatography (Fig. 1.4B).
- Take out the plate when the solvent front reaches a distance of ~1 cm from the top of the plate (5-10 min). Mark the line for the migration distance and let the plate dry at room temperature.
- Dip the plate carefully into the detection reagent for a few seconds (Fig. 4C).
- Let the plate dry using a hairdryer. Mark the spots using a pencil which appear as light spots on purple background within seconds.

Appendix A.3

Detailed description GC-FID analysis

For the analysis, TA standards and extracts are dissolved and diluted in tetrahydrofuran. The TA are derivatized with trimethylsilyldiazomethane and *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide, converting the carboxylic acid and hydroxyl groups to the corresponding methyl ester and trimethylsilyl ether. The derivatized solution is evaporated, dissolved in *n*-hexane and injected. We suggest to dissolve the extracts in tetrahydrofuran (3 mg/mL). Apple peel may be diluted because of the high TA concent (e.g. yielding 0.3 mg/mL). 20-50 µl of diluted peel samples are mixed with 25 µL MeOH, 25 µL toluene, and 25 µL $TMSCHN_2$ (2 M in *n*-hexane). Following 10 minutes of derivatization at room temperature, the samples are evaporated and reconstituted in 25 µL pyridine and 25 µL BSTFA + 1% TMCS and derivatized for 20 min. After evaporation, the residue is redissolved in 50 µL *n*-hexane and analyzed by GC-FID.

We recommened a standard GC setup, comprising a non-polar, robust standard column and detection by FID. We used a GC-FID 8860 instrument (Agilent

Technologies, Waldbronn, Germany) equipped with a DB5 capillary column (30 m, 0.25 mm ID, 0.25 µM µm film) (J&W Scientific, Folsom, CA, USA). As carrier gas H_2 and a flow rate of 1.2 mL/min were used. The oven temperature program was initial 200 °C, then 10 °C/min to 280 °C (23 min), 30 °C/min to 310 °C (2.5 min) and 30 °C/min to 330 °C (10 min). The injector temperature was 240 °C. The split mode with split ratio 1/20 and an injection volume of 1 µL were used. Detector temperature was held at 330 °C, with an air flow of 450 mL/min, H_2 fuel flow of 40 mL/min, and N_2 make-up flow of 45 mL/min. Quantification was carried out by external calibration. For this a solution of standards (10 mM in MeOH) of standards was mixed and sequentially diluted (2-500 µM) in MeOH.

Appendix A.4

Notes for instructors

Experimental procedure

The protocols described here were developed in an educational institution and have already been tested with a group of three undergraduate students. Based on their experimental results, the protocol was optimized for the educational context. To avoid common student errors, we would like to emphasize the following aspects:

The initial extraction of the apple peel was carried out in boiling water (step 1b). Pay attention to any foaming of the suspension (leading to boiling over and a spill) by adjusting the heat supply. The heating step should therefore be carried out under constant observation. After heating, the suspension should cool down before filtering.

After cold extraction in ethyl acetate, LLE extraction takes place in the separatory funnel (step 2). When combining the filter paper and the flask, ensure that the product is transferred carefully on the filter paper (step 4). The powder can easily be lost.

Materials

Apples

The use of regional, fresh apple fruit is recommended for TA extraction. The fruits are peeled, the peel can directly be extracted and the pulp can be eaten by the students. In our procedure, apples were bought from regional fruit farms in Leichlingen and Wermelskirchen, Germany. The Delbarestivale apples were bought in fall 2022 and 2023, Golden Delicious and Boskoop in 2023. Apples were peeled and cut directly after harvest and the pulp and peel were collected, then the fresh material was dried and used for extraction.

Chemicals and reagents used for experiments

For TA extraction only organic solvents are used that are suitable for students. In our work, ethanol and ethyl acetate were obtained from Fisher Scientific (Schwerte, Germany) and cyclohexane (technical grade) was destillated for extraction.

For analysis by TLC, the eluents and the detection reagent can be prepared by the students. In our experiment, $KMnO_4$ was from Janssen Chimica (Beerse, Belgium) and K_2CO_3 and NaOH from Grüssing (Filsum, Germany). The normal phase TLC plates with unmodified silica gel 60 aluminium were purchased from Merck (Darmstadt, Germany).

For GC analysis *n*-hexane, toluene, tetrahydrofurane and pyridine were obtained from Fisher Scientific (Schwerte, Germany). The chemicals (trimethylsilyl)diazomethane ($TMSCHN_2$, 2M in *n*-hexane) was bought from TCI Deutschland GmbH (Eschborn, Germany) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) from Carl Roth GmbH (Karlsruhe, Germany).

The qualitative evaluation of the TA extracts can be performed without standard compounds e.g. by comparing the provided R_f values. For quantitative evaluation standard compounds are needed e.g. for external calibration. In our work, UA was purchased from abcr GmbH (Karlsruhe, Deutschland), CA and MA from Cayman Chemical (local distributor Biomol, Hamburg Germany), BA from TCI

Deutschland GmbH (Eschborn, Germany) and PA from Merck (Darmstadt, Germany).

Appendix A.5

Results of TA extraction

In the first step of the extraction protocol, fresh apple peels are boiled in water. The crude extract of Boskoop apple peels with and without extraction in water are shown (Fig. S1). For the elution range of 5-15 minutes, the signals for early eluting matrix components decrease drastically due to prior extraction in water. At the same time, the signal heights for TA in the extract increases. This leads to the conclusion that boiling in water leads to a removal of matrix and an enrichment of the TA compounds in the extract.

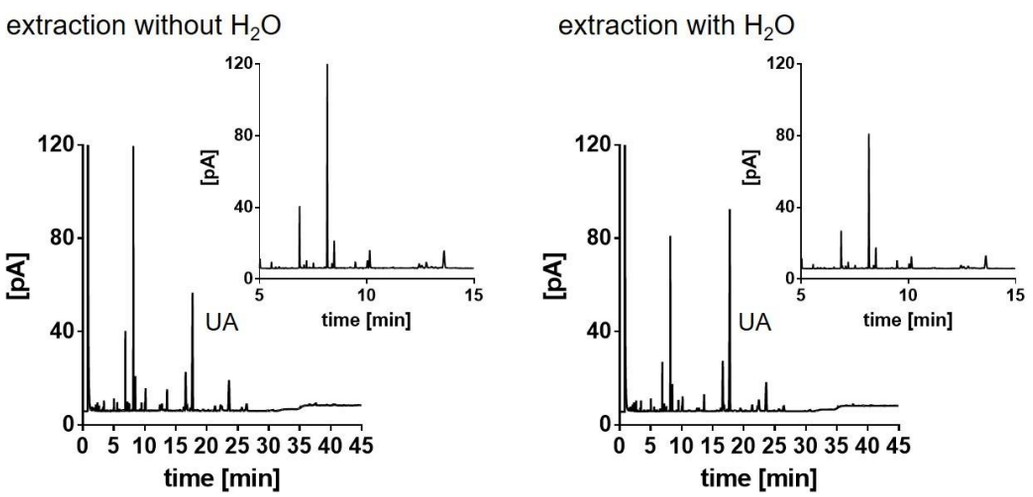


Fig. A1: GC-FID chromatograms with or without prior extraction in H_2O. Comparison of the GC-FID chromatograms of the raw extracts (3 mg/mL) of Boskoop apple peel samples with or without prior extraction in H_2O on a nonpolar (DB-5; 30 m, 0.25 mm ID, 0.25 µm film) column.

The TA extraction includes a degreasing step in cyclohexane. Chromatograms of the filtrates after boiling in cyclohexane are shown (Fig. S2). The high signals for matrix components in the elution range of 5-15 minutes can be easily seen. It can be concluded that the degreasing with cyclohexane has a high impact on the purification and concentration of TA in the extracts.

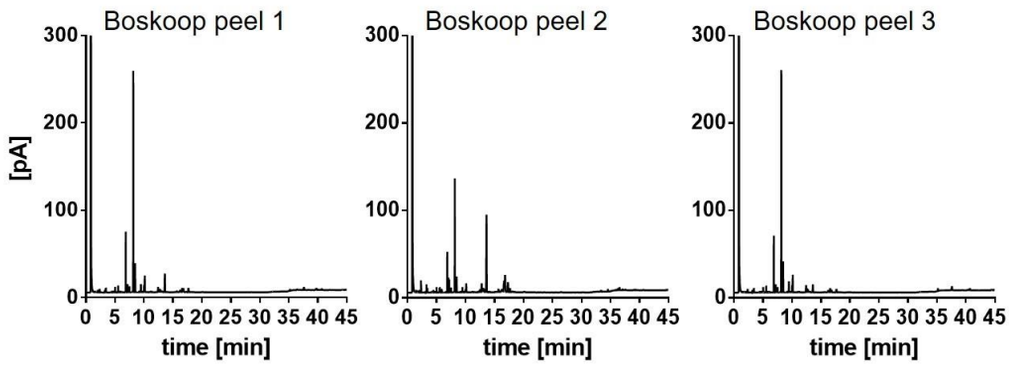


Fig. A2: GC-FID analysis of the filtrate of degreased Boskoop apple peel (3 mg/mL). Separation was carried out on on a nonpolar (DB-5; 30 m, 0.25 mm ID, 0.25 µm film) column separating the TA by boiling point.

Appendix A.6

Further educational material

A detailed and interactive student's handout and an animated GC-FID presentation illustrating the extraction and analytical procedure can be found at: https://pubs.acs.org/doi/suppl/10.1021/acs.jchemed.3c01328/suppl_file/ed3c01328_si_003.pdf (accessed 2025-11-17).

B APPENDIX FOR CHAPTER 3

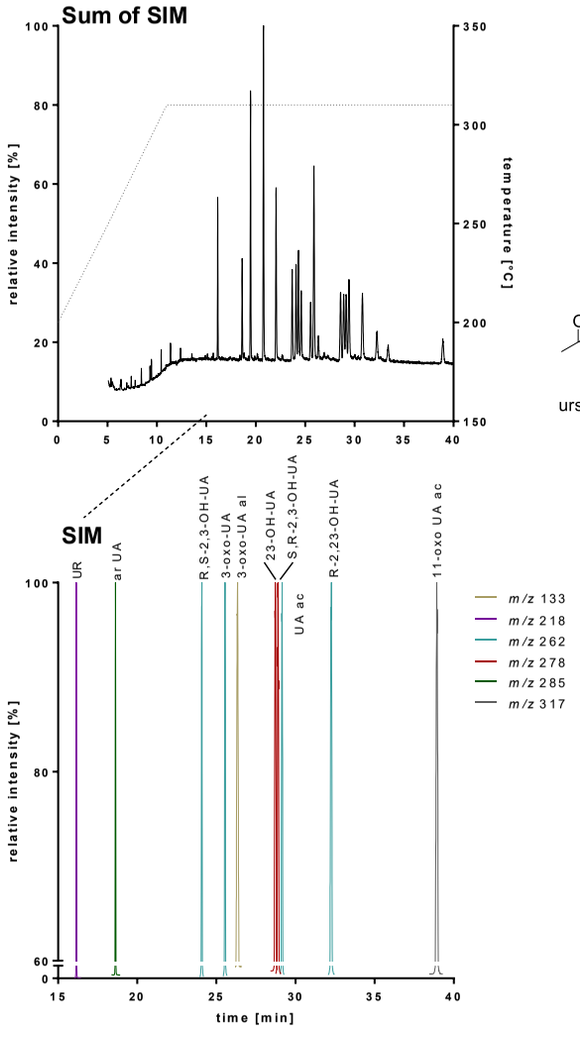
Appendix B.1

Material and Methods

GC-FID analysis

A GC-FID 8860 instrument (Agilent Technologies, Waldbronn, Germany) was equipped with a DB5 capillary column (30 m, 0.25 mm ID, 0.25 µM µm film) (J&W Scientific, Folsom, CA, USA). As carrier gas H2 and a flow rate of 1.2 mL/min were used. The oven temperature program was initial 200 °C, then 10 °C/min to 280 °C (23 min), 30 °C/min to 310 °C (2.5 min) and 30 °C/min to 330 °C (10 min). The injector temperature was 240 °C. The split mode with split ratio 1/20 and an injection volume of 1 µL were used. Detector temperature was held at 330 °C, with an air flow of 450 mL/min, H_2 fuel flow of 40 mL/min, and N_2 make-up flow of 45 mL/min.

The concentration of all TA standards was verified by GC-FID. For this, the relative response factors between OA (purity ≥ 98%) and the respective TA were used by calculating the effective carbon number (ECN), injecting them together in a concentration of 40 µM and by comparing the peak area. If the determined concentration of a TA in stock solution was not within ± 15%, a correction factor was used.



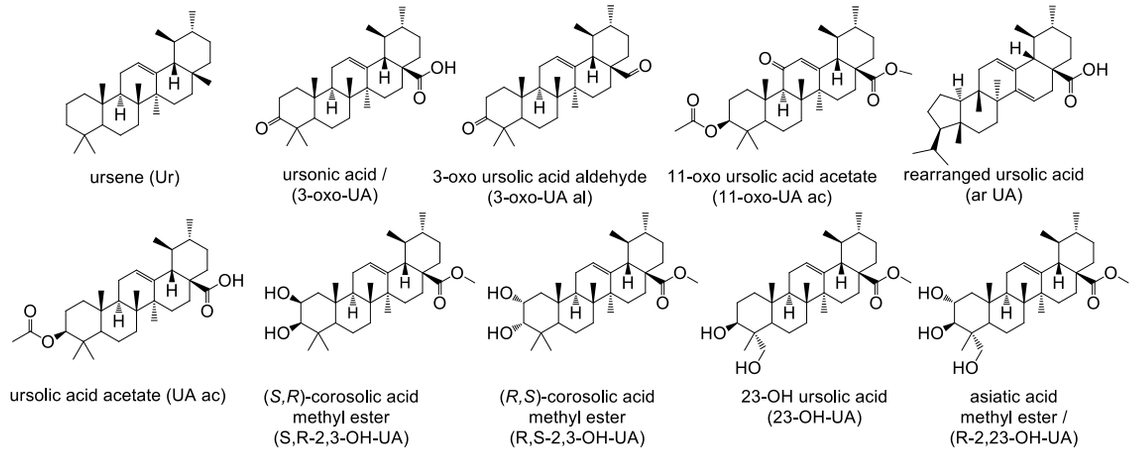


Fig. B1: Chromatographic separation of further triterpenic acid derivatives not included in Figure 3.1. Left: A mixture of all TA standards (20-100 µM) was analyzed on a 5%-phenyl/95%-dimethyl polysiloxane analytical column (DB5-MS; 40 m, 0.18 mm ID, 0.25 µm film). Shown is the sum of SIM (TOP) and the individual SIM traces of the 10 further TA used for quantification (BOTTOM). Right: Structures of the TA derivatives. The optimized separation enables the simultaneous analysis of all 20 TA derivatives in a total run time of 40 min.

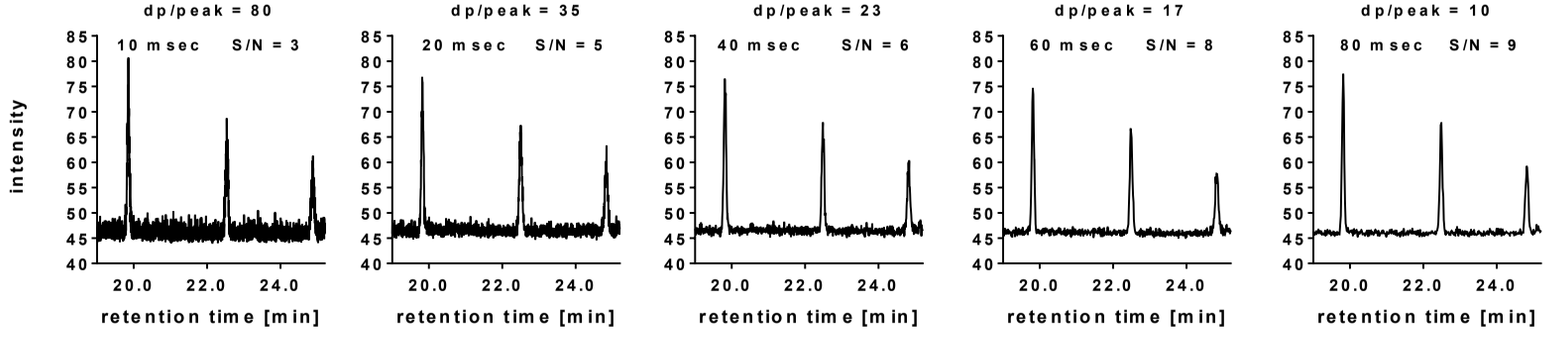


Fig. B2: Influence of the dwell time on the signal-to-noise ratio and the number of data points per peak. URA, R-UA and OA (80 nM standard solution) were analyzed in SIM mode (*m/z* 262) and the peak-to-peak signal-to-noise ratio as well as the number of data points measuring 12 parallel SIM traces across the peak were determined. A sufficient number of data points for quantification is required, with the highest possible signal-to-noise ratio (S/N). Therefore, a dwell time of 40 ms was chosen for the GC-MS method

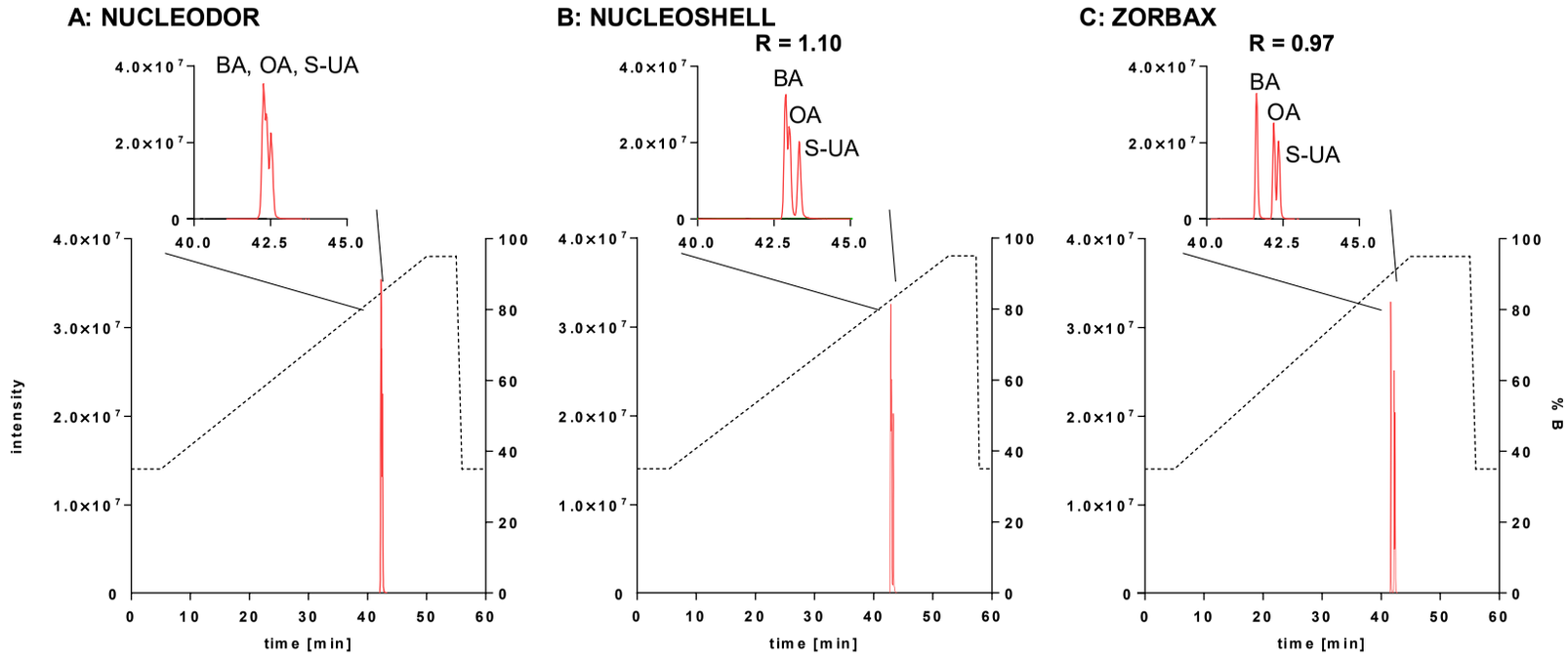


Fig. B3: LC-(ESI-)-MS analysis of triterpenic acid derivatives. The separation of S-UA, BA and OA using different LC columns and indicated gradients are shown. Shown are XIC´s of $[M-H^+]^-$ ions at *m/z* 455.3531. The mobile phase consisted of A: H_2O/MeOH/HAc (95/5/0.1 *v/v/v*) and B: MeOH + 0.1% HAc at 300 µL/min. The following columns were tested A: NUCLEODUR Sphinx C18 (Macherey-Nagel, Düren Germany), (2.0 x 150 mm, 1.8 µm), B: NUCLEOSHELL Biphenyl C18 (Macherey-Nagel, Düren Germany), (2.0 x 150 mm, 2.7 µm), C: RRHD ZORBAX Eclipse Plus C18 (Agilent, Waldbronn Germany), (2.1 x 150 mm; 1.8 µm). Standards (1 µM in MeOH, 5 µL injected) were measured using the QExactive HF Orbitrap MS (Thermo Fisher Scientific, Schwerte Germany) in ESI(-)-mode at 40 °C column temperature. Baseline separation for S-UA and OA was not achieved on any of the tested RP-C18 columns. Some studies describe the successful separation using RP-18 phases. Guo et al. showed baseline separation for S-UA, BA and OA in a total run time of 45 min resp. 75 min [1, 2]. Zhang et al. used a polymeric column and could show baseline separation for S-UA and OA in less than 20 [3]. However, the peaks showed strong fronting [3]. The use of a C30 column enabled a sufficient baseline separation for S-UA and OA [4] comparable to the GC-MS method developed here

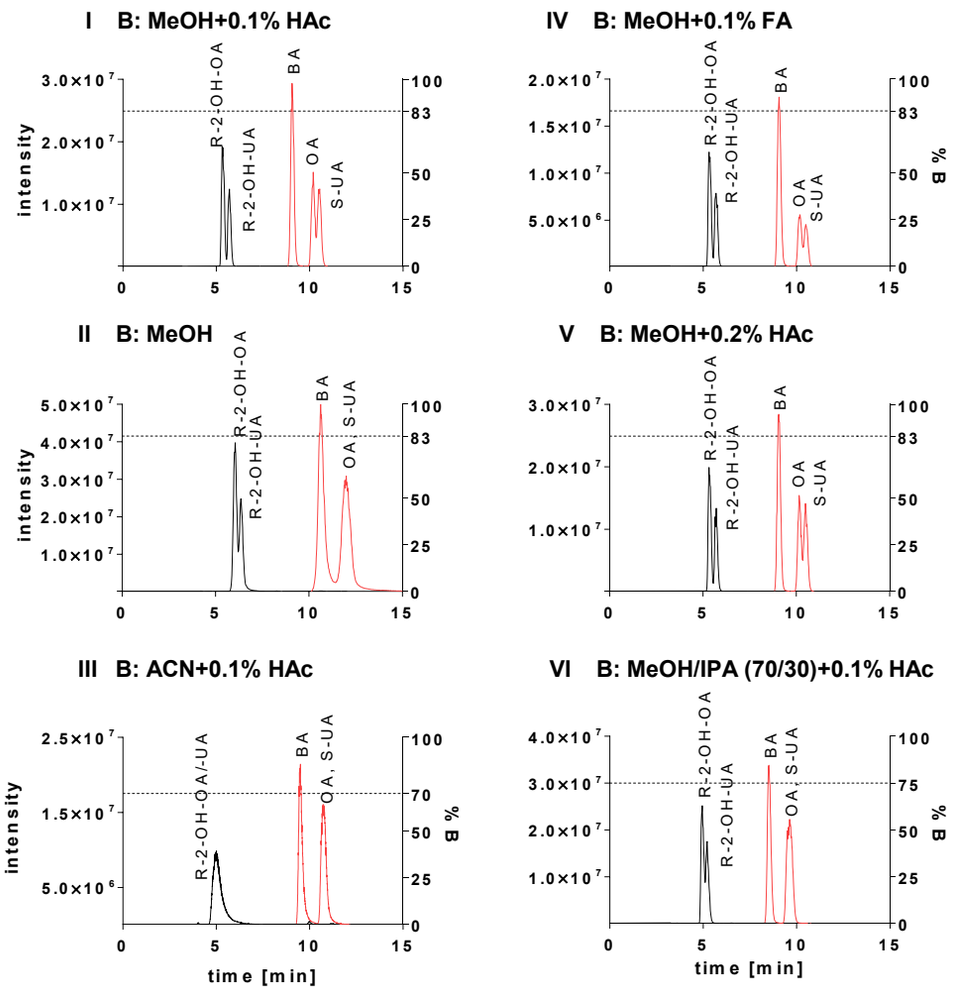


Figure B4: LC-(ESI-)-MS analysis of triterpenic acid derivatives. Shown are effects of different eluents on the separation of triterpenic acids. The XIC´s of [M-H+]- ions at m/z 455.3531 and m/z 471.3480 are shown. The following mobile phases were tested: A H2O/MeOH/HAc (95/5/0.1 *v/v/v*), B I: MeOH+0.1% HAc, II: MeOH, III: ACN+0.1% HAc, IV: MeOH+0.1% FA, V: MeOH+ 0.2% HAc, VI: MeOH/IPA (70/30)+0.1% HAc at 300 µL/min. Standards (1 µM in MeOH, 5 µL injected) were separated isocratically on column C RRHD ZORBAX Eclipse Plus C18 (2.1x150 mm; 1.8 µm) and measured using the QExactive HF Orbitrap MS in ESI(-)-mode (Thermo Fisher Scientific, Schwerte Germany) at 40 °C column temperature. The separation pair OA and S-UA shows coelution when using IPA or ACN as eluents. The best separation, but no baseline separation, was achieved with acidic MeOH.

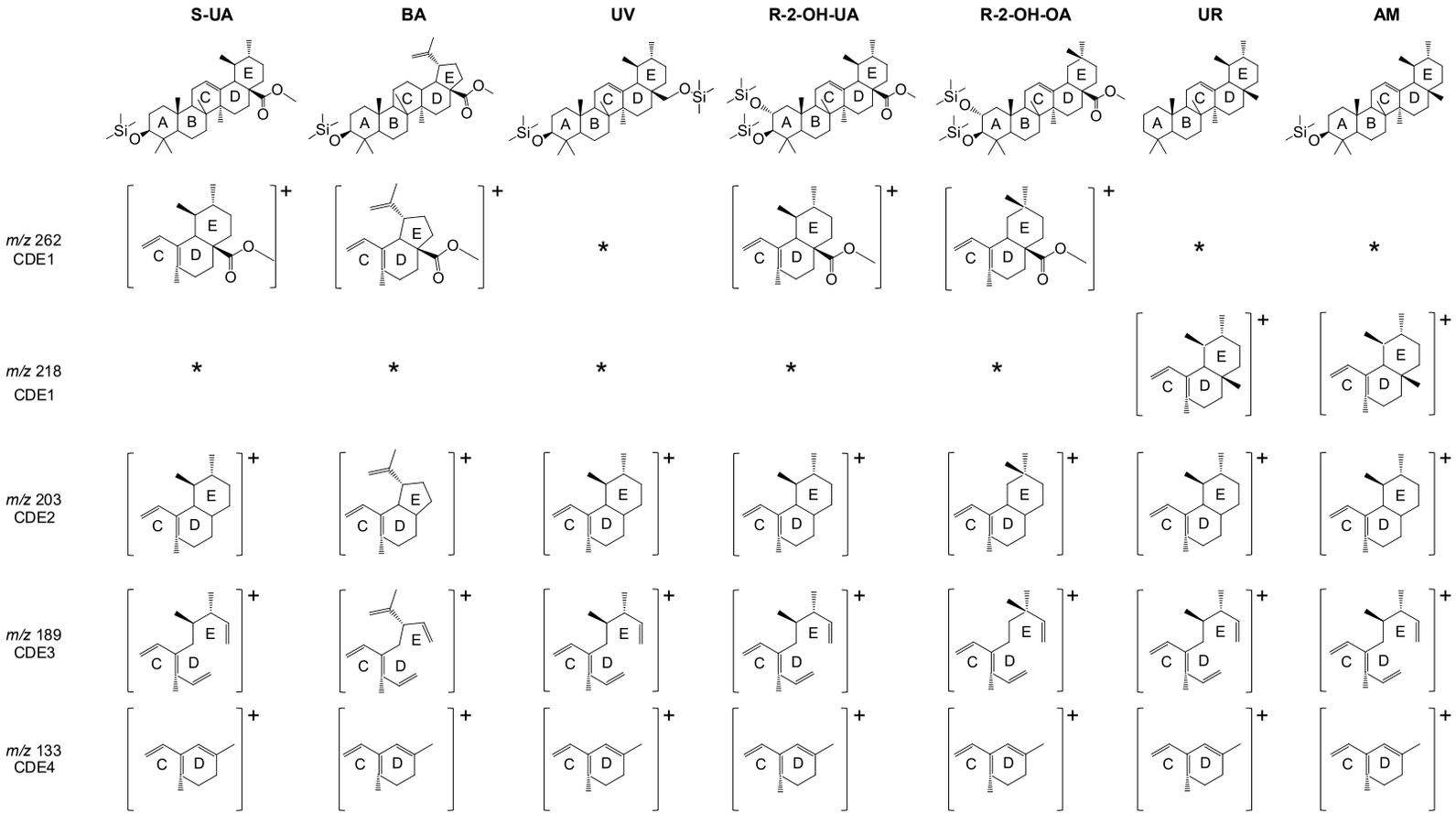


Fig B5: GC-MS analysis of TA derivatives following derivatization with $TMSCHN_2$ and BSTFA. Structures are suggested for fragments (CD1-CD4) of S-UA, BA, R-2-OH-UA, R-2-OH-OA, UV, UR and AM. *not detected. Derivatized standards with the same backbone exhibit identical fragmentation behavior and follow retro-Diels-Alder reactions. Fragment CDE1 with *m/z* 262 is detectable for carboxylic acid containing TA S-UA, BA, R-2-OH-Ua and R-2-OH-OA at ring E. A methyl group at the same position leads to the fragment with *m/z* 218 for UR and AM. Further cleavages of the functional groups and ring opening result in fragments with *m/z* 203, 189, and 133

Tab. B1: Detection of the fragments CDE1-4 in the GC-EI spectra of TA derivatives with $TMSCHN_2$ and/or BSTFA

		***m/z* 262**	***m/z* 203**	***m/z* 189**	***m/z* 133**
Analyte	derivatized with	CDE1	CDE2	CDE3	CDE4
UR	$TMSCHN_2$		×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA		×	×	×
ar UA	$TMSCHN_2$				
	BSTFA				
	$TMSCHN_2$ + BSTFA				
URA	$TMSCHN_2$	×	×	×	×
	BSTFA			×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
AM	$TMSCHN_2$		×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA		×	×	×
R-UA	$TMSCHN_2$	×	×	×	×
	BSTFA	×	×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
UV	$TMSCHN_2$		×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA		×	×	×
R,S-2,3-OH-UA	$TMSCHN_2$	×	×	×	×
	BSTFA	×	×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
OA	$TMSCHN_2$	×	×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
BA	$TMSCHN_2$	×	×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
3-oxo-UA	$TMSCHN_2$	×	×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
S-UA	$TMSCHN_2$	×	×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×

Tab. B1 (continued)

		***m/z* 262**	***m/z* 203**	***m/z* 189**	***m/z* 133**
Analyte	derivatized with	CDE1	CDE2	CDE3	CDE4
3-oxo-UA al	$TMSCHN_2$		×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA		×	×	×
23-OH-UA	$TMSCHN_2$	×	×	×	×
	BSTFA	×	×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
S,R-2,3-OH-UA	$TMSCHN_2$	×	×	×	×
	BSTFA	×	×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
UA ac	$TMSCHN_2$	×	×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
R-2-OH-OA	$TMSCHN_2$	×	×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
R-2-OH-UA	$TMSCHN_2$	×	×	×	×
	BSTFA		×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
R-2,23-OH-UA	$TMSCHN_2$				
	BSTFA	×	×	×	×
	$TMSCHN_2$ + BSTFA	×	×	×	×
S-19-OH-UA	$TMSCHN_2$			×	×
	BSTFA			×	×
	$TMSCHN_2$ + BSTFA			×	×
11-oxo-UA ac	$TMSCHN_2$			×	×
	BSTFA			×	×
	$TMSCHN_2$ + BSTFA			×	×

Tab. B2: Parameters of the GC-MS analysis of TA derivatives derivatized with $TMSCHN_2$ and BSTFA. The ions in EI are listed and qualifier (underlined) and quantifier (bold) are highlighted. The retention time (mean ± SD, n=5) and peak width at half height (FWHM, mean ± SD, n=5) were determined for standard solutions (10 µM). The limit of detection (LOD, S/N ≥ 3), lower limit of quantification (LLOQ, S/N ≥ 5, accuracy ±20%) as well as function of the linear calibration and coefficient of determination (R^2) were determined for the quantifier ions in selected ion monitoring mode with a dwell time of 40 ms.

Analyte	Retention Time [min]	*m/z ion (*relative intensity above 20%)	FWHM [s]	LOD [nM]	LLOQ [nM]	Regression equatation	R^2
UR	16.15 ± 0.01	**218** (100); 203 (20); 191 (20); 189 (15); 133 (15); 69 (25)	2.8 ± 0.1	8	24	f(x)=0.497x-0.059	0.975
ar UA	18.62 ± 0.01	452 (60); 437 (30); 393 (25); 392 (20); **285** (50); 281 (20); 241 (50); 239 (25); 213 (40); 207 (30); 173 (100); 171 (20); 169 (20); 155 (30); 145 (20); 143 (30); 137 (50); 131 (30); 109 (25); 95 (60); 81 (40); 69 (30), 67 (25); 55 (45)	3.3 ± 0.1	430	1000	f(x)=0.159x-0.375	0.989
R,S-2,3-OH-UA	24.07 ± 0.01	278 (10); 263 (25); **262** (100); 203 (70); 189 (15); 147 (45); 133 (30); 119 (20); 73 (50)	5.6 ± 0.1	80	300	f(x)=0.866x-0.002	0.989
3-oxo-UA	25.54 ± 0.01	**262** (75); 203 (100); 189 (20); 147 (30); 133 (70); 119 (25); 55 (25)	5.8 ± 0.2	32	80	f(x)=0.540x+0.002	0.989
3-oxo-UA al	26.33 ± 0.01	203 (100); 189 (5); 147 (15); **133** (50)	6.3 ± 0.2	800	1600	f(x)=0.505x-5.635	0.994
23-OH-UA	28.58 ± 0.01	**278** (15); 263 (20), 262 (100); 203 (70); 189 (15); 148 (40); 147 (35); 133 (45); 119 (20); 73 (65)	7.0 ± 0.1	800	320	f(x)=0.115x-0.670	0.994
S,R-2,3-OH-UA	28.89 ± 0.01	**278** (60); 277 (30); 263 (40); 262 (85); 203 (30); 189 (25); 147 (50); 133 (60), 131 (20); 119 (35); 73 (100)	7.3 ± 0.2	800	1000	f(x)=0.272x+0.089	0.994
UA ac	29.15 ± 0.01	**262** (80); 203 (100); 190 (25); 189 (40); 133 (80); 119 (30); 105 (25); 69 (20)	7.1 ± 0.1	32	80	f(x)=0.773x-0.339	0.910
R-2,23-OH-UA	32.25 ± 0.01	**262** (50); 203 (20); 191 (30); 147 (100); 133 (45); 73 (55)	9.1 ± 0.4	400	600	f(x)=0.580x-0.046	0.991
11-oxo-UA ac	38.92 ± 0.01	**317** (100); 276 (50); 257 (50); 248 (30); 217 (30); 208 (20); 191 (30); 189 (30); 175 (50); 173 (30); 161 (40); 159 (25); 147 (40); 135 (40); 133 (40); 121 (20), 119 (70); 107 (30); 105 (50); 95 (60); 93 (20); 91 (25); 81 (25); 73 (50); 69 (50); 55 (30)	10.4 ± 0.4	70	140	f(x)=0.315x+0.363	0.986

Tab. B3: Recovery of apple peel extracts spiked with TA (2-120 g/100 g apple peel extract) prior to the derivatization procedure and analysis by GC-FID. Results are shown as mean ± SD (n=3). The recovery of 93-112% at all spiked concentrations indicates a good accuracy of the method. Moreover, the high spiking level being more than 5 fold higher than the endogenous concentration demonstrates a sufficient excess of derivatization agents in the presence of the biological matrix leading to complete derivatization.

Concentration ± SD [g/100 g apple peel extract]			
		Mean	Mean Recovery [%]
Analyte	Added	Calculated	
S-UA	0	35 ± 1	
	24.4	66 ± 3	111 ± 4
	45.7	90 ± 10	112 ± 13
	60.9	110 ± 2	115 ± 2
	121	188 ± 6	120 ± 4
R-2-OH-OA	0	1.6 ± 0.1	
	2.5	3.8 ± 0.2	93 ± 5
	4.7	6.0 ± 0.3	95 ± 4
	6.3	7.3 ± 0.3	93 ± 3
	12.6	13.6 ± 0.4	96 ± 2

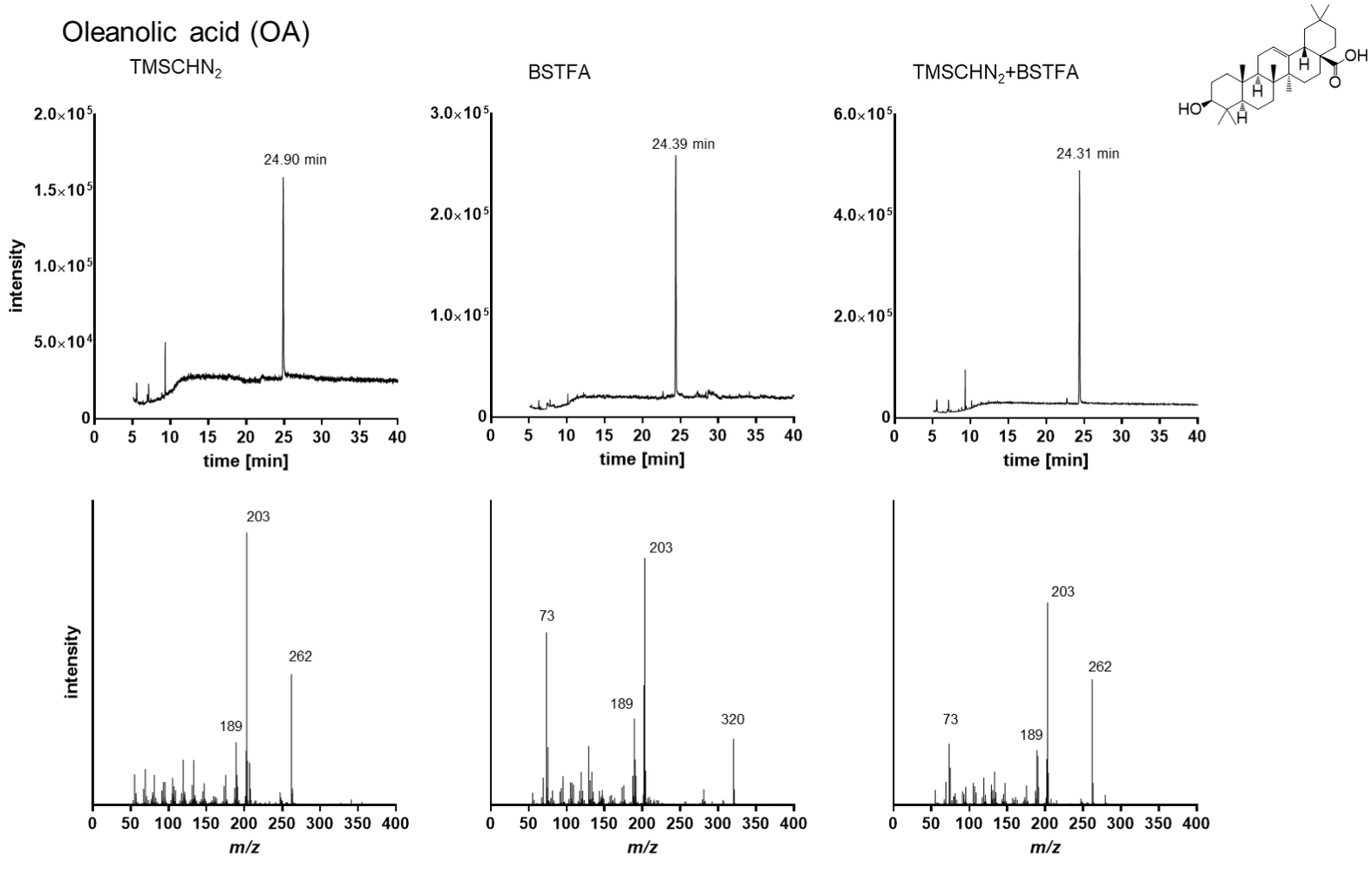


Fig. B6: MS chromatograms and spectra of TA after derivatization with $TMSCHN_2$ and/or BSTFA. Standards were analyzed in a concentration of 100 µM in full-scan mode (*m/z* 50-400).

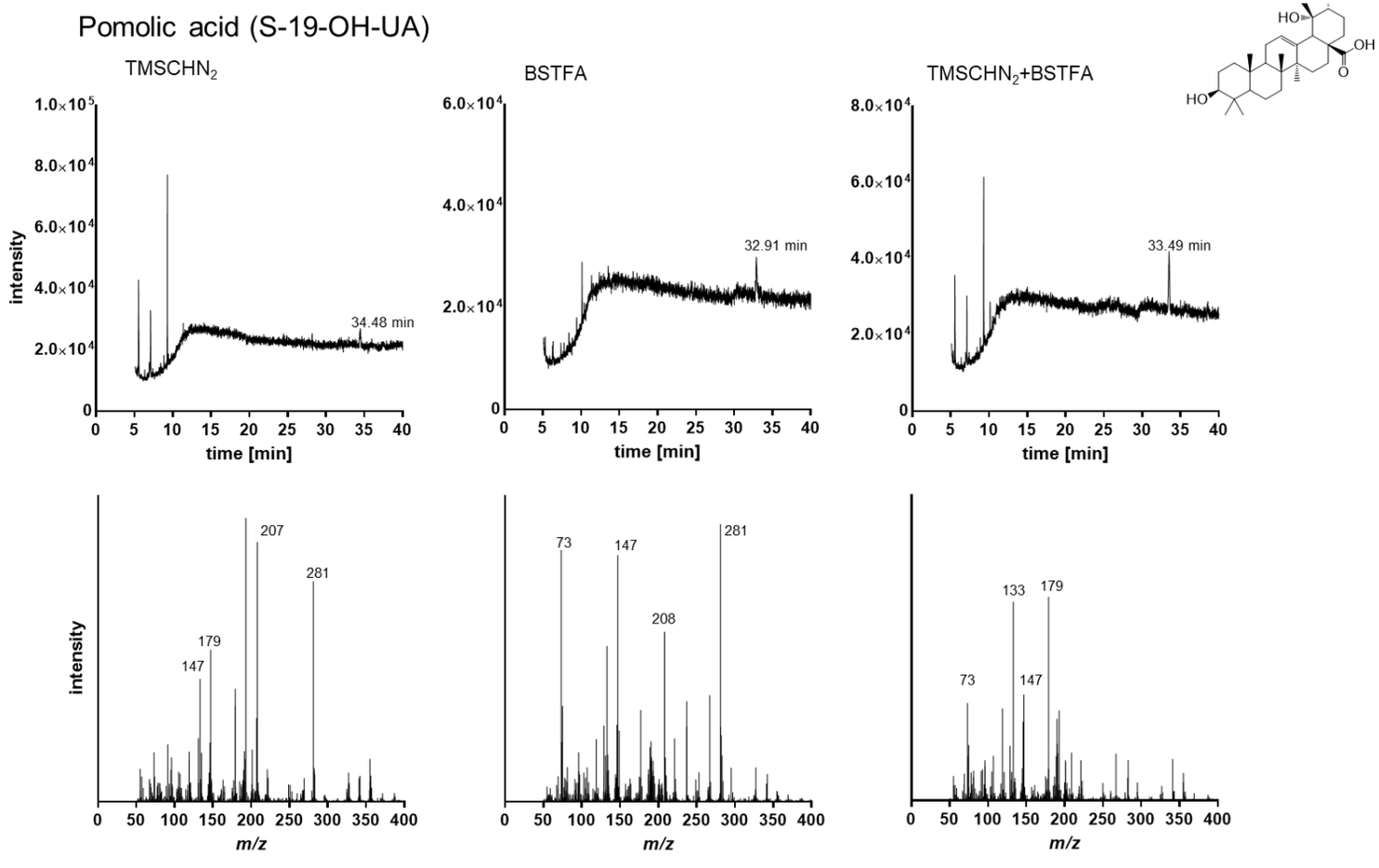
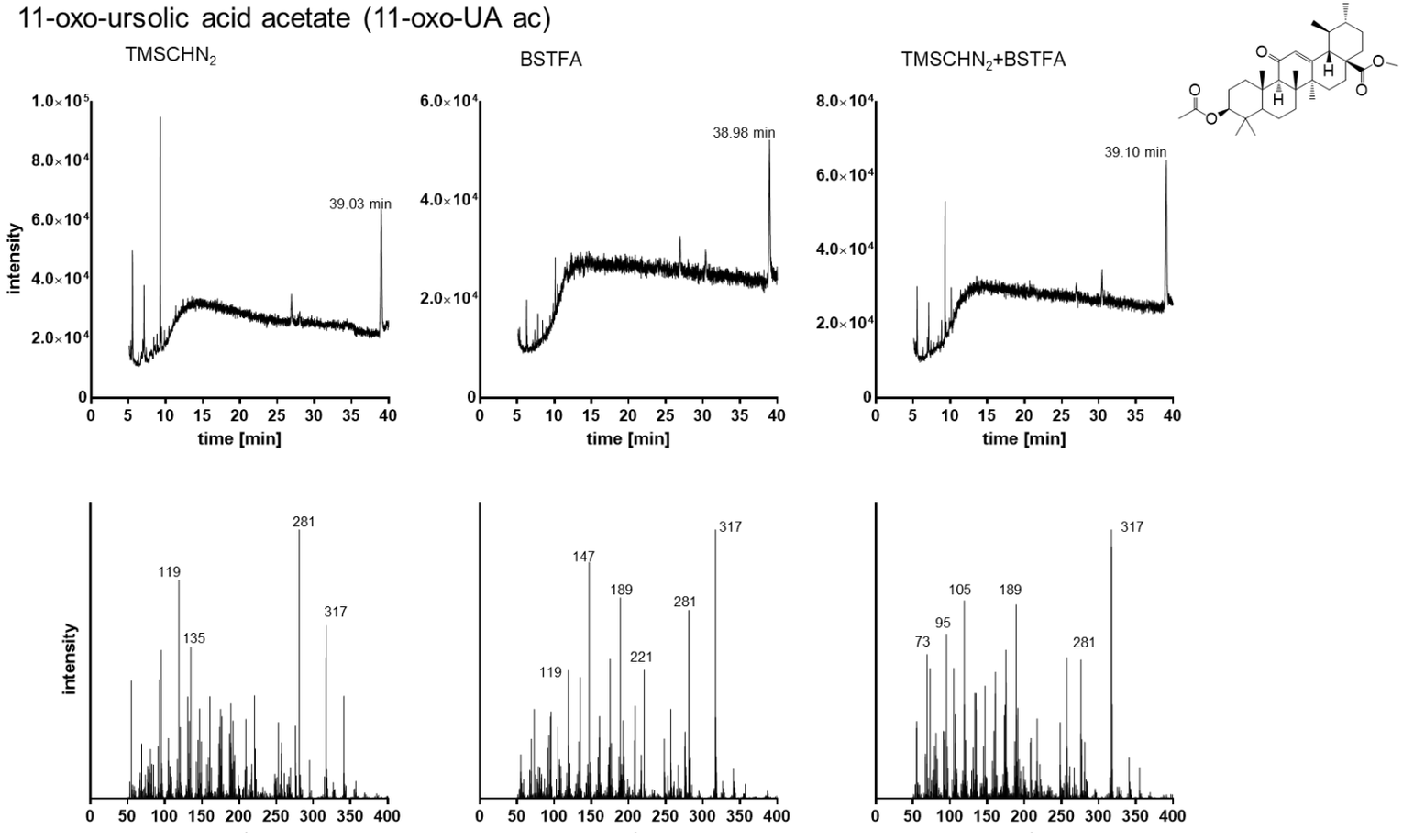


Fig. B6 (continued)



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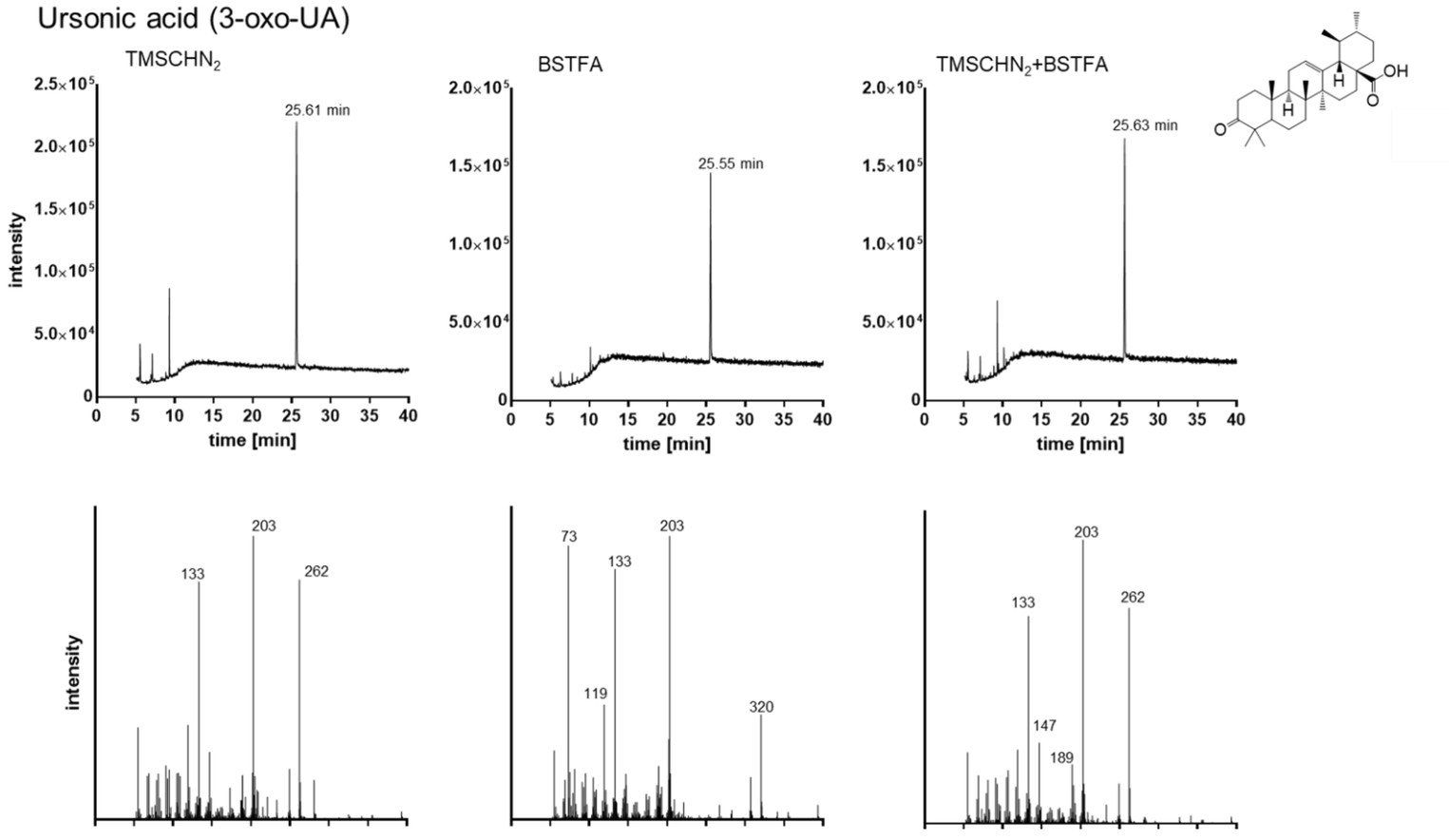

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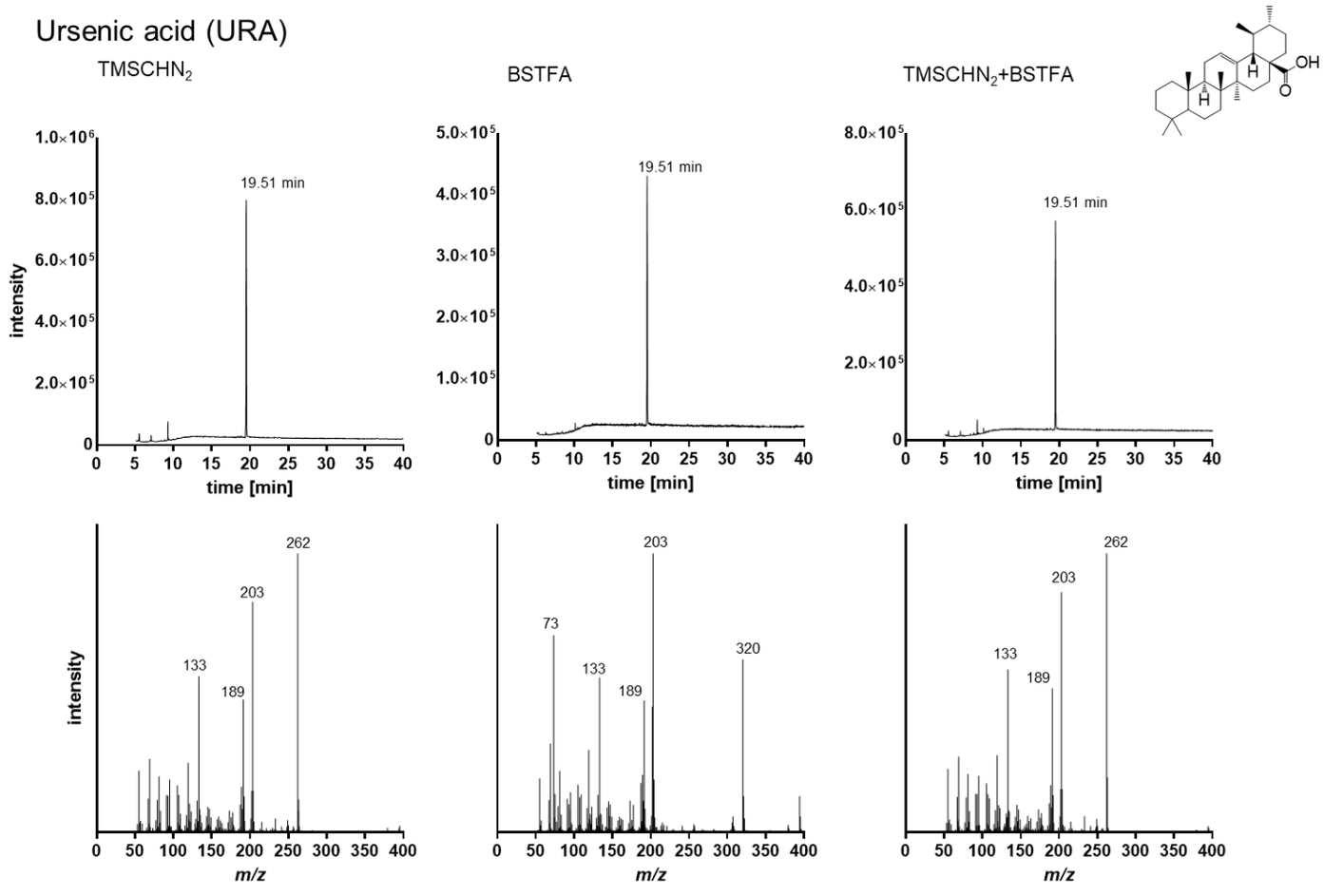

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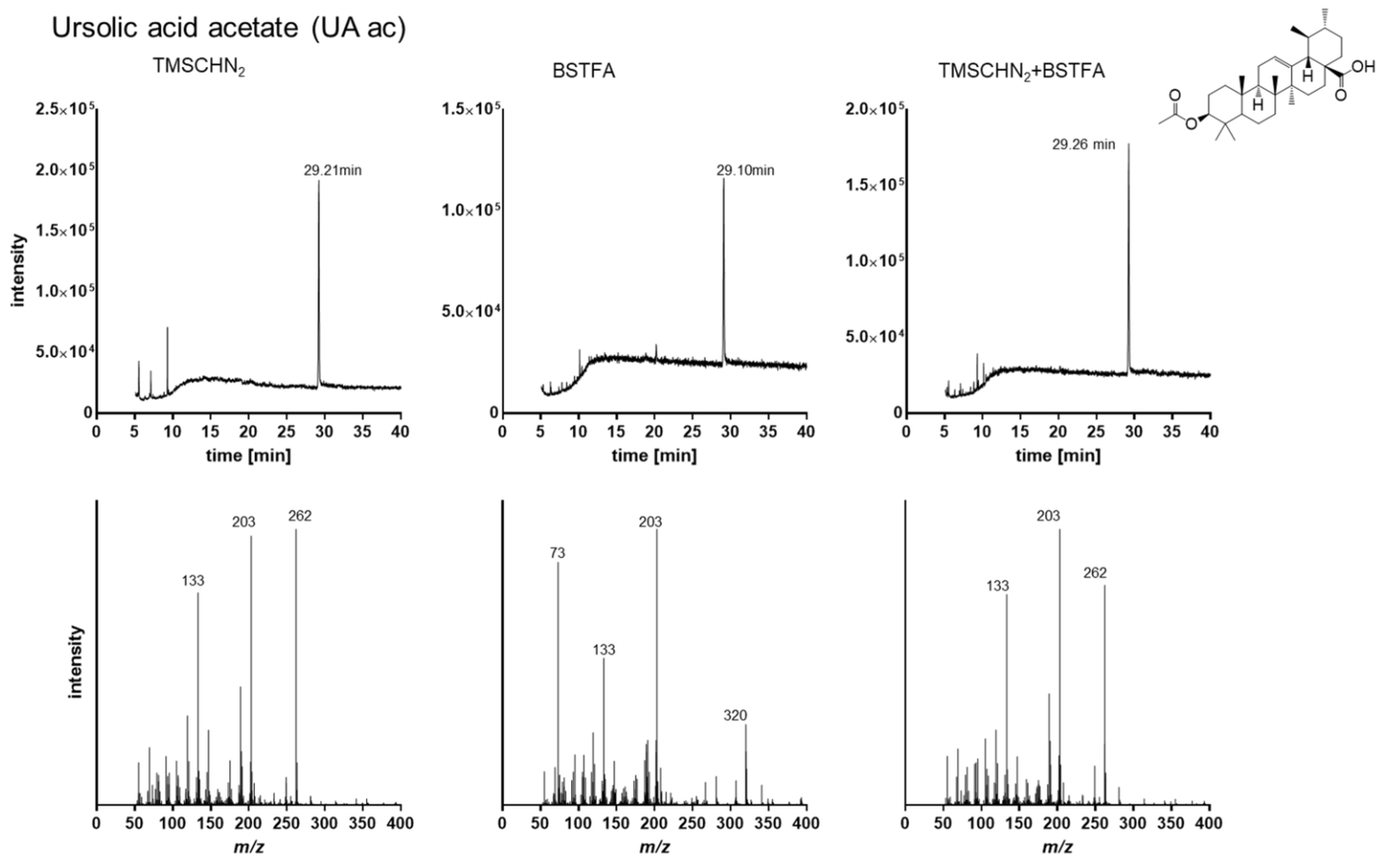
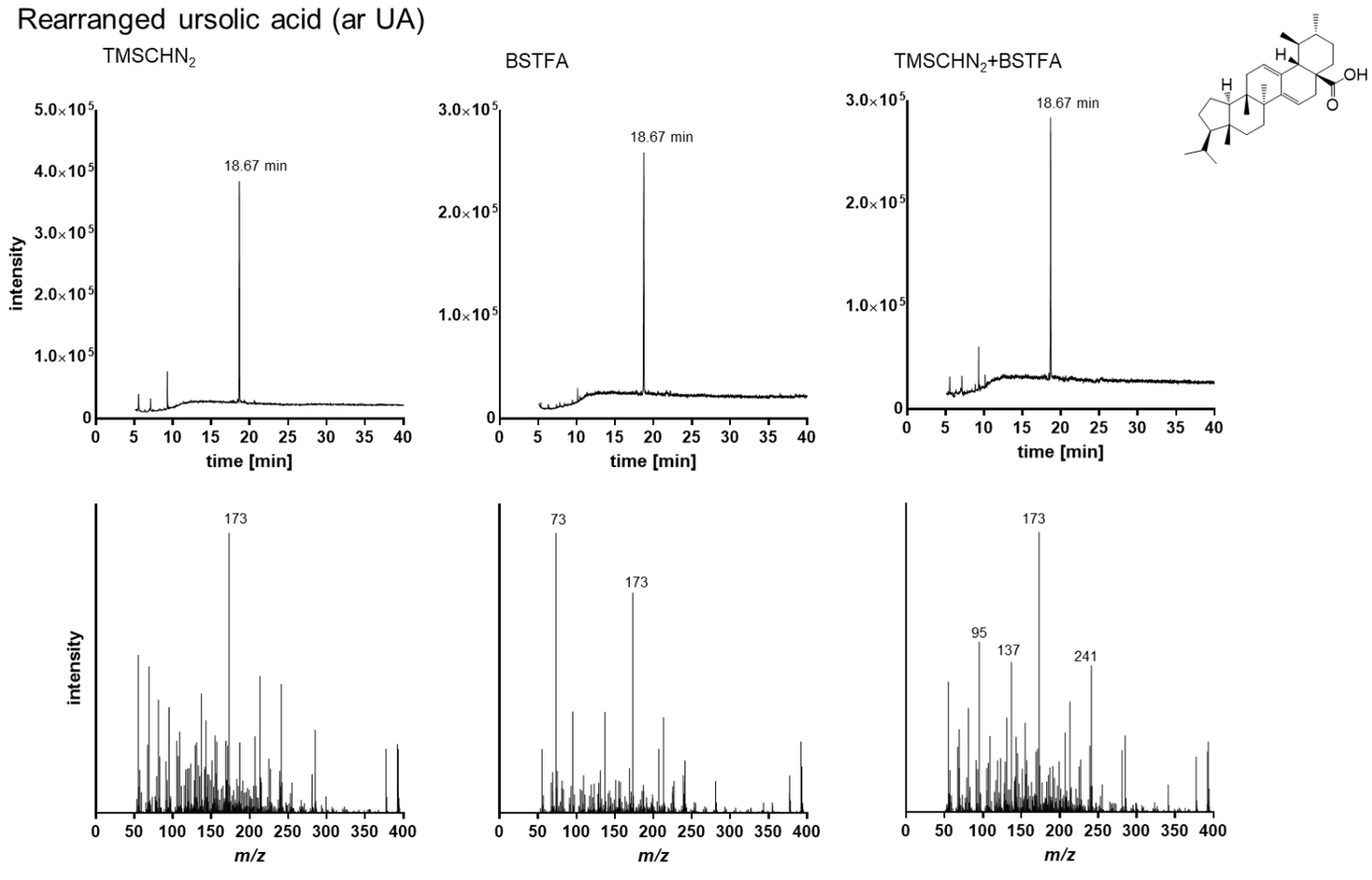


Fig. B6 (continued)



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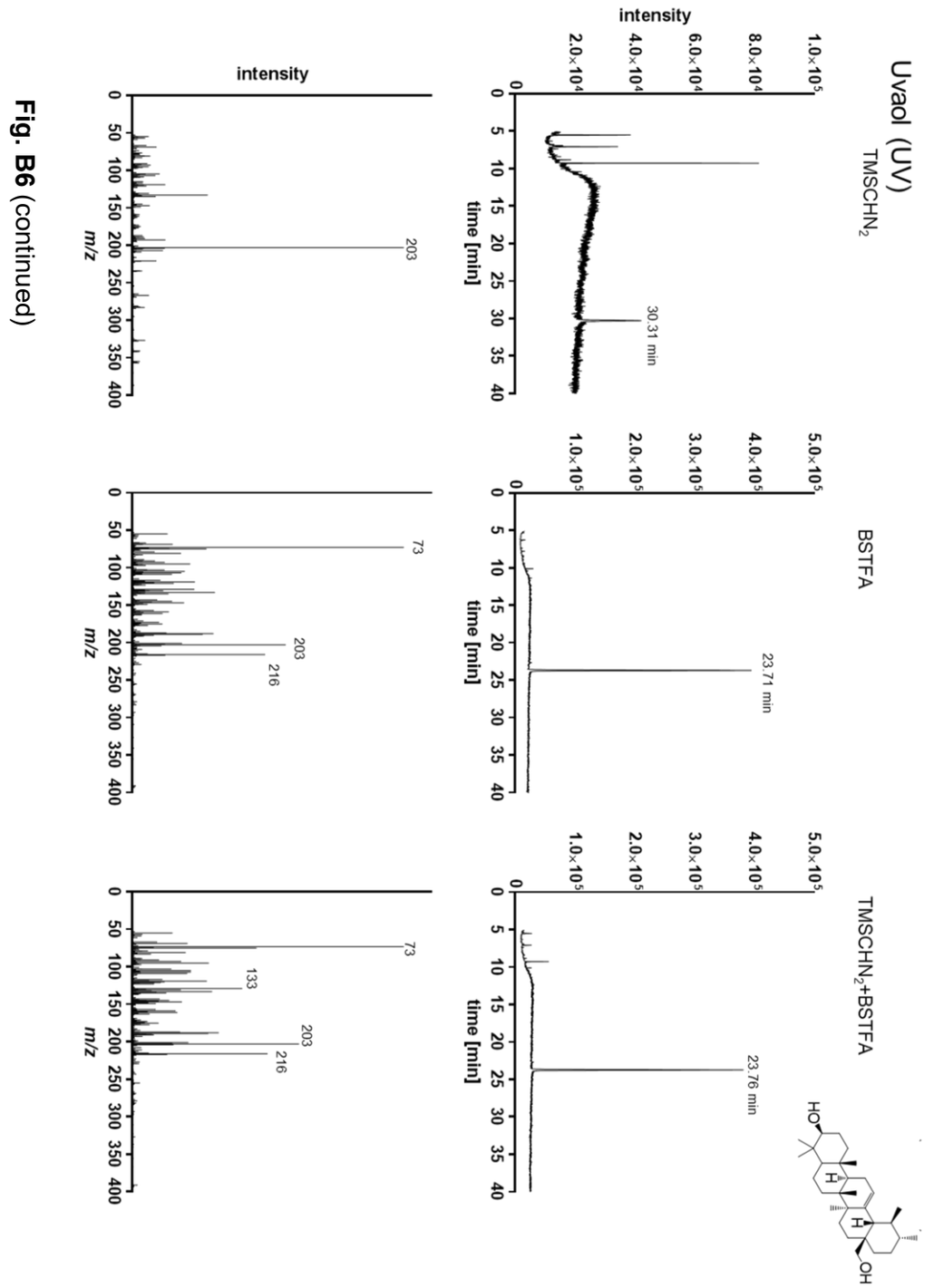

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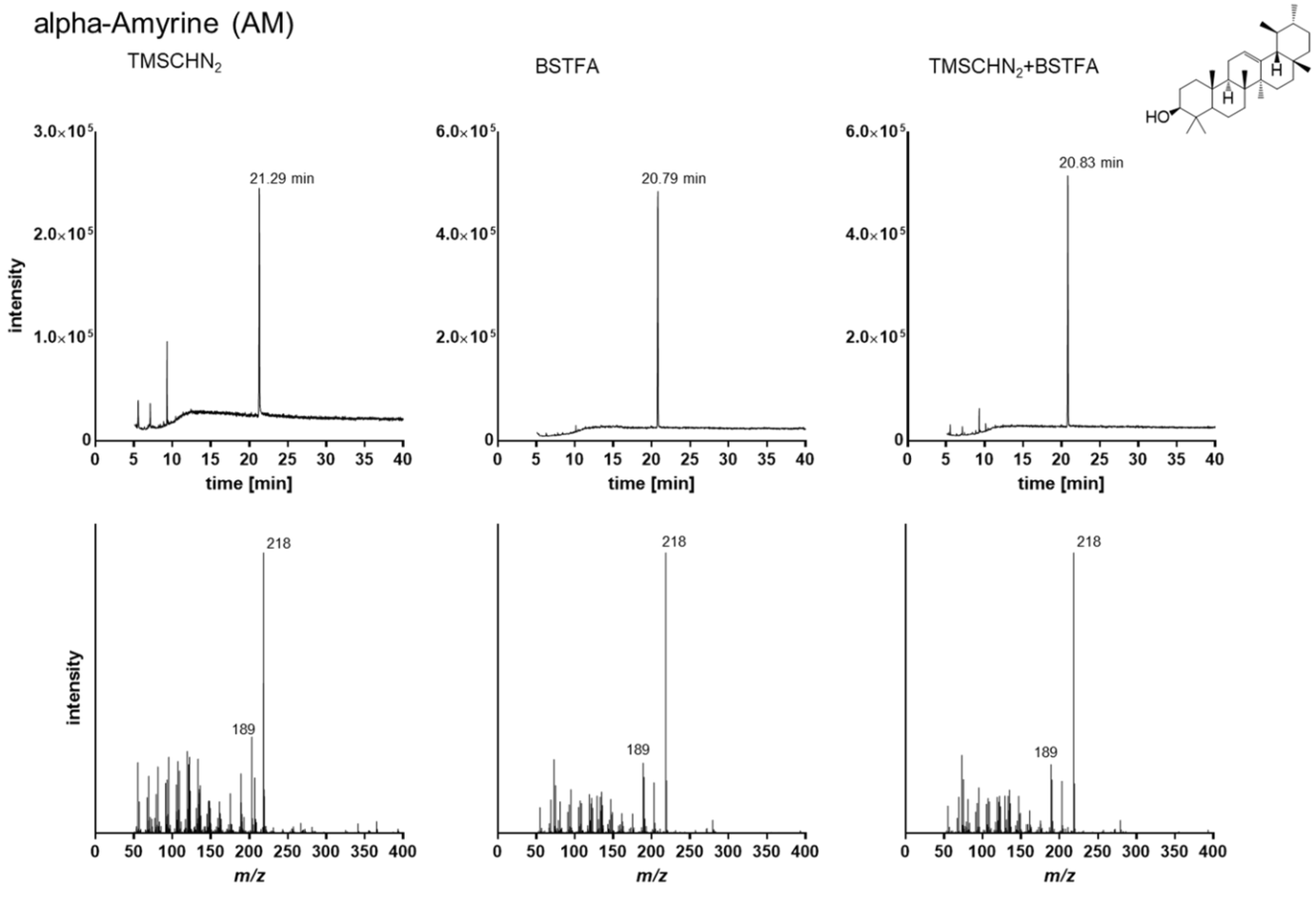

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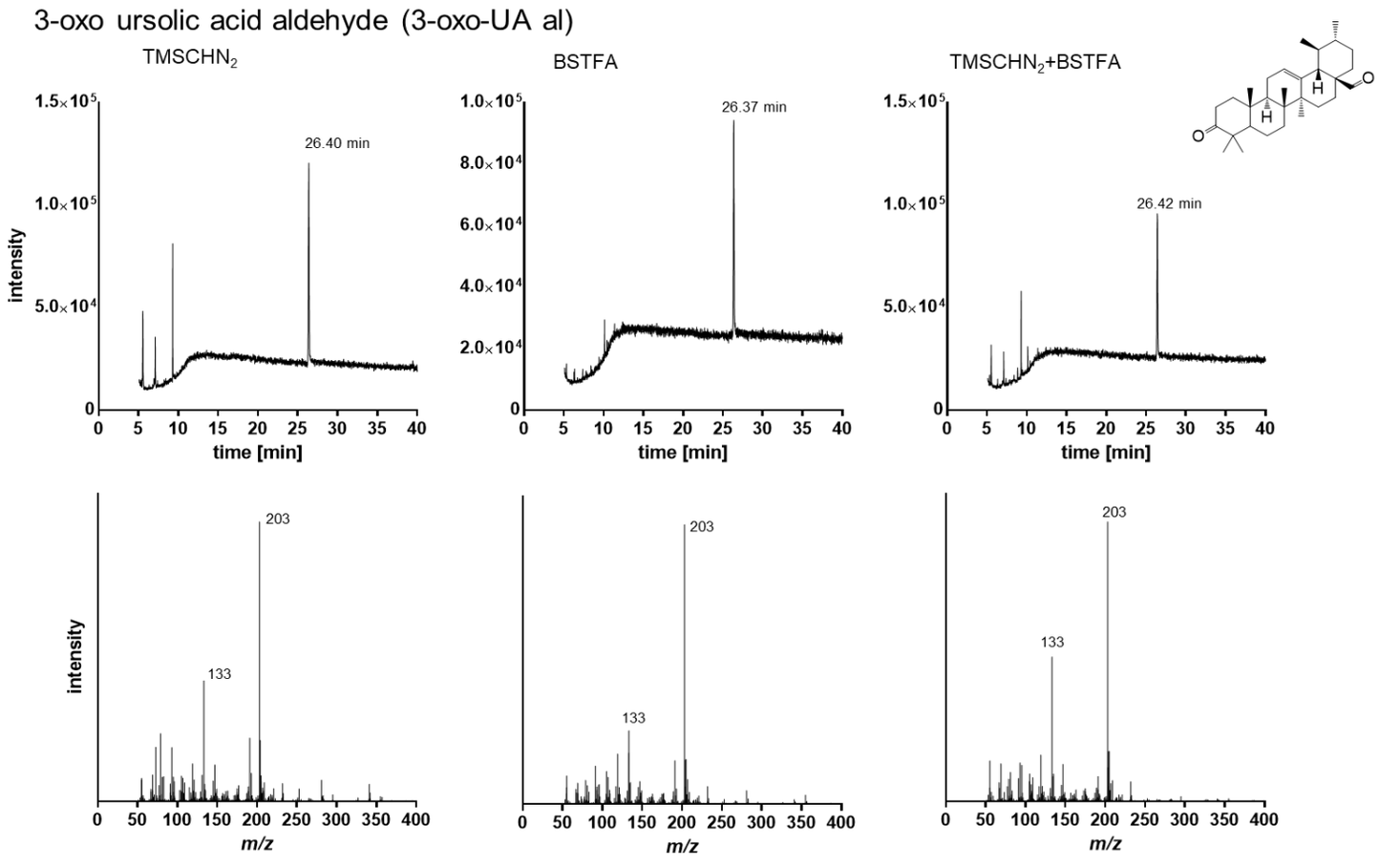

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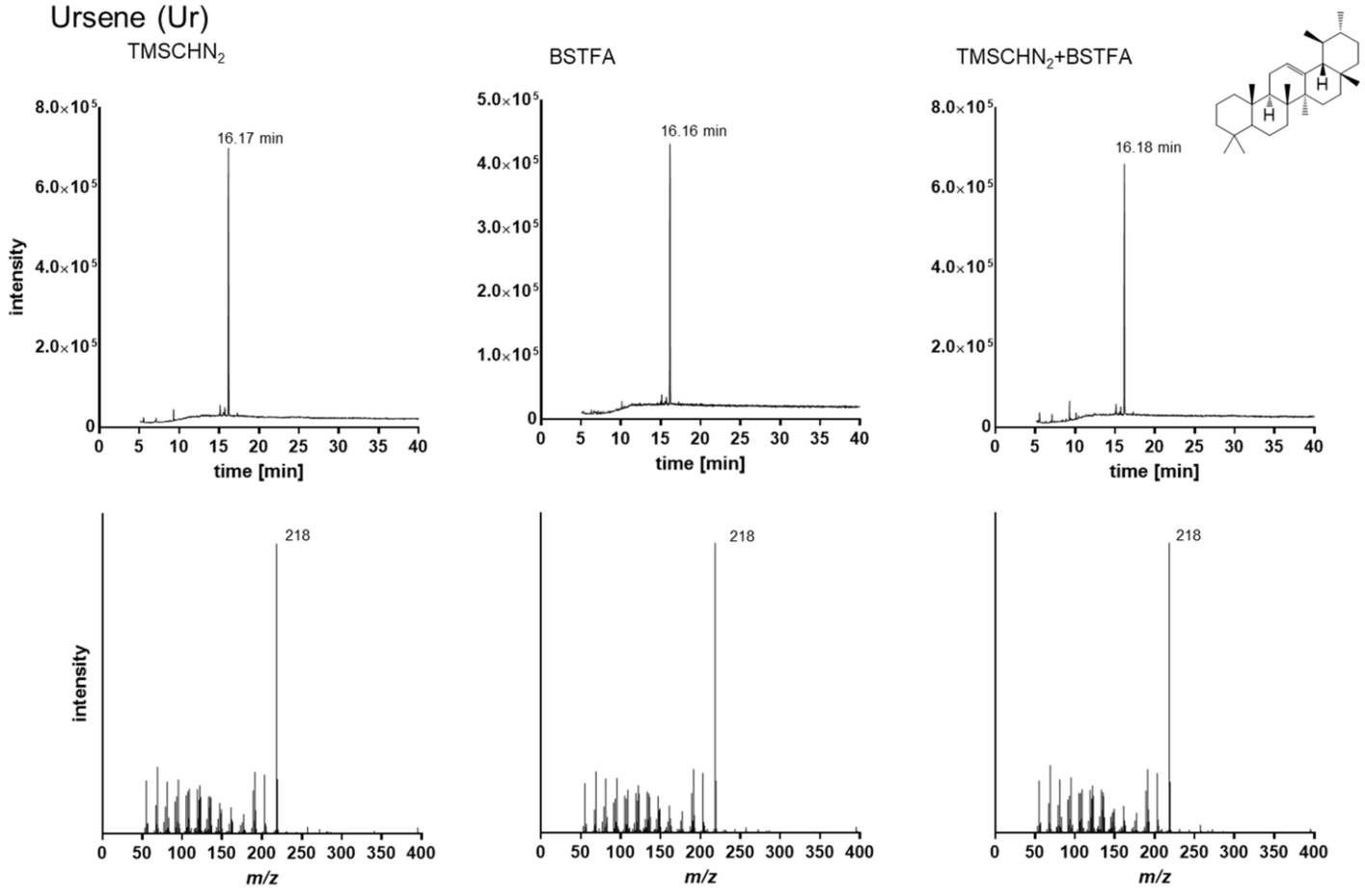

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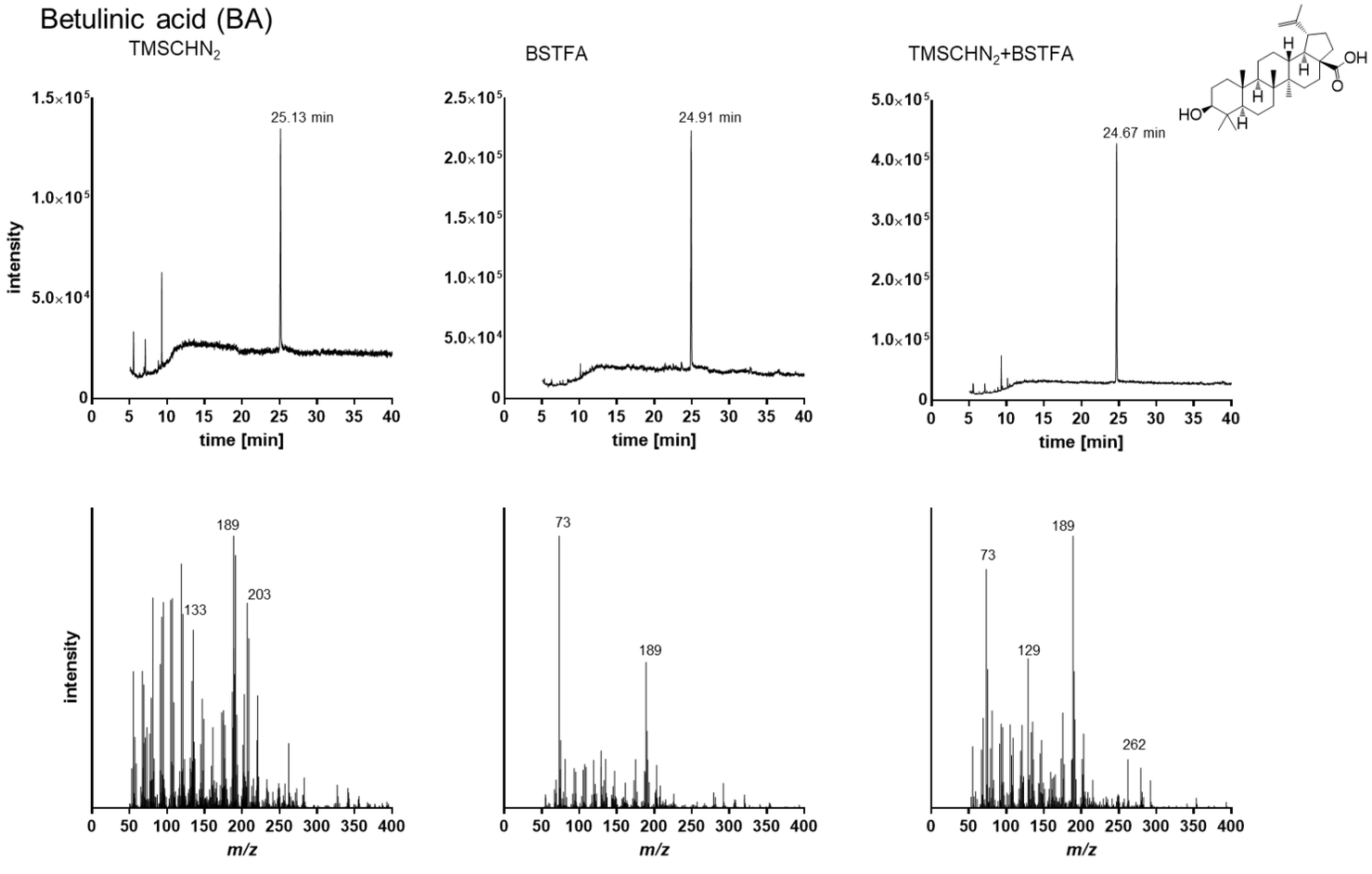

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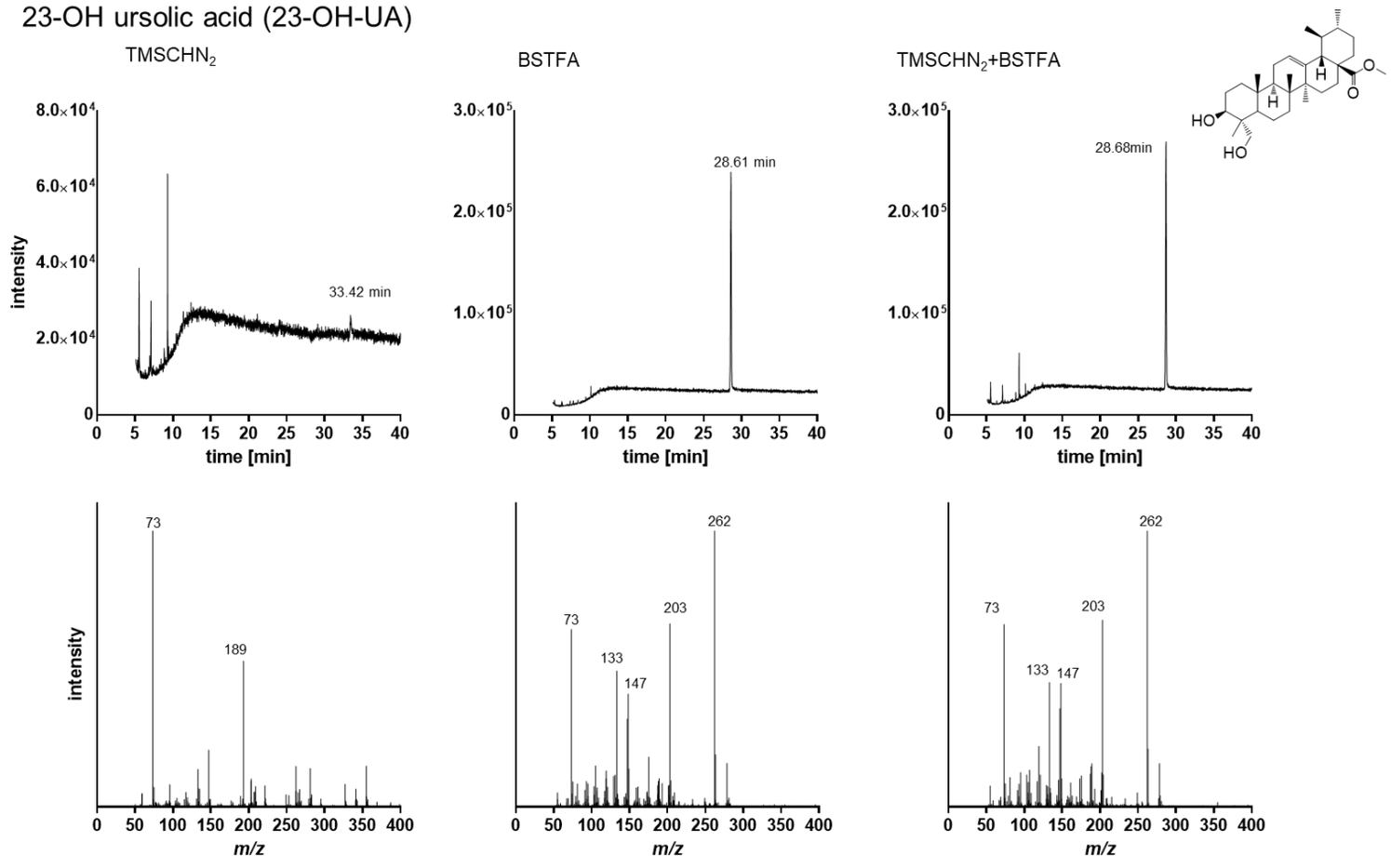

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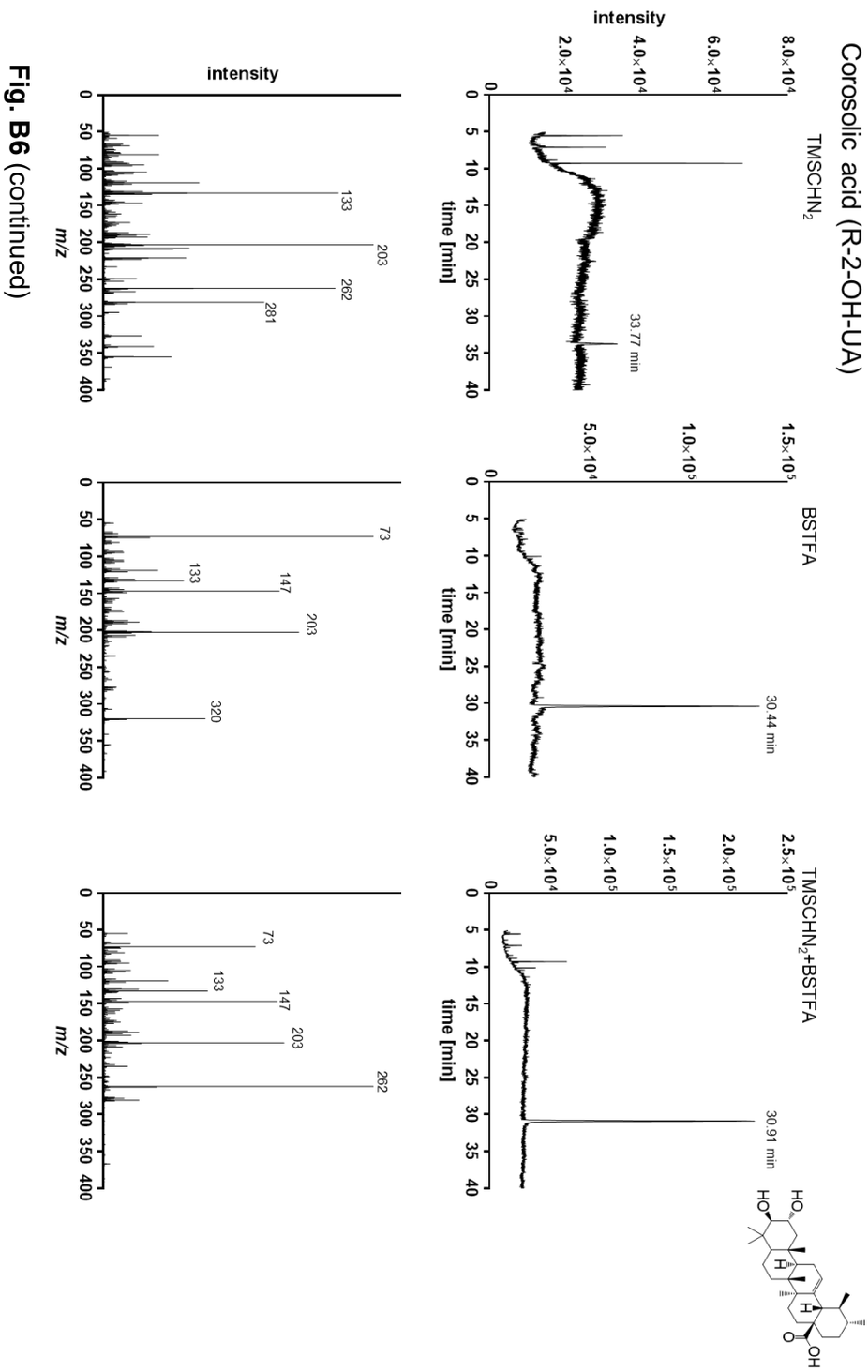

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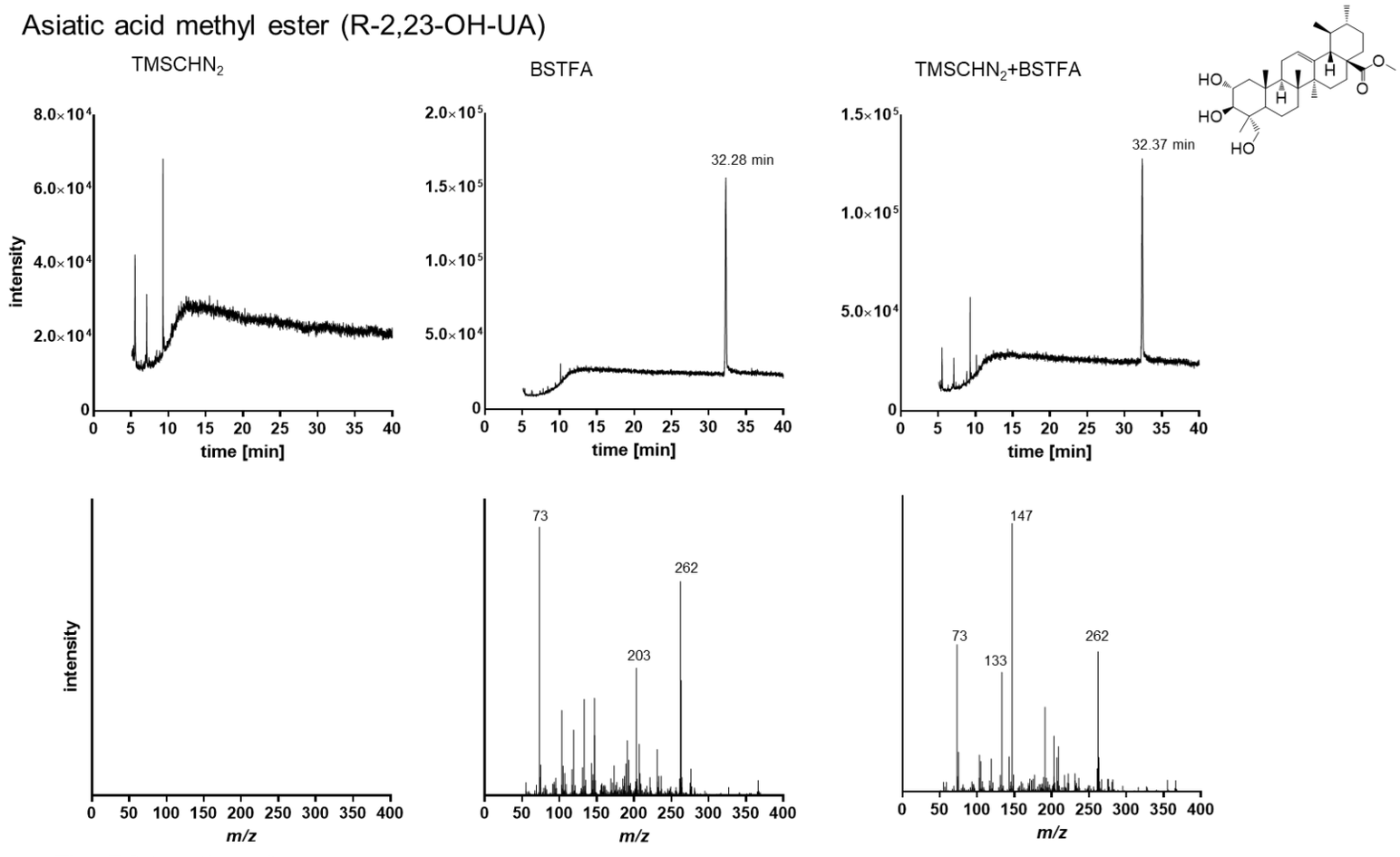

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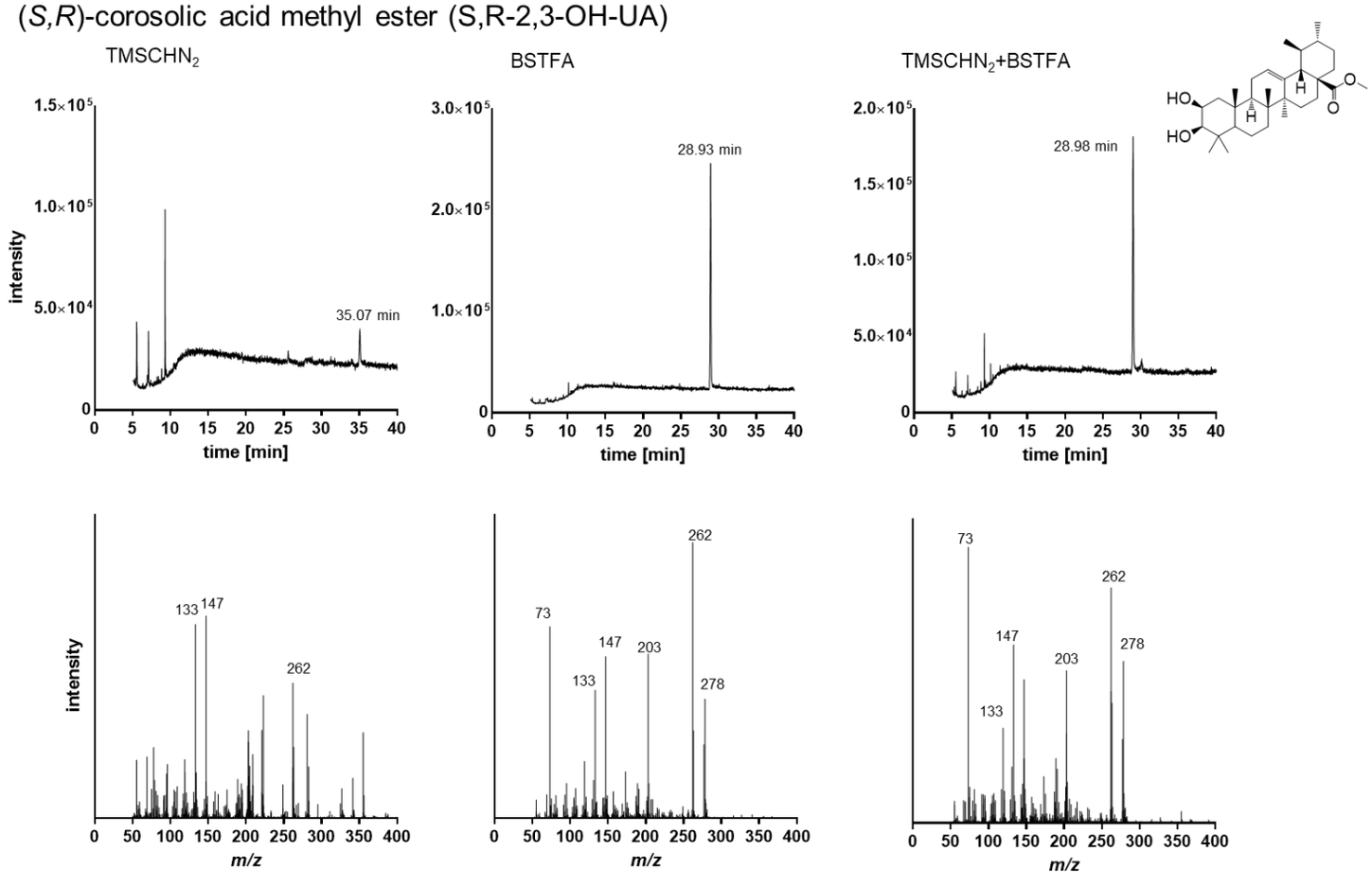
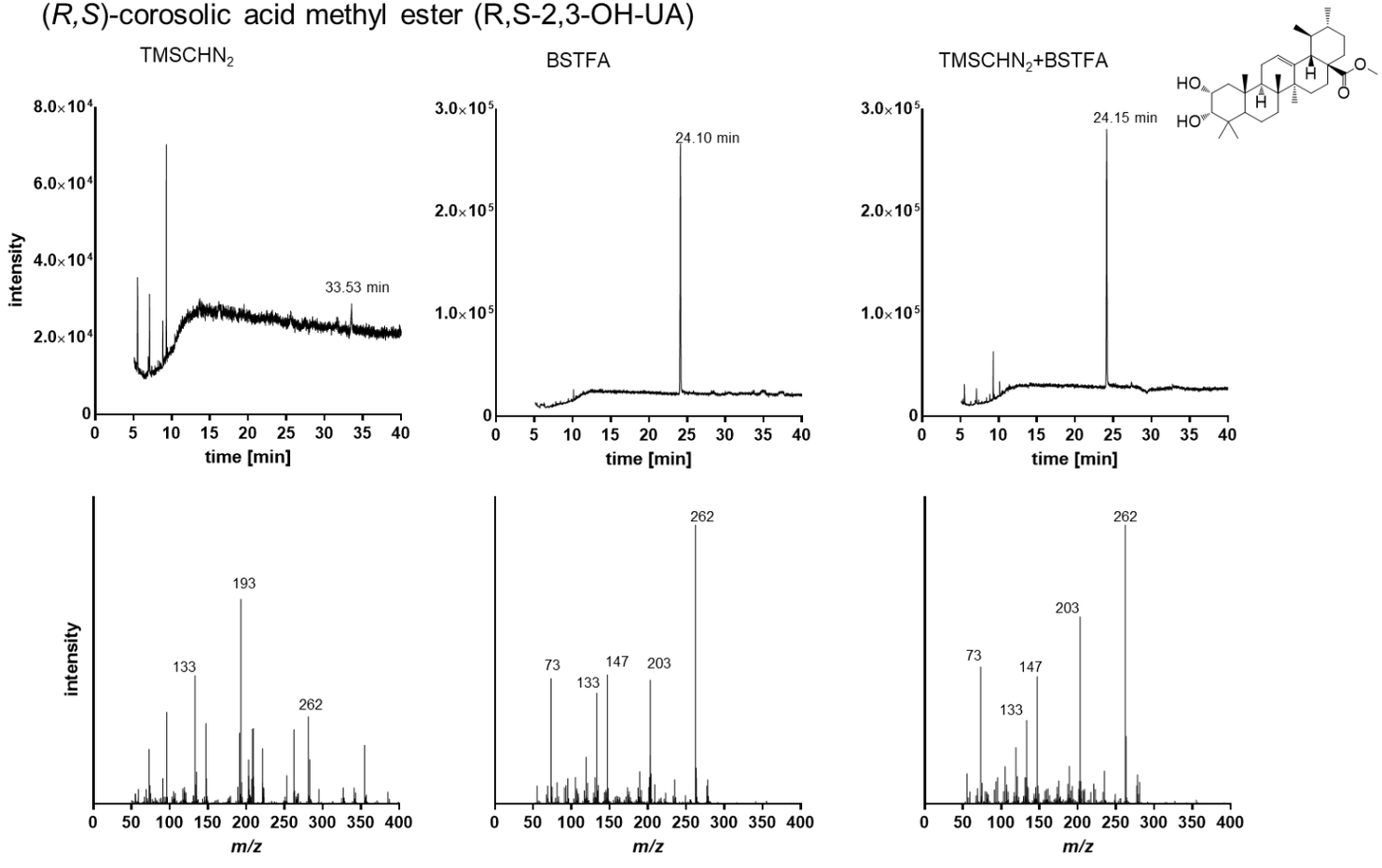


Fig. B6 (continued)

(*R,S*)-corosolic acid methyl ester (R,S-2,3-OH-UA)



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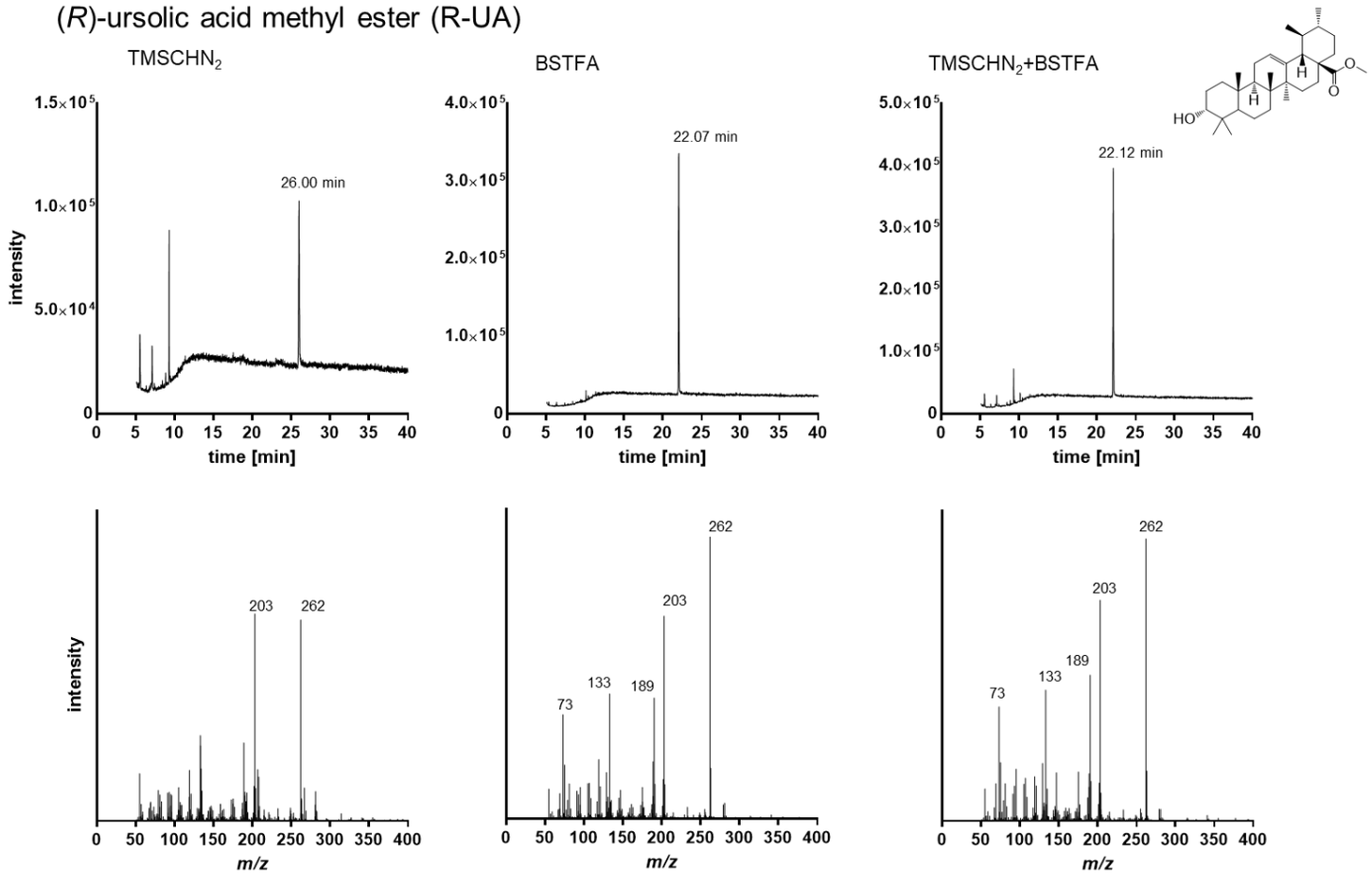

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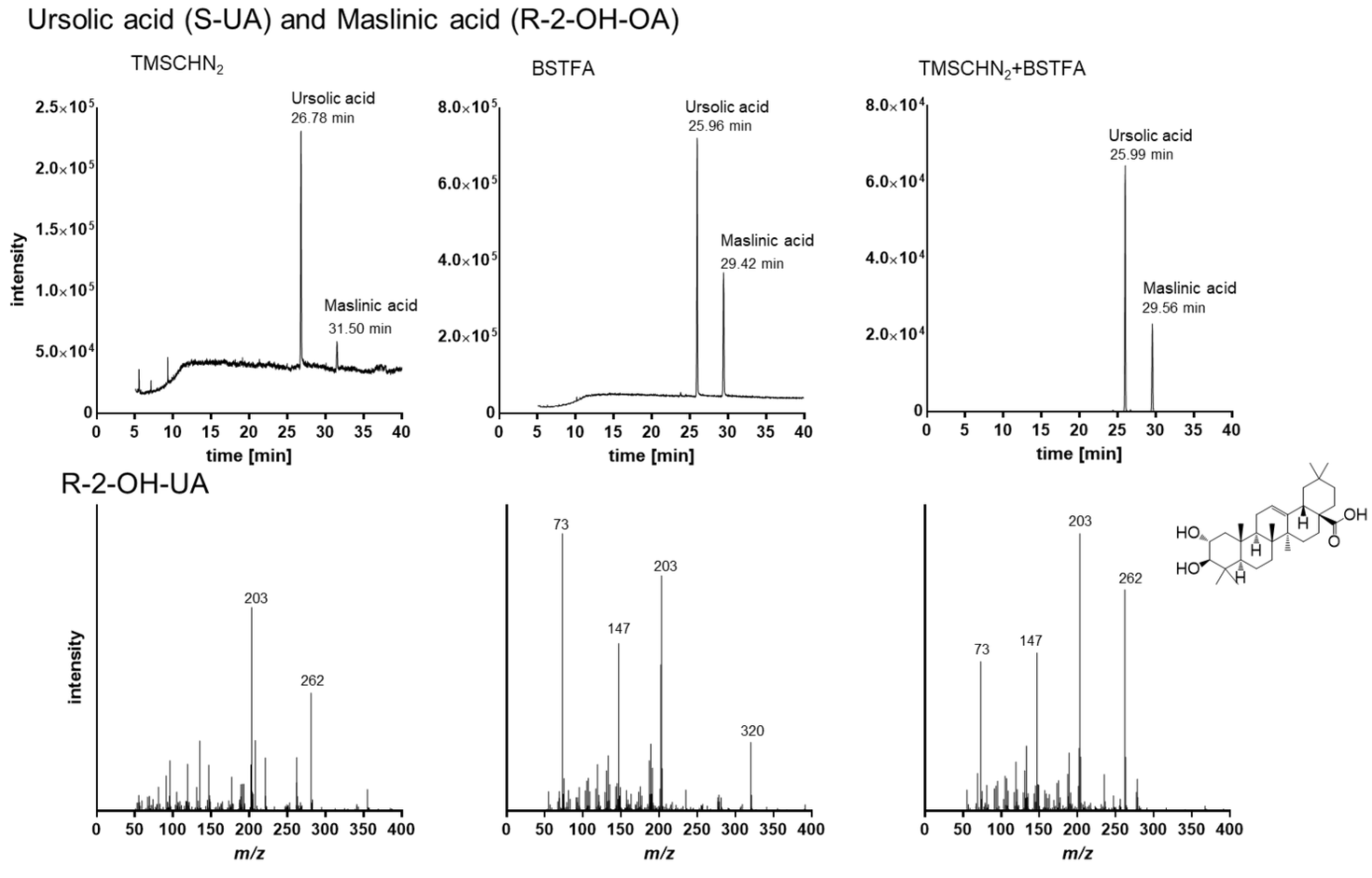


Fig. B6 (continued)

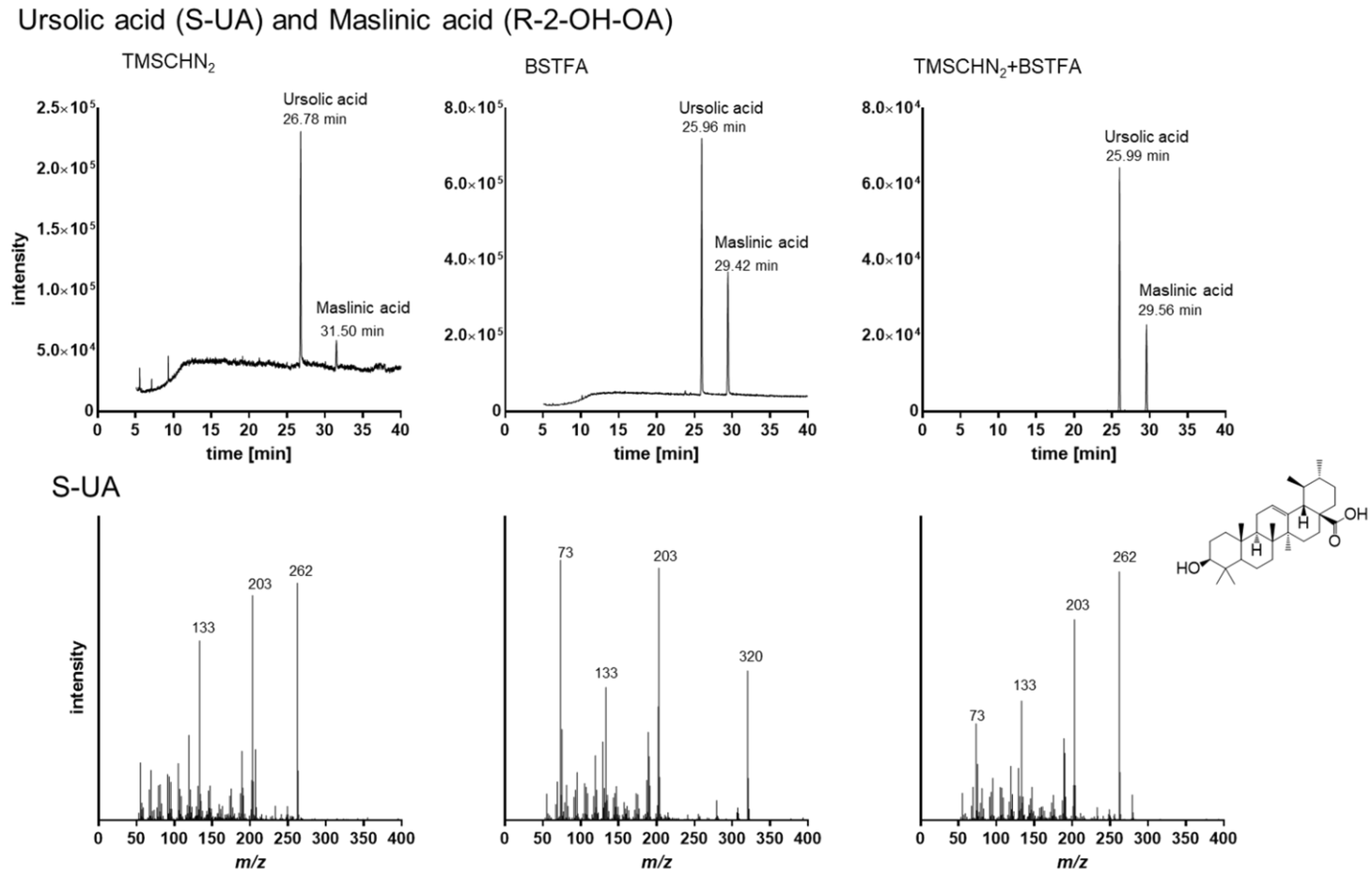
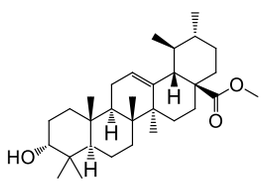


Fig. B6 (continued)

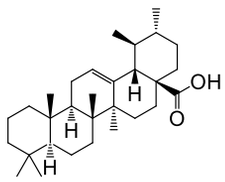
9.1 REFERENCES

[1] Guo, S., Duan, J.-A., Tang, Y.-P., Yang, N.-Y., Qian, D.-W., Su, S.-L., & Shang, E.-X.. Characterization of Triterpenic Acids in Fruits of Ziziphus Species by HPLC-ELSD-MS. *Journal of Agricultural and Food Chemistry*, **2010** *58*(10), 6285-6289.
[2] Guo, S.; Duan, J.-a.; Tang, Y.; Qian, Y.; Zhao, J.; Qian, D.; Su, S.& Shang, E., Simultaneous qualitative and quantitative analysis of triterpenic acids, saponins and flavonoids in the leaves of two Ziziphus species by HPLC–PDA–MS/ELSD. *Journal of Pharmaceutical and Biomedical Analysis,* **2011** *56* (2), 264-270.
[3] Zhang, Y., Xue, K., Zhao, E. Y., Li, Y., Yao, L., Yang, X., & Xie, X. Determination of oleanolic acid and ursolic acid in Chinese medicinal plants using HPLC with PAH polymeric C18. *Pharmacognosy magazine***, 2013** *9*(Suppl 1), S19-24.
[4] Yabukawa, K., & Izuchi, R.. Simultaneous quantitation of pentacyclic triterpenoids in fruit peels using liquid chromatography–single quadrupole mass spectrometry. *Food Chemistry*, **2025** *471*, 142781.

C APPENDIX FOR CHAPTER 4

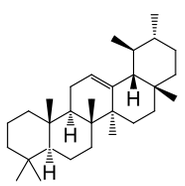


(*R*)-ursolic acid methyl ester (R-UA)

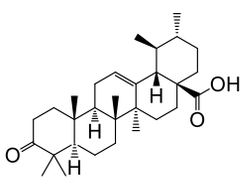


ursenic acid (URA)

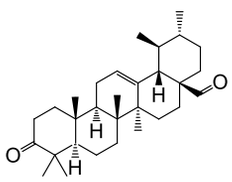
Fig. C1: TA derivatives used as internal standards for quantification.



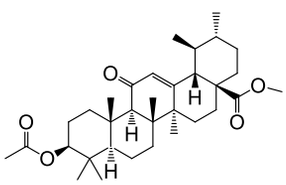
ursene (Ur)



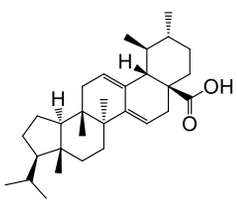
ursonic acid / (3-oxo-UA)



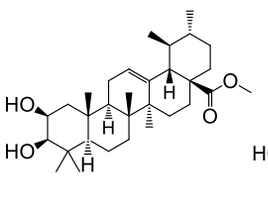
3-oxo ursolic acid aldehyde (3-oxo-UA al)



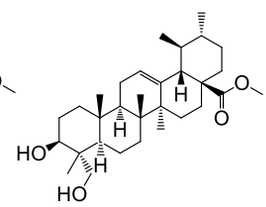
11-oxo ursolic acid acetate (11-oxo-UA ac)



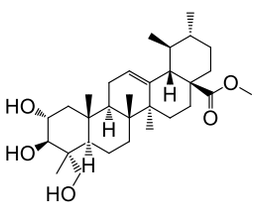
rearranged ursolic acid (ar UA)



(*S,R*)-corosolic acid methyl ester (S,R-2,3-OH-UA)



23-OH ursolic acid (23-OH-UA)



asiatic acid methyl ester / (R-2,23-OH-UA)

Fig. C2: TA derivatives which were covered by the method [1] used in this study and not detected in the analyzed *Rosaceae* fruits.

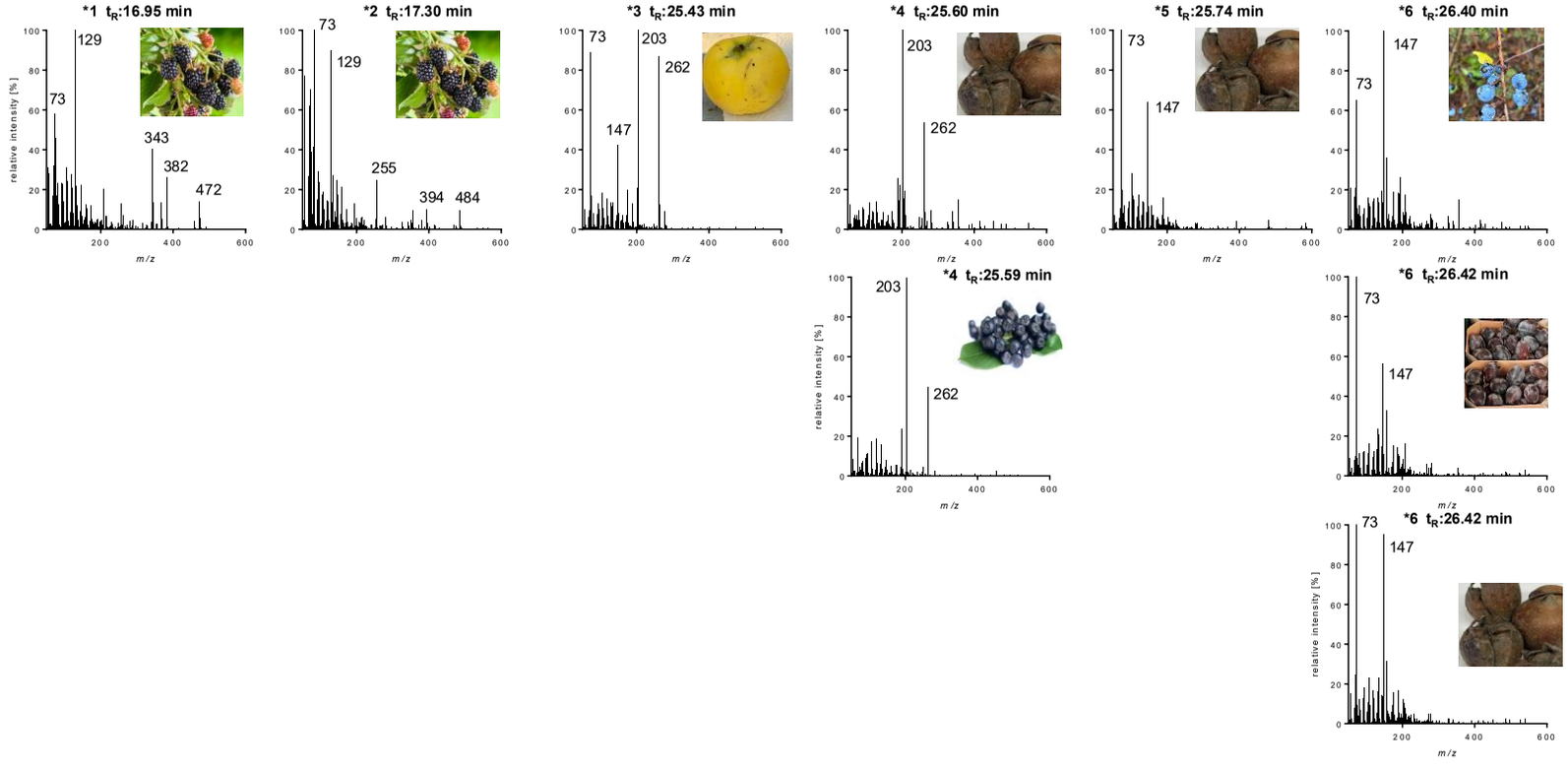


Fig. C3: GC-EI-MS spectra (*m/z* 50-600) of non-characterized TA derivatives of Rosaceae extracts from *Mespilus*, *Cydonia*, and *Aronia* (peaks *1-*6 in Fig. 4.3). Peaks *1 and *2 were exclusively found in *Rubus sect. Rubus*. Peaks *3 and *4 show a fragment ion at m/z 262, indicating the presence of methylated carboxylic acid groups and at *m/z* 73, indicating the presence of trimethylsilylated groups. Peaks *5 and *6 show a fragment ion at *m/z* 73 but not at *m/z* 262, suggesting compounds bearing a hydroxy group but no carboxy groups.

Tab. C1: Origin of *Rosaceae* species investigated: For all analyzed *Rosaceae* fruits, the month and year of harvest, the collected and analyzed material, as well as the sampling location and its coordinates are listed.

Species	Harvest month/year	Collected material	Analyzed material	Origin	Coordinates
Filipendula ulmaria (L.) Maxim.	October 2024	Dry infructescence	Dry infructescence	Wuppertal, Germany	51° 15' 38.5" N, 7° 09' 39.4" E
Rubus phoenicolasius Maxim.	June 2023	Fruit	Whole Fruit	Wetter a.d. Ruhr, Germany	51° 22' 24.8" N, 7° 22' 29.2" E
Rubus sect. *Rubus* L. 1	June 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 14' 3.36" N, 7° 11' 1.02" E
Rubus sect. *Rubus* L. 2	June 2024	Fruit	Whole Fruit	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Rubus idaeus L.	July 2024	Fruit	Whole Fruit	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Rosa canina L. 1	September 2024	Fruit	Peel	Wuppertal, Germany	51° 15' 31.4" N, 7° 11' 13.0" E
Rosa canina L. 2	September 2024	Fruit	Whole fruit	Wuppertal, Germany	51° 14' 23.1" N, 7° 09' 58.3" E
Rosa rugosa Thunb.	September 2024	Fruit	Peel	Heiligenhafen, Germany	54° 22' 09.1" N, 10° 58' 28.8" E
Rosa multiflora Thunb.	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 17' 10.9" N, 7° 10' 09.1" E
Fragaria L.	July 2024	Fruit	Whole Fruit	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Alchemilla L.	October 2024	Dry infructescence	Dry infructescence	Velbert, Germany	51° 19' 40.1" N, 7° 01' 04.7" E
Potentilla fruticosa L.	October 2024	Dry infructescence	Dry infructescence	Wuppertal, Germany	51° 17' 00.9" N, 7° 09' 41.3" E
Agrimonia eupatoria L.	October 2024	Dry infructescence	Dry infructescence	Wuppertal, Germany	51° 15' 38.5" N, 7° 09' 39.4" E
Prunus spinosa L.	October 2024	Fruit	Peel	Wuppertal, Germany	51° 14' 22.4" N, 7° 09' 16.5" E
Prunus avium L.	July 2024	Fruit	Peel	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Prunus domestica subsp. d*omestica* L.	September 2023	Fruit	Peel	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Prunus domestica subsp. s*yriaca* (Borkh.) Janch. ex Mansf.	September 2024	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Mespilus germanica L.	October 2024	Fruit	Peel	Wuppertal, Germany	51° 15' 38.5" N, 7° 09' 39.4" E
Crataegus monogyna Jacq.	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 15' 33.9" N, 7° 11' 04.5" E
Cydonia oblonga Mill.	October 2024	Fruit	Peel	Wuppertal, Germany	51° 15' 31.4" N, 7° 11' 13.0" E

Tab. C1 (continued)

Species	Harvest month/year	Collected material	Analyzed material	Origin	Coordinates
Sorbus aucuparia L. 1	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 14' 59.0" N, 7° 15' 19.6" E
Sorbus aucuparia L. 2	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 14' 43.3" N, 7° 08' 54.1" E
Sorbus aria (L.) Crantz	September 2022	Fruit	Whole Fruit	Wuppertal, Germany	51° 14' 43.3" N, 7° 08' 54.1" E
Pyrus communis L. cv. Williams Christ	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Pyrus pyraster (L.) Du Roi	September 2024	Fruit	Peel	Wuppertal, Germany	51° 14' 43.3" N, 7° 08' 54.1" E
Pyrus communis L. cv. Köstliche von Charneux	September 2023	Fruit	Peel	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Pyrus communis L. cv. Doyenné du Comice	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. (Apple pomace)	October 2023	Fruit	Peel	Remscheid, Germany	51° 10' 49.5" N, 7° 14' 35.7" E
Malus domestica Borkh. cv. Galmac	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Santana	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Elstar 1	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Elstar 2	September 2023	Fruit	Peel	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Malus domestica Borkh. cv. Elstar 3	September 2023	Fruit	Peel	Leichlingen, Germany	51° 05' 51.9" N, 7° 02' 12.9" E
Malus domestica Borkh. cv. Gala 1	September 2023	Fruit	Peel	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Malus domestica Borkh. cv. Gala 2	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Rubinette	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Golden Delicious 1	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Golden Delicious 2	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Topaz	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E

Tab. C1 (continued)

Species	Harvest month/year	Collected material	Analyzed material	Origin	Coordinates
Malus domestica Borkh. cv. Delbarestivale	August 2023	Fruit	Peel	Solingen, Germany	51° 08' 29.9" N, 7° 00' 54.9" E
Malus domestica Borkh. cv. Wellant 1	September 2023	Fruit	Peel	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Malus domestica Borkh. cv. Wellant 2	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Wellant 3	September 2023	Fruit	Peel	Leichlingen, Germany	51° 05' 51.9" N, 7° 02' 12.9" E
Malus domestica Borkh. cv. Fuji	March 2024	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Boskoop 1	September 2023	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Boskoop 2	September 2023	Fruit	Peel	Leichlingen, Germany	51° 05' 51.9" N, 7° 02' 12.9" E
Malus domestica Borkh. cv. Boskoop 3	September 2023	Fruit	Peel	Wermelskirchen, Germany	51° 6' 37.5" N, 7° 10' 28.56" E
Malus domestica Borkh. cv. Falstaf	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. (cv. unknown)	August 2024	Fruit	Peel	Remscheid, Germany	51° 11' 47.8" N 7° 10' 15.1" E
Malus domestica Borkh. cv. Mairac	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. Berlepsch	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Malus domestica Borkh. cv. James Grieve	September 2022	Fruit	Peel	Leichlingen, Germany	51° 5′ 55.7004″ N, 7° 3′ 16.7789″ E
Cotoneaster horizontalis Decne.	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 17' 00.9" N, 7° 09' 41.3" E
Cotoneaster dammeri C.K.Schneid.	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 17' 00.9" N, 7° 09' 41.3" E
Cotoneaster bullatus Bois	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 17' 00.9" N, 7° 09' 41.3" E
Cotoneaster dielsianus E.Pritz.	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 15' 32.0" N, 7° 11' 04.3" E
Pyracantha M.Roem.	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 15' 31.4" N, 7° 11' 13.0" E
Pyracantha M.Roem.	September 2024	Fruit	Whole Fruit	Wuppertal, Germany	51° 12' 26.7" N, 7° 11' 07.7" E

Tab. C1 (continued)

Species	Harvest month/year	Collected material	Analyzed material	Origin	Coordinates
Pyracantha M.Roem.	September 2023	Fruit	Whole Fruit	Wuppertal, Germany	51° 14' 03.3" N, 7° 09' 08.9" E
Aronia Medik.	June 2022	Fruit	Whole Fruit	Leichlingen, Germany	51° 05' 51.5" N, 7° 00' 11.8" E

Tab. C2: Ratios of TA concentrations relative to S-UA (mean ± SD [%], n=3): The ratios differ both when comparing genera and when comparing different species within a genus. However, the ratios of different cultivars within a species of *Malus* are comparable.

Species	*Filipendula ulmaria* (L.) Maxim.	*Rubus phoenicolasius* Maxim.	*Rubus* sect. *Rubus* L. Sample 1	*Rubus* sect. *Rubus* L. Sample 2	*Rubus idaeus* L.
Analyzed	Dry infructescence	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit
OA	<LLOQ	<LLOQ	151 ± 8	154 ± 5	182 ± 4
BA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
R-2-OH-UA	160 ± 30 (16)	<LLOQ	<LLOQ	<LLOQ	<LLOQ
R-2-OH-OA	110 ± 10 (8)	<LLOQ	<LLOQ	1110 ± 40	<LLOQ
S-19-OH-UA	<LLOQ	<LLOQ	157 ± 17	143 ± 1	<LLOQ
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	<LLOQ	41 ± 3	41 ± 9	41 ± 5	37 ± 2
UV	6.7 ± 0,4	<LLOQ	<LLOQ	<LLOQ	<LLOQ
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Species	*Rosa canina* L. 1	*Rosa canina* L. 2	*Rosa rugosa* Thunb.	*Rosa multiflora* Thunb.	*Fragaria* L.
Analyzed	Peel	Whole Fruit	Peel	Whole Fruit	Whole Fruit
OA	76 ± 10	350 ±20	17,8 ± 0.7	100 ± 15	<LLOQ
BA	<LLOQ	<LLOQ	4,5 ± 0.4	120 ± 12	<LLOQ
R-2-OH-UA	<LLOQ	19 ± 13	3,2 ± 0.7	15.3 ± 0.9	<LLOQ
R-2-OH-OA	<LLOQ	23 ± 3	2,1 ± 0.8	93 ± 26	<LLOQ
S-19-OH-UA	170 ± 30	<LLOQ	7,5 ±1.2	<LLOQ	<LLOQ
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	5.2 ± 0.5	0.75 ± 0.01	0,08 ± 0.01	1.9 ±0.3	<LLOQ
UV	<LLOQ	<LLOQ	0,71 ±0.05	<LLOQ	<LLOQ
UA ac	<LLOQ	<LLOQ	<LLOQ	150 ± 70	<LLOQ

Tab. C2 (continued)

Species	*Alchemilla* L.	*Potentilla fruticosa* L.	*Agrimonia eupatoria* L.	*Prunus spinosa* L.	*Prunus avium* L.
Analyzed	Dry infructescence	Dry infructescence	Dry infructescence	Peel	Peel
OA	30 ± 4	29 ± 3	<LLOQ	22.4 ± 0.9	12.9 ± 0.2
BA	<LLOQ	<LLOQ	<LLOQ	32 ± 4	<LLOQ
R-2-OH-UA	204 ± 8	72 ± 6	30 ±3	0.9 ± 0.2	16 ± 1
R-2-OH-OA	36 ± 6	25 ± 4	130 ± 10	<LLOQ	<LLOQ
S-19-OH-UA	160 ± 40	7,9 ±2.9	<LLOQ	8.7 ± 1.4	<LLOQ
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	0.39 ± 0.07	0,50 ±0.07	3,2 ± 0.5	0.29 ± 0.04	<LLOQ
UV	3.2 ± 0.9	1.5 ± 0.2	<LLOQ	<LLOQ	<LLOQ
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Species	*Prunus domestica* subsp. *domestica* L.	*Prunus domestica* subsp. *syriaca* (Borkh.) Janch. ex Mansf.	*Mespilus germanica* L.	*Crataegus monogyna* Jacq.	*Cydonia oblonga* Mill.
Analyzed	Peel	Peel	Peel	Whole Fruit	Peel
OA	760 ± 90	250 ± 30	70 ± 20	17.5 ± 0.7	12 ± 1
BA	<LLOQ	90 ± 8	120 ± 40	<LLOQ	<LLOQ
R-2-OH-UA	<LLOQ	<LLOQ	10 ± 3	29 ±3	21 ± 6
R-2-OH-OA	460 ± 40	180 ± 20	80 ± 20	33 ± 7	6.8 ±1.0
S-19-OH-UA	<LLOQ	490 ± 100	380 ± 150	47 ± 7	7.0 ±0.2
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	24 ±2
AM	<LLOQ	<LLOQ	1.1 ± 0.3	<LLOQ	0.57 ± 0.05
UV	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.93 ± 0.06
UA ac	<LLOQ	<LLOQ	13 ± 9	<LLOQ	<LLOQ

Tab. C2 (continued)

Species	*Sorbus aucuparia* L. 1	*Sorbus aucuparia* L. 2	*Sorbus aria* (L.) Crantz	*Pyrus communis* L. cv. Williams Christ	*Pyrus pyraster* (L.) Du Roi
Analyzed	Whole Fruit	Whole Fruit	Whole Fruit	Peel	Peel
OA	66 ± 9	31 ± 1	59 ± 6	23 ± 3	49 ± 1
BA	<LLOQ	<LLOQ	450 ± 80	22 ± 4	15 ± 1
R-2-OH-UA	9 ± 2	3.3 ± 0.3	<LLOQ	<LLOQ	2.6 ± 0.1
R-2-OH-OA	10 ± 3	12 ± 3	<LLOQ	9.4 ± 1.4	14.2 ± 0.9
S-19-OH-UA	140 ±30	46 ± 5	<LLOQ	32 ± 2	74 ± 4
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	0.5 ± 0.2	1.2 ± 0.1	<LLOQ	0.5 ± 0.2	0.32 ± 0.03
UV	6.2 ± 1.5	4.4 ± 0.2	<LLOQ	2.0 ± 0.2	2.5 ± 0.1
UA ac	4.3 ± 0.1	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Species	*Pyrus communis* L. cv. Köstliche von Charneux	*Pyrus communis* L. cv. Doyenné du Comice	*Malus domestica* Borkh. (Apple pomace)	*Malus domestica* Borkh. cv. Galmac	*Malus domestica* Borkh. cv. Santana
Analyzed	Peel	Peel	Peel	Peel	Peel
OA	15.0 ± 0.2	38 ± 2	13.7 ± 0.4	28.4 ± 0.4	14.8 ± 0.1
BA	4.9 ± 0.4	<LLOQ	<LLOQ	4.3 ± 0.6	<LLOQ
R-2-OH-UA	3.2 ± 0.4	2.9 ± 0.2	12 ± 1	6.1 ± 0.4	2.9 ± 0.1
R-2-OH-OA	2.8 ± 0.2	19 ± 3	5.4 ± 0.5	7.0 ± 0.7	1.29 ± 0.04
S-19-OH-UA	12.6 ± 0.3	33 ± 4	12.0 ± 0.3	<LLOQ	8.1 ± 0.2
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	0.12 ± 0.01	0.48 ± 0.04	0.076 ± 0.003	0.074 ± 0.009	0.061 ± 0.013
UV	0.93 ± 0.05	1.9 ± 0.1	0.94 ± 0.03	0.63 ± 0.05	0.90 ± 0.11
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

Tab. C2 (continued)

Species	*Malus domestica* Borkh. cv. Elstar 1	*Malus domestica* Borkh. cv. Elstar 2	*Malus domestica* Borkh. cv. Elstar 3	*Malus domestica* Borkh. cv. Gala 1	*Malus domestica* Borkh. cv. Gala 2
Analyzed	Peel	Peel	Peel	Peel	Peel
OA	16 ± 2	16.1 ± 0.4	17 ± 1	15.6 ± 0.6	15.0 ± 0.9
BA	3.1 ± 0.9	<LLOQ	<LLOQ	<LLOQ	2.6 ± 0.3
R-2-OH-UA	4.4 ± 0.7	4.7 ± 0.8	3.2 ± 0.3	6.8 ± 0.6	4.2 ± 0.2
R-2-OH-OA	3.6 ± 1.1	2.8 ±0.3	2.1 ± 0.2	4.4 ± 0.9	2.9 ± 0.5
S-19-OH-UA	12 ± 4	17 ± 2	12.0 ± 0.1	17 ± 2	12.6 ± 0.9
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	0.11 ± 0.04	0.078 ± 0.012	0.082 ± 0.005	0.049 ± 0.009	0.060 ± 0.005
UV	1.0 ± 0.2	0.84 ± 0.06	1.3 ± 0.1	0.82 ± 0.06	0.81 ± 0.05
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Species	*Malus domestica* Borkh. cv. Rubinette	*Malus domestica* Borkh. cv. Golden Delicious 1	*Malus domestica* Borkh. cv. Golden Delicious 2	*Malus domestica* Borkh. cv. Topaz	*Malus domestica* Borkh. cv. Delbarestivale
Analyzed	Peel	Peel	Peel	Peel	Peel
OA	20 ± 1	13.9 ± 0.2	14 ± 1	14 ± 1	15 ± 2
BA	3.5 ± 0.8	2.0 ±0.2	2.2 ± 0.6	2.3 ± 0.3	2.2 ± 0.6
R-2-OH-UA	5.6 ± 0.1	4.6 ± 0.3	4.1 ± 0.5	6.4 ± 0.1	2.2 ± 0.4
R-2-OH-OA	6.9 ± 0.7	2.7 ± 0.3	2.5 ± 0.3	3.4 ± 0.3	2.0 ± 0.6
S-19-OH-UA	24 ± 2	5.6 ± 0.7	7.0 ± 1	6.0 ± 0.5	11 ± 3
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	0.10 ± 0.02	0.11 ± 0.01	0.11 ± 0.02	0.055 ± 0.005	0.072 ± 0.012
UV	1.13 ± 0.08	2.1 ± 0.1	1.8 ± 0.3	1.0 ± 0.1	0.83 ± 0.13
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

Tab. C2 (continued)

Species	*Malus domestica* Borkh. cv. Wellant 1	*Malus domestica* Borkh. cv. Wellant 2	*Malus domestica* Borkh. cv. Wellant 3	*Malus domestica* Borkh. cv. Fuji	*Malus domestica* Borkh. cv. Boskoop 1
Analyzed	Peel	Peel	Peel	Peel	Peel
OA	21 ± 1	34 ± 2	21 ± 1	13 ± 1	24 ± 2
BA	4.5 ± 0.7	5.2 ± 0.8	3.8 ± 0.1	2.1 ± 0.6	9.0 ± 1
R-2-OH-UA	7.6 ± 0.3	10 ± 1	10 ± 1	7.1 ± 1.0	12 ± 1
R-2-OH-OA	8.3 ± 0.2	8.7 ± 0.8	8.3 ± 0.4	3.9 ± 0.8	9.0 ± 1
S-19-OH-UA	28 ± 4	28 ± 3	33 ± 4	2.8 ± 0.4	26 ± 2
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	0.11 ± 0.02	0.12 ± 0.02	0.10 ± 0.01	0.092 ± 0.015	0.35 ± 0.02
UV	0.71 ± 0.06	0.80 ± 0.05	0.64 ± 0.04	2.6 ± 0.2	0.91 ± 0.11
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Species	*Malus domestica* Borkh. cv. Boskoop 2	*Malus domestica* Borkh. cv. Boskoop 3	*Malus domestica* Borkh. cv. Falstaf	*Malus domestica* Borkh. (cv. Unknown)	*Malus domestica* Borkh. cv. Mairac
Analyzed	Peel	Peel	Peel	Peel	Peel
OA	29 ± 1	18.1 ± 0.4	14.8 ± 0.3	19 ± 1	23 ± 2
BA	57 ± 6	4.4 ± 0.3	2.3 ± 0.1	3.5 ± 0.6	5.3 ± 1.2
R-2-OH-UA	5.3 ± 0.3	8.8 ± 0.4	5.8 ± 0.2	3.7 ± 0.2	18 ± 3
R-2-OH-OA	28 ± 5	6.6 ± 0.5	3.0 ± 0.2	2.9 ± 0.4	9.7 ± 0.7
S-19-OH-UA	80 ± 8	17 ± 2	6.2 ± 0.4	35 ± 7	35 ± 3
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AM	3.1 ± 0.5	0.15 ± 0.03	0.063 ± 0.003	0.070 ± 0.007	0.087 ± 0.017
UV	3.2 ± 0.5	0.66 ± 0.01	0.85 ± 0.03	0.96 ± 0.07	0.73 ± 0.09
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

Tab. C2 (continued)

Species	*Malus domestica* Borkh. cv. Berlepsch	*Malus domestica* Borkh. cv. James Grieve	*Cotoneaster horizontalis* Decne.	*Cotoneaster dammeri* C.K.Schneid.	*Cotoneaster bullatus* Bois
Analyzed	Peel	Peel	Whole Fruit	Whole Fruit	Whole Fruit
OA	21 ± 2	15 ± 1	11.5 ± 0.3	32 ± 2	12.1 ± 0.2
BA	5.2 ± 1.5	2.6 ± 0.5	<LLOQ	<LLOQ	<LLOQ
R-2-OH-UA	4.6 ± 0.2	6.2 ± 0.8	10.8 ± 0.9	60 ± 10	12 ± 3
R-2-OH-OA	7.7 ± 1.7	3.2 ± 0.6	4.6 ± 0.4	54 ± 8	7.3 ± 2.3
S-19-OH-UA	27 ± 5	5.0 ± 1.0	<LLOQ	100 ± 10	<LLOQ
R,S-2,3-OH-UA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	1.3 ± 0.3
AM	0.10 ± 0.03	0.047 ± 0.007	0.28 ± 0.02	2.3 ± 0.2	0.63 ± 0.05
UV	0.73 ± 0.10	0.41 ± 0.04	1.6 ± 0.1	11 ± 1	3.2 ± 0.5
UA ac	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Species	*Cotoneaster dielsianus* E.Pritz.	*Aronia* Medik.	*Pyracantha* M.ROEM.	*Pyracantha* M.ROEM.	*Pyracantha* M.ROEM.
Analyzed	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit	Whole Fruit
OA	23 ± 1	18.2 ± 0.2	18 ± 1	18 ± 1	<LLOQ
BA	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
R-2-OH-UA	6.5 ± 0.5	15 ± 1	440 ± 50	440 ± 50	230 ± 20
R-2-OH-OA	15 ± 3	21.4 ± 0.2	104 ± 5	120 ± 20	89 ± 3
S-19-OH-UA	5.4 ± 0.4	<LLOQ	<LLOQ	<LLOQ	<LLOQ
R,S-2,3-OH-UA	<LLOQ	4.4 ± 0.2	<LLOQ	<LLOQ	<LLOQ
AM	0.69 ± 0.08	0.36 ± 0.01	2.2 ± 0.1	3.1 ± 0.2	2.5 ± 0.2
UV	3.0 ± 0.3	2.1 ± 0.2	20 ± 1	18 ± 3	6.8 ± 0.3
UA ac	<LLOQ	40 ± 1	<LLOQ	<LLOQ	<LLOQ

Tab. C3: *m/z* values of the ions monitored in SIM to quantify TA. For the different TA derivatives detected in the analyzed *Rosaceae* samples, it is indicated which ions are formed by the derivatized molecules and which are used as quantifier (**bold**). [1] The *m/z* were monitored to detect rearranged ursolic acid and 11-oxo-ursolic acid acetate [1] which were not detected in the analyzed *Rosaceae* fruits.

m/z ion	ursolic acid (S-UA)	oleanolic acid (OA)	betulinic acid (BA)	corosolic acid (R-2-OH-UA)	maslinic acid (R-2-OH-OA)	pomolic acid (S-19-OH-UA)	(R,S)-corosolic acid methyl ester/ (R,S)-2,3-OH-UA	alpha-amyrine (AM)	uvaol (UV)	ursolic acid acetate (UA ac)	ursenic acid (URA)	*(*R)-ursolic acid methyl ester (R-UA)
129	X		X			X						X
133	X	X	X	X	X	X	X	X	X	X	X	X
147			X	X	X	X	X					
173												
179						**X**						
189	X	X	**X**	X	X	X	X	X	X	X	X	X
216									**X**			
218								**X**				
262	**X**	**X**	X	**X**	**X**	X	**X**			**X**	**X**	**X**
278	X			X	X		X					
285[1]												
317[1]												

Tab. C4: Concentration (mean ± SD) of TA in apple peel used as quality control for TA extraction. To control the performance of the extraction procedure, apple peel with known TA amount was extracted in each batch of sample preparation and the TA concentration determined. Interday mean values and relative SD (RSD) were calculated for repeated (n=2) analysis on six different days. The RSD values below 20% indicate a reproducible sample preparation for all extraction batches.

		TA concentration [µmol/g dry extract]				
Apple peel QC Sample		S-UA	OA	R-2-OH-OA	R-2-OH-UA	S-19-OH-UA
Day 1	1	4.48	0.84	0.20	0.22	1.04
	2	5.67	1.14	0.23	0.27	1.24
Day 2	1	4.07	0.87	0.20	0.22	0.83
	2	4.89	0.94	0.20	0.23	1.04
Day 3	1	3.16	0.68	0.15	0.19	0.55
	2	3.87	0.80	0.17	0.21	0.84
Day 4	1	4.70	1.01	0.24	0.26	1.16
	2	3.47	0.73	0.18	0.19	0.84
Day 5	1	6.00	1.08	0.24	0.22	1.00
	2	4.70	0.84	0.20	0.18	0.82
Day 6	1	4.57	0.89	0.26	0.19	1.21
	2	4.33	0.87	0.26	0.20	1.15
Mean interday		4.49	0.89	0.21	0.21	0.98
SD		0.66	0.09	0.03	0.02	0.18
RSD [%]		15	10	14	9	18

10.1 REFERENCES

[1] Wiebel, M., Wende, L., Bensberg, K., Zschau, T., Kirsch, S. F., & Schebb, N. H. Development of a gas chromatography–mass spectrometry (GC–MS) method for the characterization and quantification of triterpenic acids. *Food Chemistry*, **2025** *14601*

LIST OF ABBREVIATIONS

11-oxo-UA ac	11-oxo-ursolic acid acetate
3-oxo-UA	Ursonic acid
AM	alpha-amyrin
ar UA	Rearranged ursolic acid
BA	Betulinic acid
CA	Corosolic acid
CYP	CYP P450 monooxygenase
DW	Dry weight
EI	Electron ionization
FID	Flame Ionization Detection
GC	Gas chromatography
IS	Internal standard
LC	Liquid chromatography
LLOQ	Lower Limit of quantification
LOD	Limit of detection
MA	Maslinic acid
MS	Mass spectrometry
OA	Oleanolic acid
OSC	Oxidosqualene cyclase
PA	Pomolic acid
R,S-2,3-OH-UA	(*R,S*)-corosolic acid methyl ester
R-2,23-OH-UA	Asiatic acid methyl ester
R-2-OH-OA	Maslinic acid

R-2-OH-UA	Corosolic acid
R-UA	(*R*)-ursolic acid methyl ester
S,R-2,3-OH-UA	(*S,R*)-corosolic methyl ester
S-19-OH-UA	Pomolic acid
S-UA	Ursolic acid
TA	Triterpenic acid
TLC	Thin-layer chromatography
UA ac	Ursolic acid acetate
UR	Ursene
URA	Ursenic acid
UV	Uvaol

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CURRICULUM VITAE

The Curriculum Vitae is not available on the online version.

LIST OF PUBLICATIONS

14.1 PUBLICATIONS IN PEER-REVIEWED JOURNALS

Within the scope of this thesis

Wiebel, M.; Bensberg, K.; Wende, L.; Grandrath, R.; Plitzko, K.; Bohrmann-Linde, C.; Kirsch, S. F.; Schebb, N. H., Efficient and Simple Extraction Protocol for Triterpenic Acids from Apples. *Journal of Chemical Education* **2024**, *101* (5), 2087-2093.

Wiebel, M.; Wende, L.; Bensberg, K.; Zschau, T.; Kirsch, S. F.; Schebb, N. H., Development of a gas chromatography–mass spectrometry (GC–MS) method for the characterization and quantification of triterpenic acids. *Food Chemistry* **2025**, 146012.

Wiebel, M.; Wende, L. M.; Bensberg, K.; Zschau, T.; Kirsch, S. F.; Schebb, N. H., Genera specific occurrence of triterpenic acids in edible fruits of the *Rosaceae* family. [Manuscript submitted for publication.]

Further publications

Koch, E.; Wiebel, M.; Hopmann, C.; Kampschulte, N.; Schebb, N. H., Rapid quantification of fatty acids in plant oils and biological samples by LC-MS. *Analytical and bioanalytical chemistry* **2021**, *413* (21), 5439-5451.

Koch, E.; Wiebel, M.; Löwen, A.; Willenberg, I.; Schebb, N. H., Characterization of the oxylipin pattern and other fatty acid oxidation products in freshly pressed and stored plant oils. *Journal of Agricultural and Food Chemistry* **2022**, *70* (40), 12935-12945.

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Seidel, U.; Eberhardt, K.; Wiebel, M.; Luersen, K.; Ipharraguerre, I. R.; Haegele, F. A.; Winterhalter, P.; Bosy-Westphal, A.; Schebb, N. H.; Rimbach, G., Stearidonic acid improves eicosapentaenoic acid status: studies in humans and cultured hepatocytes. *Frontiers in Nutrition* **2024**, *11*, 1359958.

Rohwer, N.; Sander, A.; Ocyirk, S.; Wiebel, M.; Kühl, A.; Schebb, N. H., Grune, T.; Weylandt, K.-H., Ketone ester supplementation protects from experimental colitis via improved goblet cell differentiation and function. *European Journal of Nutrition* **2025**. [Accepted for publication.]

14.2 NON-PEER REVIEWED SCIENTIFIC ARTICLE

Grandrath, R.; Wiebel, M.; Bensberg, K.; Schebb, N. H.; Kirsch, S. F.; Bohrmann-Linde, C., Aus der Schale in die Schule. *Nachrichten aus der Chemie* **2025**, *73* (3), 10-12.

14.3 CONFERENCE CONTRIBUTIONS

Wiebel, M.; Koch, E.; Kampschulte, N.; Schebb, N. H., Combined sample preparation and LC-MS-based analysis of oxylipins and their precursor fatty acids in biological samples. Poster presented at *8th European Workshop on Lipid Mediators*; **2022** Jun 29 - Jul 1; Stockholm, Sweden.

Wiebel, M.; Bensberg, K.; Kirsch, S. F.; Schebb, N. H., Development of a gas chromatography-mass spectrometry (GC-MS) method for the analysis of triterpenic acids. Poster presented at *50th Deutscher Lebensmittelchemikertag*; **2022** Sep 19-21; Hamburg, Germany.

Wiebel, M.; Bensberg, K.; Kirsch, S. F.; Schebb, N. H., Analysis of triterpenic acids in apple pomace using gas chromatography-mass spectrometry (GC-MS). Poster presented at *Regionalverbandstagung NRW Lebensmittelchemische Gesellschaft*; **2023** Mar 15; Duisburg, Germany.

Wiebel, M.; Bensberg, K.; Grandrath, R.; Bohrmann-Linde, C.; Kirsch, S. F.; Schebb, N. H., Extraction and gas chromatographic analysis of triterpenic acids in apples. Poster presented at *51st Deutsche Lebensmittelchemietage*; **2023** Aug 21-23; Bonn, Germany.

Wiebel, M., Entwicklung einer GC-MS-Methode zur Analyse von Triterpensäuren in Pflanzen. Talk presented at *34th Dotorandenseminar of AK Separation Science GDCh*; **2024** Jan 07-09; Hohenroda, Germany.

Wiebel, M.; Bensberg, K.; Kirsch, S. F.; Schebb, N. H., Analyse von Triterpensäuren in Rosaceae-Früchten. Talk presented at *Regionalverbandstagung NRW Lebensmittelchemische Gesellschaft*; **2024** Mar 13; Münster, Germany.

Wiebel, M.; Schebb, N. H., Gas chromatographic analysis of triterpenic acids in Rosaceae fruits. Poster presented at *52nd Deutsche Lebensmittelchemietage*; **2024** Sep 16-18; Freising, Germany.