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Molecular Characterization and Quantification of Saponins from Sugar Beet (*Beta vulgaris* ssp. *vulgaris* var. *altissima*)

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„Gebildet ist,

wer Parallelen sieht,

wo andere

völlig Neues erblicken.“

Anton Graff
1736 – 1813
schweizerischer Maler

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The extracts produced during the course of this work (see Section 4.1) were characterized in close cooperation with the Department of Food Physics and Meat Science (Professor Jochen Weiss, Dr. Hanna Salminen, and Dr. Theo Ralla) at the University of Hohenheim.

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List of Publications

Peer-reviewed publications

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Ralla, T., Salminen, H., Braun, K., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Investigations into the Structure-Function Relationship of the Naturally-Derived Surfactant Glycyrrhizin: Emulsion Stability. *Food Biophysics* 2020, *15* (3), 288-296.

Ralla, T., Salminen, H., Braun, K., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Investigations into the structure-function relationship of plant-based surfactant glycyrrhizin: Interfacial behavior & emulsion formation. *LWT - Food Science and Technology* 2020, *120*, 108910.

Ralla, T., Salminen, H., Wolfangel, Timo, **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Value Addition of Red Beet (*Beta vulgaris* L.) By-Products: Emulsion formation and stability. *International Journal of Food Science and Technology* 2018*, 54* (3), 619-625.

Ralla, T., Salminen, H., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Oat bran extract (*Avena sativa* L.) from food by-product streams as new natural emulsifier. *Food Hydrocolloids* 2018, *81*, 253-262.

Ralla, T., Herz, E., Salminen, H., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Emulsifying Properties of Natural Extracts from *Panax ginseng* L. *Food Biophysics* 2017*, 12* (4), 479-490.

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Oral presentations (given by Theo Ralla)

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Ralla, T., Salminen, H., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Influence of the Structure on the Function of Naturally-Derived Saponins Used as New Surfactants. 3rd Food Structure and Functionality Forum Symposium 2018, Montreal, Canada.

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Ralla, T., Salminen, H., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Plant Saponin Extracts from Food By-Products as Natural Emulsifier. 2nd BioEconomy Congress 2017, Hohenheim, Germany.

Poster presentations

Ralla, T., Salminen, H., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Interfacial and Emulsifying Properties of Oat Bran Saponin Extract. 7th International Symposium on Delivery of Functionality in Complex Food Systems 2017, Auckland, New Zealand.

Ralla, T., Salminen, H., **Edelmann, M.**, Dawid, C., Hofmann, T., Weiss, J., Using Extracts from By-Products of Plant Stuff Processing as Natural Emulsifier: Saponins. Reverse Engineering of Processed Foods 2017, Campinas, Brazil.

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Abstract

Consumer demand for sustainably and ecologically produced food has grown in recent years. However, for numerous reasons, this demand has not always been met. New solutions are still being sought, particularly in the area of emulsifiers, an integral ingredient of many food products. The biggest challenge is the limited scope of application offered by current alternatives. While some progress has been made using foam active quillaja saponins, they are neither pleasant-tasting nor sustainably produced. Only very little is known about other alternatives, and especially the behavior of saponins, particularly on a molecular level, is not very well understood. Moreover, it is often not known which parts of a plant contain the highest levels of saponins and are therefore most suitable for extraction.

To expand the current level of knowledge about emulsifying and foam active extracts, saponin extracts were made from oat bran, beetroot as well as sugar beet and characterized in close cooperation with the Department of Food Physics and Meat Science at the University of Hohenheim. Measurements conducted on these extracts showed that foam activity is a good indicator of their emulsifying ability. The most promising one − sugar beet extract − was examined in more detail using taste dilution analysis, which revealed saponin fractions with a slight off-taste and high foam activity. A series of eight saponins was obtained from these fractions and further characterized, together with three commercially available ones that were identified using non-targeted screening. The unequivocal identification and structure elucidation was performed using a combination of liquid chromatography−tandem mass spectrometry (LC−MS/MS) and both one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy. For most substances, this was the first time that a complete assignment of ^{1}H and ^{13}C NMR signals had been made. All of these saponins possess taste recognition values above 1000 μmol/L, which is higher than most values for the taste-active saponins reported in the literature. The foam activity of these substances was measured using a self-developed small-scale foam activity assay. It was measured for different concentrations of saponins in a buffer solution. The foam activity of numerous other saponins was also determined, resulting in the most comprehensive overview of the foam activities of individual saponins.

In addition to these isolation and characterization activities, an LC−MS/MS method of performing simultaneous quantitative analyses of all the obtained sugar beet saponins was developed and validated. These were quantified in several sugar beet varieties (root and leaves), different sugar beet compartments, and side streams originating during the sugar beet converting process. Great variations in mass fractions were found in these materials, ranging from 862 mg/kg to 2,452 mg/kg for the various sugar beet root varieties and from 907 mg/kg to 5,398 mg/kg for the sugar beet leave varieties. Sugar beet fiber was identified as the best saponin source, with a total saponin quantity of 12.7 g/kg, followed by dried sugar beet pulp, with a total quantity of 10.3 g/kg. As a by-product stream, dried sugar beet pulp is highly suitable for the sustainable manufacture of saponins.

The results obtained are of key significance to the utilization of sugar beet saponins as well as saponins extracted from other plant materials or by-product streams for use as food additives.

Kurzfassung

Die Kundennachfrage nach natürlichen Lebensmitteln, die nachhaltig und ökologisch produziert werden, ist in den letzten Jahren gestiegen, wird jedoch aus zahlreichen Gründen nicht immer erfüllt. Insbesondere im Bereich der Emulgatoren wird nach wie vor nach neuen Lösungen gesucht, da sie ein integraler Bestandteil vieler Lebensmittel sind. Die größte Herausforderung ist der begrenzte Anwendungsbereich derzeitiger Alternativen. Mit schaumaktiven Quillaja-Saponinen wurden bereits einige Fortschritte erzielt, diese sind allerdings weder wohlschmeckend noch nachhaltig produziert. Über weitere Alternativen ist nur sehr wenig bekannt und insbesondere das Verhalten der Saponine auf molekularer Ebene ist nicht besonders gut erforscht. Zudem ist oft nicht bekannt, welche Pflanzenteile besonders viele Saponine enthalten und daher für die Gewinnung von Saponinen besonders geeignet sind.

Um das Wissen über emulgierende und schaumaktive Extrakte zu erweitern, wurden Saponinextrakte aus Haferkleie, Roter Bete sowie Zuckerrüben hergestellt und in enger Zusammenarbeit mit dem Fachgebiet für Lebensmittelphysik und Fleischwissenschaft der Universität Hohenheim charakterisiert. Schaumaktivitätsmessungen an diesen Extrakten zeigten, dass die Schaumaktivität ein guter Indikator für die Emulgierfähigkeit dieser Extrakte ist. Der vielversprechendste Extrakt – Zuckerrübenextrakt – wurde mithilfe der Geschmacksverdünnungsanalyse genauer untersucht und wies saponinhaltige Fraktionen mit leichtem Fehlgeschmack sowie hohen Schaumaktivitäten auf. Aus diesen Fraktionen wurden insgesamt acht Saponine gewonnen und zusammen mit drei kommerziell erhältlichen Saponinen, die durch ein nicht zielgerichtetes Screening identifiziert wurden, weiter charakterisiert. Die zweifelsfreie Identifizierung und Strukturaufklärung erfolgte durch eine Kombination aus Flüssigchromatographie mit Tandem-Massenspektrometrie-Kopplung (LC-MS/MS) und eindimensionaler (1D) sowie zweidimensionaler (2D) Kernspinresonanzspektroskopie (NMR). Für die meisten Substanzen konnte erstmals eine vollständige Zuordnung der ^{1}H- und ^{13}C-NMR-Signale vorgenommen werden. Alle untersuchten Saponine besitzen Geschmackserkennungsschwellenwerte von mehr als 1000 μmol/l, welche größer als die meisten in der Literatur berichteten Werte für geschmacksaktive Saponine sind. Die Schaumaktivität dieser Substanzen wurde mit einem selbst entwickelten, miniaturisierten Schaumaktivitätstest für verschiedene Konzentrationen von Saponinen in einer Pufferlösung gemessen. Zudem wurde die Schaumaktivität zahlreicher weiterer Saponine bestimmt, wodurch der umfassendste Überblick über die Schaumaktivitäten einzelner Saponine erhalten wurde.

Neben der Isolierung und Charakterisierung wurde eine LC-MS/MS-Methode für die simultane quantitative Analyse aller gewonnenen Zuckerrübensaponine entwickelt und validiert. Diese wurden in verschiedenen Zuckerrübensorten (Wurzeln und Blättern), verschiedenen Zuckerrübenkompartimenten und Nebenproduktströmen, die während des Zuckerrübenverarbeitungsprozesses entstehen, quantifiziert. Es wurden sehr unterschiedliche Massenanteile in diesen Materialien gefunden. Sie reichen von 862 mg/kg bis 2.452 mg/kg für verschiedene Zuckerrübenwurzelsorten und von 907 mg/kg bis 5.398 mg/kg für verschiedene Zuckerrübenblättersorten. Als beste Saponinquelle wurden Zuckerrübenfasern mit einer Gesamtsaponinmenge von 12,7 g/kg identifiziert, gefolgt von Zuckerrübentrockenschnitzeln mit einer Gesamtmenge von 10,3 g/kg. Zuckerrübentrockenschnitzel eignen sich als Nebenproduktstrom besonders gut für die nachhaltige Gewinnung von Saponinen.

Die erzielten Ergebnisse sind von wesentlicher Bedeutung für die Erschließung von Zuckerrübensaponinen und Saponinen aus anderen Pflanzenmaterialien oder Nebenproduktströmen für den Einsatz als Lebensmittelzusatzstoffe.

Table of Contents

Acronyms and Symbols

Ara	Arabinose
B.C.	Before Christ
°C	Degrees Celsius
CaO	Calcium oxide
$Ca(OH)_2$	Calcium hydroxide
CCS	Collision Cross Section
^{13}C-NMR	^{13}C-Nuclear Magnetic Resonance
CO_2	Carbon dioxide
CoA	Coenzyme A
COSY	COrrelated SpectroscopY
DEPT	Distortionless Enhancement by Polarization Transfer
extract FB	Ginseng extract obtained from Finzelberg GmbH & Co. KG
extract CJ	Ginseng extract obtained from CheilJedang
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
Glc	Glucose
GlcA	Glucuronic acid
GPC	Gel Permeation Chromatography
H^+	Proton
ha	Hectare
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
^{1}H-NMR	^{1}H-Nuclear Magnetic Resonance

H_2O	Water
HPLC	High-Performance Liquid Chromatography
HSQC	Heteronuclear Single-Quantum Correlation
J	Coupling constant, [Hz]
K	Kelvin
KBBE	Knowledge-Based Bio-Economy
kg/(ha×a))	Kilogram per year and hectare
K_2HPO_4	Dipotassium phosphate
μL	Micro liter
L.	Linnaeus (Carl von Linné)
LC-HRMS	High resolution mass spectrometry coupled with liquid chromatography
LC–MS/MS	Tandem mass spectrometry coupled with liquid chromatography
LD_{50}	Median lethal dose
LiI	Lithium iodide
μm	Micrometer
M	Mole
MHz	Megahertz
min	Minutes
mL	Milliliter
mm	Millimeter
MPLC	Medium Pressure Liquid Chromatography
MRM	Multiple Reaction Monitoring
NaCl	Sodium chloride
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate

NADPH	Reduced form of $NADP^+$
nm	Nanometer
NMR	Nuclear Magnetic Resonance
O_2	Oxygen
O/W emulsions	Oil-in-Water emulsions
ppm	Parts per million
pyridine-D_5	Pyridine, deuterated
qNMR	Quantitative NMR
Rha	Rhamnose
ROESY	Rotating frame Overhauser Enhancement (Effect) SpectroscopY
SPE	Solid Phase Extraction
ssp.	Subspecies
t	Ton
TAS2R	Taste receptor 2 family
TDA	Taste Dilution Analysis
TD factor	Taste Dilution factor
Torr.	Torr. (John Torrey)
UPLC	Ultra-high-Performance Liquid Chromatography
UPLC-ToF-MS	Ultra-high-Performance Liquid Chromatography Time-of-Flight Mass Spectrometry
UV	Ultraviolet
v/v	Volume/Volume
W/O emulsions	Water-in-oil emulsions
Xyl	Xylose

1 Introduction

Consumers show an increasing expectation that foods are as natural as possible; they should be organically grown and tasty, and not cause any allergic or other unpleasant reactions. Some prefer foods that do not contain lactose or gluten, while others choose not to eat meat, and so on (Traitler et al. 2016). At the same time, consumer interest in sustainable food has also grown in recent years. (Baldwin 2011).

However, fulfilling these consumer wishes is not an easy task. Some food companies are already working towards reducing their ingredient lists to avoid E numbers (Rützler and Reiter 2017) but there is still a long way to go, especially when it comes to emulsifiers, which are an integral ingredient of many food products. There are already a few natural substitutes for emulsifiers available, such as proteins, polysaccharides and phospholipids, but all of them are limited in their scope of application (McClements and Gumus 2016). A promising alternative could be quillaja saponins (Yang et al. 2013). However, their use in large amounts is not sustainable (Oleszek and Marston 2013) and they possess a bitter off-taste (Waller and Yamasaki 2013).

Although this bitter taste is a common feature of saponins (Hoffmann 2003), there are exceptions, like glycyrrhizin, the major saponin in licorice, which is around 150 times sweeter than sucrose (Schmid 2018). A method of isolating pleasant-tasting saponins from by-product streams that arise during food production has not yet been established, but it could lead to more sustainability, along with an increased scope of application through enhanced functional performance (McClements and Gumus 2016).

1.1 Bioeconomy

The growing world population requires new ways to ensure sustainable nutrition of expected nine billion people (Godfray et al. 2010). The knowledge-based bio-economy (KBBE) offers the opportunity to make an important contribution to solving these challenges (*Nationale Politikstrategie Bioökonomie* 2014). However, a uniform description of this concept does not yet exist (McCormick and Kautto 2013). It denotes the targeted use of biological resources, such as plants, animals, residues and natural organisms, as well as enzymes, proteins and other biological molecules (*Forschung für eine biobasierte Wirtschaft* 2017) and covers all economic sectors and services in which biological resources are produced, processed or used (*Prioritäten in der Bioökonomie-Forschung* 2011).

The KBBE and the use and advancement of biological processes and resources forms a bridge between technology, economics, and ecology. This leads to more efficient use and increased sustainability. Not only does the KBBE replaces fossil resources, but it also develops entirely new processes and products. It has the potential to become a significant part of a sustainable economic system (*Nationale Politikstrategie Bioökonomie* 2014). Furthermore, it is often considered a more effective way of producing food and feedstuffs, primarily for attaining food security (Pietzsch et al. 2017).

Moreover, the KBBE can open up considerable value-creation and employment potential in food and feed production among other areas. Indeed, the potential offered by high-quality valorization of residual and waste materials is significantly high (*Nationale Politikstrategie Bioökonomie* 2014). However, in line with Frewer and Gremmen, residues and waste are not designated as such in the following, but as by-products or by-product streams, as long as it might be possible to make use of them (Waldron 2007). Germany, in particular, could benefit from the valorization of high-quality products, since it is well known for its high processing quality (*Nationale Politikstrategie Bioökonomie* 2014). Above all small and medium-sized companies can profit from this trend, considering that 75% of companies in the German food industry have fewer than 100 employees (Meyer 2010). Finally, the utilization of by-product streams is in accordance with the United Nations' Sustainable Development Goals (Pietzsch et al. 2017).

1.2 By-Product Streams

Global agriculture is responsible for 70-85% of the water footprint and 30% of greenhouse gas emissions (Smetana et al. 2015). Despite this, over 220 million tons of food-related waste are disposed of annually in Europe, of which more than ten million tons are food-processing waste (Waldron 2007). These processed raw-material residues, often referred to as “waste” rather than by-product streams, could be utilized to yield value-added products (Galanakis 2015). They are created during the main product generation process (Chandrasekaran 2012).

Food waste was not recognized as a matter of concern until the end of the twentieth century. While food production had priority, increasing efficiency was not considered so important (Galanakis 2015). This was supported by the fact that disposal was cost-efficient (Waldron 2007). The situation changed at the beginning of the 21st century (Galanakis 2015). In particular, European Union legislation made the disposal of waste more expensive (Waldron 2007). Additionally, as mentioned above, consumer interest in sustainable food started to grow (Baldwin 2011). However, since most food-processing systems were developed at least 30 to 40 (or more) years ago, there are many unused by-product streams that are still being generated today (Waldron 2007).

Dieu states five reasons why some by-product streams containing valuable components that are produced in industrial activities are still being wasted (Baldwin 2011):

1. Companies do not know how to separate these valuable components.
2. Their reuse and recovery are not economical.
3. Neither do waste exchanges exist nor is there any knowledge of how they work, and it is consequently unknown who might be interested in reusing any recovered components of value.
4. Lack of incentives and legislation discourages reuse and recycling as well as resource conservation.
5. Companies are uninterested in the volume of waste they generate since the costs and fines incurred for waste discharge/disposal are low.

Another problem is that utilizing by-product streams requires the formulation of new products for current or new markets (Waldron 2007). Other problems include the lack of communication of research findings to industry, the absence of adequate technology, and up-scaling challenges (Waldron 2009). For some time, it has become an increasingly attractive idea to make use of by-product streams. In addition to increased legislative pressure and a more conscious consumer demand (Waldron 2007), by-products constitute an increasingly substantial negative cost to the food industry (Waldron 2009).

Two examples of by-product streams – whey from the dairy industry and starch from potato processing – demonstrate how they can be utilized despite the high research and development costs. Their implementation, including the development of new technologies and markets, took between 10 to 20 years. The subject of by-product streams is set to gain increasing importance in the future, as can be seen by the following examples (Waldron 2007). Sensorial aspects are among the most limiting factors (Waldron 2009), which is not surprising, since taste is an essential element of consumer acceptance. Finally, it would be important to know whether consumers acknowledge the utilization of by-product streams as a way of increasing sustainability, or whether they are concerned due to the increase in profitability (Waldron 2007).

Other by-product streams also exist in addition to the ones mentioned above. Extensive literature about plant-based by-product streams has been published by Anal, Chandrasekaran, Oreopoulou et al., Schieber and Schieber et al. (Schieber et al. 2001;

Oreopoulou and Russ 2006; Chandrasekaran 2012; Anal 2017; Schieber 2017). More and more methods besides landfill, composting and reuse as feed are now being devised for using such by-product streams, such as the production of biofuels, (fine) chemicals and energy (Chandrasekaran 2012). However, their use as food for human nutrition should have the highest priority (Galanakis 2015). By-product streams are able to provide not only carbohydrates, proteins and fat but also antioxidants, carotenoids, sterols, etc., which can be used as ingredients in conventional food products or as functional ingredients for nutraceuticals (Herrero et al. 2006; Anal 2017). Phenols and carotenoids, for example, could be applied as natural food preservatives, since they extend the shelf life of the product (Oreopoulou and Russ 2006; Poltronieri and D'Urso 2016).

An example of different by-product streams arising from a single plant (sugar beet) is given in Section 1.4. Finally, it should be mentioned that the enormous potential of side streams originating from by-product streams has not been exploited to date (Schieber 2017). A possible new way of using them on an industrial scale could be to replace artificial food additives such as emulsifiers with substances generated from by-product streams, as supported by consumer wishes.

1.3 (Natural) Emulsifiers

Emulsions mostly consist of two immiscible liquids forming a disperse system. Liquid droplets (the disperse phase) are surrounded by a liquid medium (the continuous phase). Emulsions are typically either oil-in-water (O/W) or water-in-oil (W/O) types (Tadros 2009; Köhler and Schuchmann 2012). Examples of emulsion-based foods are milk, dressings, sauces and mayonnaise (Köhler and Schuchmann 2012), all of which contain emulsifiers (Hasenhuettl and Hartel 2008). Furthermore, emulsifiers provide desirable physicochemical and sensory attributes to foods, affecting their appearance, texture, mouthfeel, and flavor (McClements 2015a; McClements et al. 2017). Another application is the encapsulation, protection and delivery of bioactive compounds like nutraceuticals, antimicrobials, antioxidants as well as colors, flavors, nutrients, and vitamins (Velikov and Pelan 2008; McClements 2010; Sagalowicz and Leser 2010; Fathi et al. 2012; Ozturk and McClements 2016).

The size of emulsion droplets typically ranges between 0.1 and 100 μm. Emulsions are not thermodynamically stable, which means they tend to separate back into their oil and water phases over time. However, their stability can be extended by adding an emulsifier (Köhler and Schuchmann 2012; McClements et al. 2017). These do not only promote the emulsion’s stability, but also facilitate its formation (Ozturk and McClements 2016). Many of the most effective emulsifiers currently used in food products are not of natural origin but synthetically produced, like sorbitan or sucrose esters (Stauffer 1999; Hasenhuettl and Hartel 2008; McClements et al. 2017). Although they are harmless, consumers are increasingly interested in all-natural products and therefore prefer naturally-derived food additives such as natural emulsifiers (Matissek and Baltes 2015; Ozturk and McClements 2016). This trend (cf. Section 1.2) can lead to an increase in the market value of by-product streams from which natural emulsifiers might be obtained.

Emulsifiers always have the same fundamental mode of action, regardless of their origin (synthetic or natural). Homogenization facilitates the formation of fine droplets and enhances the stability of the lipid droplets. One of the most common mechanical devices used to create emulsions with nano-sized droplets on an industrial scale is the high-pressure homogenizer. First, a coarse emulsion is formed by combining the aqueous phase, in which the emulsifier is (mostly) dissolved, with the oil phase, using a high-shear mixer. This low-energy input results in relatively large droplets (typically, $d > 1$ μm) that are coated by emulsifier, while any additional emulsifier molecules remain within the aqueous phase (McClements and Gumus 2016). Second, the coarse emulsion is pumped at high pressure through a small nozzle in the homogenizer that facilitates further droplet disruption due to such disruptive forces as cavitation, turbulence, and shearing, eventually leading to the formation of nano-sized emulsion droplets (Dumay et al. 2013; McClements and Gumus 2016).

Two parameters are particularly important in the emulsification process: (i) sufficient emulsifier molecules are required at any one time to fully cover the droplet surfaces and decrease the interfacial tension. Moreover, (ii) adsorption to the interface should be fast enough during the homogenization process to form a protective coating (Köhler and Schuchmann 2012; McClements and Gumus 2016). Otherwise, small droplets will be formed which coalesce immediately (Köhler and Schuchmann 2012; Lee et al. 2013; McClements and Gumus 2016; Martínez-Monteagudo et al. 2017).

1.3.1 Breakdown Processes in Emulsions

Several other breakdown processes besides coalescence may occur during the storage of emulsions (Tadros 2009; Köhler and Schuchmann 2012); these are illustrated in Figure 1.

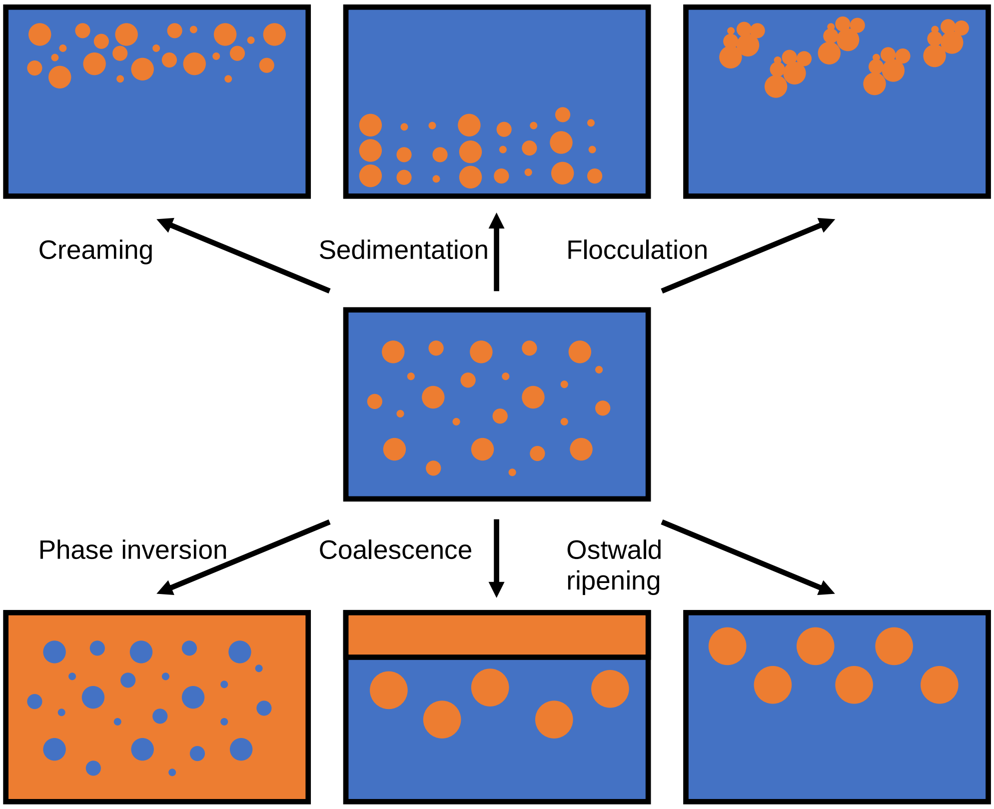


Figure 1: *Breakdown processes in emulsions. Based on Tadros (Tadros 2009).*

Creaming and sedimentation are two degradation mechanisms that are based on the same physical effect, the density difference. This difference between the dispersed and continuous phases leads to movement of the dispersed phase either towards the top (if the density is less than that of the medium) or the bottom (if the density is greater than that of the medium). Destabilization arises when this effect is undercompensated by Brownian motion. However, similar densities or small droplets delay this process (Tadros 2009; Köhler and Schuchmann 2012).

Flocculation is another degradation mechanism that occurs through aggregation of droplets (without any change in primary droplet size), forming larger units. It is caused by van der Waals attractions when there is insufficient repulsion to keep the droplets apart from each other (Tadros 2009).

Ostwald ripening refers to the growing of larger droplets at the expense of smaller ones and is caused by diffusive mass transport through the continuous phase, typically occurring during longer storage times. The mean droplet size distribution shifts to larger values over time (Tadros 2009; Köhler and Schuchmann 2012).

Coalescence occurs when two droplets are in close proximity to each other, forming a liquid film between the droplets. This destabilization mechanism is a reversible process which has three possible outcomes: The first is that droplets are separated if the repulsive forces are stronger than the attractive forces. Second, if there is a balance between repulsive and attractive forces, flocculation could occur. Third, thinning and disruption can take place if the attractive forces predominate. This leads to the fusion of two or more droplets, possibly causing complete separation of the emulsion into the distinct liquid phases (Tadros 2009; Köhler and Schuchmann 2012).

Phase inversion describes a change in the continuous phase, for example, an oil-in-water emulsion changing to a water-in-oil emulsion. This mechanism is possible under the influence of energy input, temperature changes or changes in composition (Tadros 2009; Köhler and Schuchmann 2012).

As mentioned above, these processes are not fast, which is why external stress tests are required for the development of new emulsions (Köhler and Schuchmann 2012). These include thermal processing, pH adjustment, the addition of salt, and long-term storage (Yang et al. 2013). Furthermore, the investigation of freeze-thaw cycles can deliver important information that can be used in the development of frozen food products with enhanced properties (Thanasukarn et al. 2004).

A broad knowledge of emulsifier performance is especially important for the development of new ‘natural’ food products with naturally-derived emulsifiers. As stated above, this is of particular interest to the food industry, since one of its main trends is the replacement of synthetic or animal-based emulsifiers with naturally-derived, plant-based alternatives (McClements and Gumus 2016; McClements et al. 2017). An overview of established, naturally-derived emulsifiers is given in the following (cf. Section 1.3.2). Nevertheless, further research is needed to identify and characterize viable natural emulsifiers for food products (McClements et al. 2017).

1.3.2 Natural Emulsifiers

The most common natural emulsifiers are proteins, polysaccharides, phospholipids and perhaps soon saponins (Ozturk and McClements 2016). Furthermore, food-grade colloidal particles that stabilize food emulsions by way of a Pickering mechanism are also sometimes used (Dickinson 2012; Lam et al. 2014; Rayner et al. 2014; Berton-Carabin and Schroën 2015; McClements and Gumus 2016). Other, less frequently used emulsifiers are protein-saccharide conjugates (Hettiarachchy et al. 2012). All these emulsifiers display huge differences in terms of their performance and molecular structure (McClements and Gumus 2016; Ozturk and McClements 2016; McClements et al. 2017). The most common ones are described in the following (Section 1.3.2.1 to 1.3.2.4).

1.3.2.1 Proteins

Proteins display considerable differences in their emulsifying properties. A protein that is a good emulsifier contains balanced amounts of polar and non-polar amino acids. The number of polar and non-polar amino acids on the surfaces of proteins determines their emulsifying properties. Too many nonpolar ones lead to poor water solubility, while decreased surface activity will result if only very few are present (McClements and Gumus 2016; Ozturk and McClements 2016; McClements et al. 2017).

Proteins’ electrical properties also have a major influence on their functional characteristics in emulsions. In particular, electrostatic repulsion plays a key role in preventing protein-coated oil droplets from aggregating (Dickinson 2010; Lam and Nickerson 2013; McClements 2015b; McClements and Gumus 2016). Electrostatic repulsion arises through positive (NH_3^+) and negative charges (COO^-), but it is absent at the isoelectric point. Emulsions stabilized with proteins tend to flocculate at pH values close to the isoelectric point and are sensitive to high salt concentrations due to the screening of electrostatic forces (McClements 2004; Dickinson 2010; Ozturk and McClements 2016; McClements et al. 2017).

Common proteins used as emulsifiers are isolated or extracted from either bovine milk, as are whey proteins and caseins (Dalgleish 2006; McClements and Gumus 2016) or plants such as soy and peas (Lam and Nickerson 2013). Despite the advantages of animal-based proteins, demand for plant-based proteins is rising in the industry, as they tend to be cheaper and more widely available (Can Karaca et al. 2015; McClements and Gumus 2016).

1.3.2.2 Polysaccharides

The main stabilizing action for most food polysaccharides is quite different to that of proteins, since most of them are composed of hydrophilic molecules that are not particularly surface-active. Their emulsifying properties are usually based on viscosity modification or gelation of the aqueous continuous phase, which inhibits droplet movement (Dickinson 2003; Ozturk and McClements 2016). Chemical or enzymatical modifications are possible and can lead to increased surface activity if non-polar groups or proteins are attached to the hydrophilic backbone of the polysaccharides. However, the resulting emulsifier is no longer considered natural (Ozturk and McClements 2016).

Some naturally-derived polysaccharides have non-polar groups or proteins attached to their hydrophilic carbohydrate chains, which leads to their remarkable emulsifying properties (Dickinson 2003; Ozturk and McClements 2016). The most widely used emulsifier of this type in the food industry is gum arabic, which is a complex mixture of glycoproteins and polysaccharides, especially arabinose and ribose. Another example is pectin, which is a polymer of mainly D-galacturonic acid and galactomannans (Ozturk and McClements 2016). However, a disadvantage of gum arabic is the relatively high emulsifier-to-oil ratio (≈1:1) required to form stable emulsions (Charoen et al. 2011; Qian et al. 2011; Ozturk and McClements 2016).

1.3.2.3 Phospholipids

Phospholipids are natural amphiphilic molecules found as major components of biological membranes in animals, plants, and microorganisms (Akoh 2017; McClements et al. 2017). They consist of a glycerol backbone that is covalently bound to two fatty acids and one phosphoric acid moiety (McClements and Gumus 2016; Akoh 2017). Common sources are soy (~92%), sunflower (~5%), rapeseed (~2%), and, to a small extent, egg (~1%). In commercial contexts, the mixture of phospholipids derived from different sources is always called ‘lecithin’. However, care must been taken, since in scientific literature, ‘lecithin’ is often regarded as referring to a phospholipid that contains choline (Norn 2015).

Many lecithins are too lipophilic, which results in poor emulsifying properties when used alone (McClements et al. 2017). Additionally, they are susceptible to salt addition, since they are primarily stabilized by electrostatic repulsion (Washington 1996; Komaiko et al. 2016; McClements et al. 2017).

1.3.2.4 Saponins

Saponins are sterol or triterpene glycosides that are surface active (Oakenfull 1981). They can be isolated from various natural sources and plants which typically contain a series of closely related saponins that may differ only slightly in structure (Osbourn et al. 2011; Oleszek and Marston 2013; Ozturk and McClements 2016). More details regarding saponins are provided in Section 1.6. Two of the most prominent sources of saponins are the Chilean tree *Quillaja saponaria* Molina L. and the Californian tree *Yucca schidigera* L. (Wojciechowski 2013). Quillaja bark saponin extract is an approved ingredient in the European Union for water-based non-alcoholic drinks and ciders, and in the US for beverages (Wojciechowski 2013; de Faria et al. 2017). Yucca saponin extract is classified as a food additive in the US (Güçlü-Üstündağ and Mazza 2007). However, very little is known about the emulsifying properties of yucca (Ralla et al. 2018b).

Quillaja saponin extract, in particular, is an effective, naturally-derived surfactant and well suited to use as an emulsifier, with or without co-surfactants depending on the application (Yang et al. 2013; Yang and McClements 2013; Ozturk et al. 2014; Salminen et al. 2014; Uluata et al. 2015; Zhang et al. 2015; Ozturk and McClements 2016; Zhang et al. 2016a;

McClements et al. 2017; Reichert et al. 2018). Emulsions stabilized with quillaja saponin extract are stable not only to salt addition (0 to 300 mM NaCl, pH 7.0) but also to thermal processing for 30 min up to 90 °C. Additionally, emulsions have been observed to be physically stable over a wide pH range (3−8), although droplet flocculation has been observed under highly acidic (pH 2) conditions or high ionic strength (Yang et al. 2013; Uluata et al. 2015). Consequently, quillaja saponin extract may be able to replace synthetic surfactants in selected emulsion-based food and beverage products (Yang et al. 2013).

Unfortunately, the use of large quantities of quillaja saponins is not sustainable (Oleszek and Marston 2013), and extracts typically possess an unpleasant bitter off-taste (Waller and Yamasaki 2013). Therefore, further sources need to be investigated as a potential source of natural emulsifiers, especially the by-product streams of plants that accumulate in large amounts during the processing of plant-derived products. It would also be desirable to find a source which contains saponins with no off-taste, since sensorial aspects limit their applicability (Waldron 2009). One possible by-product stream bearing a high number of saponins is sugar beet pulp, and this is described in the following (Section 1.4).

1.4 Sugar Beet

Sugar beet is one of the ten most widely grown crop plants worldwide, with a yield of 278 million tons in 2019 (FAOSTAT 2020) and is known to contain non-toxic saponins (Yoshikawa et al. 1996b). As a result of its large production quantities, considerable amounts of by-product streams are generated. Nowadays, it is mainly cultivated for the production of sucrose (Asadi 2006).

Origin of Sugar Beet

All cultivated beet species derive from the sea beet (Biancardi 2005), which grows along the Mediterranean and North Atlantic coasts from the British Isles to the Canary Islands (Stevanato et al. 2001) and is classified as *Beta Vulgaris* L. ssp. *Maritima* (L.) Arcang (Lange et al. 1999; Biancardi 2005). It is assumed that the domestication of the sea beet started about 12,000 years ago around the Persian Gulf. Biennial individuals were selected by the earliest growers, since they allowed a longer time for leaf production, which was the only part used as food. The oldest written references to beet usage date back to Aristophanes (445−385 B.C.) and Euripides (480−406 B.C.). The plant subsequently spread from Greece to modern-day Italy, where the ancestors of the modern-day garden beet, with a sweet taste and low fiber content, first appeared (Biancardi 2005). The plant continued to spread throughout Europe, favored by the expansion of the Roman Empire, with plenty of biotypes occurring over time (Cooke and Scott 1993; Biancardi 2005).

At the turn of the seventeenth century, the French agronomist Olivier de Serres described that several types of beet produced a juice similar to sugar syrup after cooking. This is often regarded as the beginning of sugar beet history (Biancardi 2005; von Lippmann 2013). The next major development was in the year 1747, when Andreas Siegmund Marggraf discovered that locally-grown beets contained a crystalline substance similar to cane sugar (Kappert et al. 1958; Biancardi 2005). Franz Carl Achard, a scholar of Marggraf, bred the “White Silesian“, a progenitor of all sugar beets, and built the first sugar-beet factory in 1802 (Cooke and Scott 1993; Biancardi 2005). Further systematic selection led to the breeding of sugar beet that contained much larger amounts of sucrose (van der Poel and Schwartz 2000). More details regarding the origin of sugar beet as well as the development of sugar beet processing can be found elsewhere (van der Poel and Schwartz 2000; Biancardi 2005).

Cultivation of Sugar Beet

The main growing areas of sugar beet are in the temperate climate zones of the northern hemisphere, with the main concentration in Europe (Hoffmann et al. 2002). The five biggest producers in 2016 were the Russian Federation (51 million tons), France (34 million tons), the United States of America (33 million tons), Germany (25 million tons) and Turkey (19 million tons) (FAOSTAT 2020).

Sugar beet yields are determined by variety, duration of growth, soil, fertilizing program, weather conditions, plant density, and harvesting method (Hoffmann et al. 2002). Conventional agriculture produces on average 67 t/ha, whereas organic farming produces 40 t/ha (Pietzsch et al. 2017). This can be explained by the fact that sugar beet needs large amounts of nitrogen (120 to 160 kg/(ha×a)), phosphorus (35 to 70 kg/(ha×a)), magnesium (35 to 50 kg/(ha×a)) and potassium (approximately 170 kg/(ha×a)). Herbicides, insecticides and fungicides are also used extensively. Moreover, crop rotation should not exceed 25%, as frequent cultivation can increase the occurrence of diseases (Kaltschmitt et al. 2016).

Composition of Sugar Beet

The medium length of a sugar beet root is around 34 cm, including the crown, neck and root (van der Poel and Schwartz 2000; Biancardi 2005). The mass ratio between the leaves and root is around 0.8 to 1.2 (Kaltschmitt et al. 2016). A typical sugar beet is shown in Figure 2. The total length of the crown and neck gives the plant height above the soil without leaves (van der Poel and Schwartz 2000).

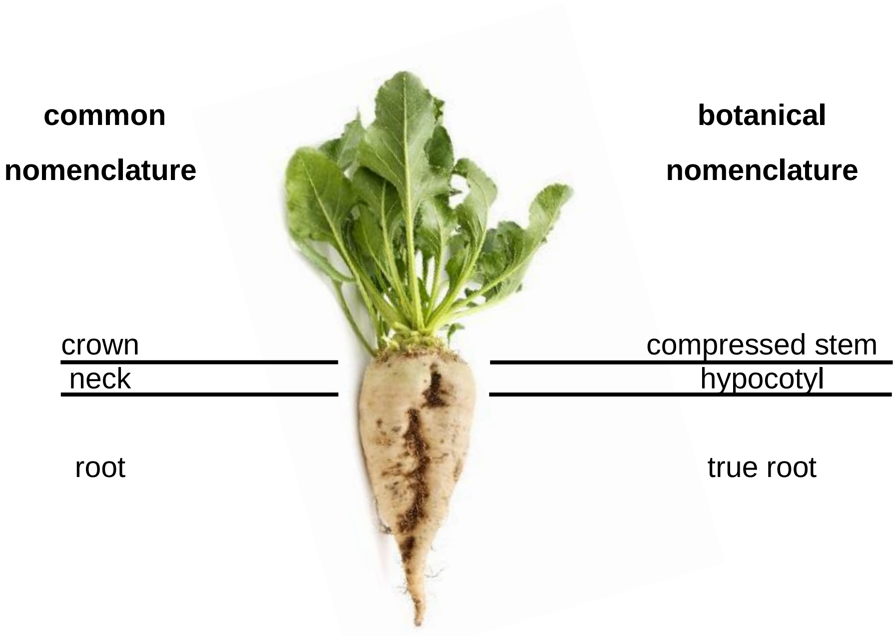


Figure 2: *Typical sugar beet with leaves and root. Labelling according to Biancardi (Biancardi 2005).*

The color of the root is creamy-white, due to the periderm, a thin cork layer that is five to eight cells deep. It protects the surface of the root and gradually the neck through its suberized cell walls and lignified middle lamella. The root comprises alternating concentric rings of vascular and parenchymal tissue internal to the periderm that surrounds a central star-shaped core, the vascular cylinder. It develops mainly during primary growth of the root and contains the vascular and ground tissue (Artschwager 1926; Biancardi 2005). The parenchymal cell vacuole stores sucrose, which typically comprises about 95% of the cell volume (Biancardi 2005). The different parts of the sugar beet are shown in Figure 3.

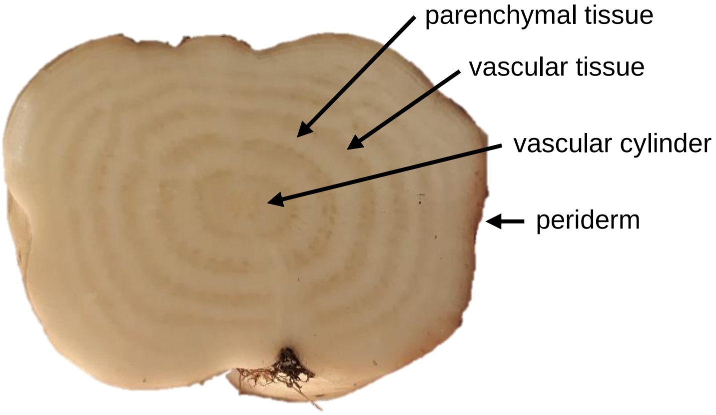


Figure 3: *Cross-section of a sugar beet with periderm and alternating concentric rings of vascular and parenchymal tissue surrounding a central vascular cylinder. Labelling according to Biancardi (Biancardi 2005).*

The average composition of sugar-beet roots is shown in Table 1. More details of the composition of the sugar beet can be found elsewhere (van der Poel and Schwartz 2000).

***Table 1:** Average composition of sugar beet roots. Selected from van der Poel et al. (van der Poel and Schwartz 2000).*

Water content	73.0 – 76.5%
Dry matter content	23.5 – 27.0%
Sucrose content	14.0 – 20.0%
Cellulose	1.1 – 1.5%
Pectin	1.0 – 2.7%
Proteins	0.5 – 1.1%
Lipids	0.1 – 0.2%
Saponins	~0.2%
Inorganics	0.4 – 0.5%

Only very little is known about sugar-beet leaves. They contain high mass fractions of protein, potassium and phosphorus (Kaltschmitt et al. 2016) as well as small amounts of saponins (Massiot et al. 1994; Yoshikawa et al. 1996b; Yoshikawa et al. 1998), in addition to other substances. While sugar beet leaves are primarily used as fertilizer or protein-rich fodder, sugar beet roots are mostly used for sucrose extraction (Kaltschmitt et al. 2016).

Sugar-Beet Processing

The first sugar-beet factory commenced operations in as early as 1802 (Biancardi 2005) with several process optimizations subsequently being implemented, culminating in today’s sugar-beet conversion process, which is shown in Figure 4 (van der Poel and Schwartz 2000). Nevertheless, process innovations are still possible today, such as the development of sugar-beet fibers like Fibrex (Hertsburg 2010).

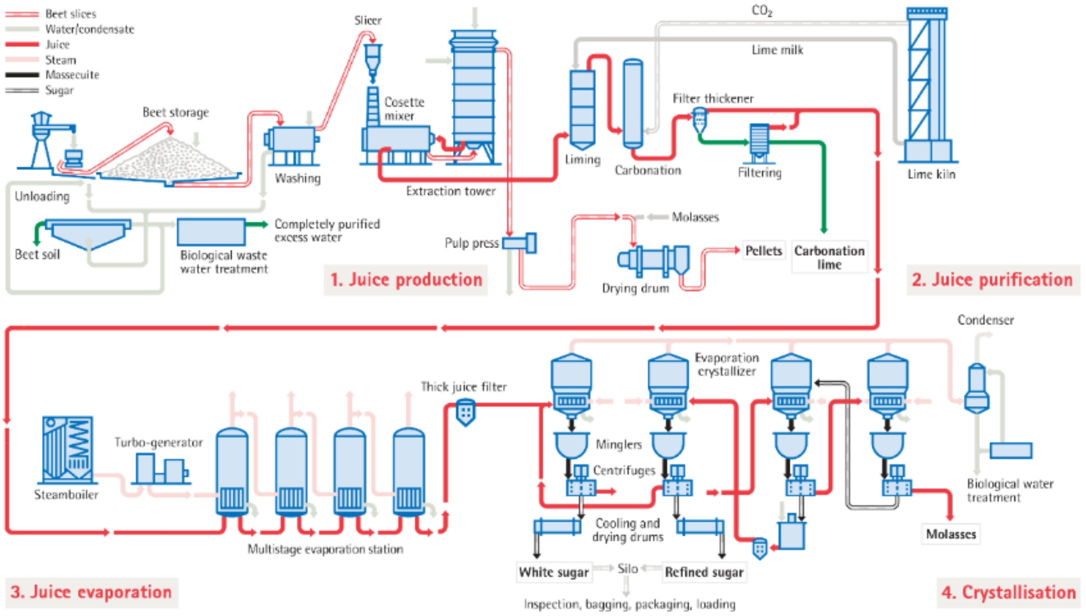


***Figure 4:** Diagram showing sugar-beet processing. Source: Kissner Group Inc. (Kissner Group Inc. 2018).*

Several preliminary steps precede the first part of the sugar-production process. The sugar-beet roots are transported to the factory without leaves and stored after unloading, which takes place without the use of water, quite in contrast to Figure 4 (van der Poel and Schwartz 2000; Asadi 2006). Storage time is strongly dependent on the climatic conditions of the beet-growing areas, and ranges from less than 20 hours in countries like Greece, over three to six weeks in Western Europe, to over 200 days in Finland, parts of Russia and China (van der Poel and Schwartz 2000). The duration of the sugar-beet processing period, or beet campaign, is three to seven months, depending on the plant availability and weather conditions. It usually starts in mid-September in Western Europe and lasts for three to four months. During this time, all factories work continuously, 24 hours a day, seven days a week, until the end of the campaign. All the time, new beets are continuously cleaned in a multi-stage process, ending in a beet washer (Asadi 2006). Washed beets are sliced into cossettes and preheated in a cossette mixer before they are extracted in the extraction tower (Südzucker AG 2018). Extraction is carried out in countercurrent at 65 to 70 °C. The resulting raw juice enters part two, the purification step. Wet pulp rises from the tower into pulp presses, which reduce the water content from 90 to 75%. It is then dried and pressed into pellets. Molasses can be added before drying to produce pressed-molasses pulp (Asadi 2006).

Juice purification (second part) begins with the liming process; this requires lime milk, which is produced by the calcination of limestone in the lime kiln and provides CaO and CO_2. Calcium oxide is dissolved in water to produce lime milk (a mixture of water and $Ca(OH)_2$), which is added to the raw juice (Asadi 2006). This process is shown in Figure 4 in a greatly simplified form and starts with liming. The gradual increase in alkalinity is important for good precipitation and flocculation of the proteins and pectin along with organic and inorganic salts of calcium. In addition, the mixture is heated to reduce invert sugars and other unstable components. CO_2 is then introduced to remove the added calcium hydroxide. The insoluble calcium carbonate which is formed in this reaction serves both as a filtration aid and as an adsorbent. The resulting mod is separated by a filter-thickener and additional filters (van der Poel and Schwartz 2000). The resulting carbonation lime is sold as soil fertilizer (Asadi 2006). Several substances are removed by the juice purification process. These include colloids like pectin, hemicellulose, proteins, and colorants, anions like phosphate, malate, oxalate and others, invert sugars, amides (formed through alkaline degradation) and last but not least some juice ingredients by adsorption (van der Poel and Schwartz 2000).

The third part of the sugar production process is where juice evaporation takes place. This is the part of the process that requires the most energy. The energy is transferred by means of steam, which is generated in a steam boiler, and transferred to the evaporation stations, which are operated sequentially. Thin juice with a dry-substance content of 15% is concentrated into thick juice with a dry-substance content of around 60%. In order to save energy, the boiling point is lowered by reducing the pressure. The thick juice is stored in large reservoirs prior to the final crystallization step. To ensure its stability, it is filtered to remove any microbes not yet killed by heat (Asadi 2006).

The final stage of the process is crystallization. Figure 4 shows one of a few possible arrangements (von Lippmann 2013). Basically, market-quality sugar is produced during the first stage, which starts with thick juice. During crystallization, water is removed by evaporation under vacuum, which produces massecuite, a semisolid mixture consisting of sugar crystals and the surrounding solution. The sugar crystals are separated in centrifuges and then dried. Industrial sugar beet processing involves a multiple crystallization process to increase the yield. At the end of the process, the ratio of sucrose to non-sucrose reaches a point where effective crystallization is no longer possible. The resulting syrup is called molasses and must be separated to prevent non-sugar accumulation (Asadi 2006).

By-Product Streams from Sugar Beet

As sugar production is a complex process, the following section focuses on the arising by-product streams. Familiar by-product streams from sugar beet processing are molasses (38 kg per ton of sugar-beet root), beet leaves and stems, carbonation sludge (60 kg) and beet pulp (50 kg) (Russ and Meyer-Pittroff 2004; Waldron 2009). Lesser-known commercial by-products are sugar-beet pectin (Williams et al. 2005) and dietary fiber (Hertsburg 2010). In addition, sugar-beet roots and leaves can be used for biogas production. However, this is not yet widespread (Kaltschmitt et al. 2016). More common is the production of alcohol by fermentation using molasses and yielding ethanol, which can be used as a basis for other chemicals. Sugar beet components can also be used as cattle feed (Anal 2017).

Other by-products also have potential for utilization, and some suggestions for this have already been made. Two examples are ferulic acid and oxalic acid, which can be isolated from sugar-beet pulp (Anal 2017). Molasses has been used for some time as a carbon source in fermentation processes. Not only ethanol can be produced, but also other chemicals such as citric acid, L-lysine, L-glutamic acid and β-carotene (van der Poel and Schwartz 2000; Schieber et al. 2001; Roukas et al. 2003; Waldron 2009). Another biochemical approach to the utilization of by-products is the enzymatic saccharification of sugar-beet pulp (Micard et al. 1996; Moloney et al. 2004). Sugar-beet pulp was also considered as a biodegradable thermoplastic material (Rouilly et al. 2006b, 2006a; Galanakis 2015). The production of carboxymethyl cellulose has also been shown to be possible (Toğrul and Arslan 2003). Xanthan gum production can be improved by supplementing with sugar-beet pulp (Yoo and Harcum 1999; Rouilly et al. 2006b), while the same material is able to bind divalent metal cations (Dronnet et al. 1997; Reddad et al. 2002; Rouilly et al. 2006b). Finally, it can be used as a reactant to produce urethanes and polyurethanes (Pavier and Gandini 2000a, 2000b; Rouilly et al. 2006b).

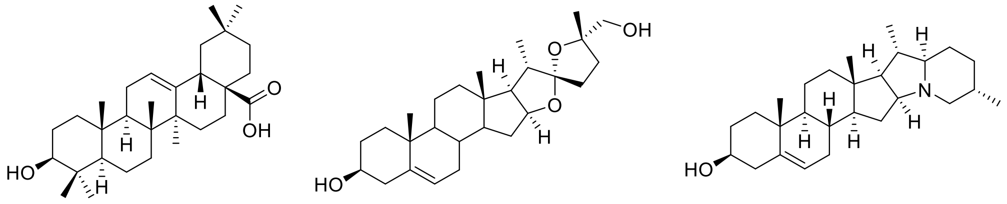
Another interesting component of sugar beet which, however, has until now not been considered suitable either as a food ingredient or for any other application are sugar-beet saponins.

1.5 Other Important Plant Materials

Besides the by-product streams from sugar beet, as well as sugar beet itself, two other plant materials are considered in this work. These are beetroot and oat bran. Oat bran consists primarily of germ and marginal layers, which represent a significant proportion of the grain (Miedaner 2014). With an annual worldwide production of more than 23 million tons in 2019, oats are significantly more important than beetroot, from which only peels are produced as a by-product stream during food manufacturing and whose annual production quantity is unknown (FAOSTAT 2020). Like sugar beet, oats and beetroot both contain saponins (Günther-Jordanland et al. 2016; Mikołajczyk-Bator et al. 2016a). However, their mass fraction is unknown.

1.6 Saponins

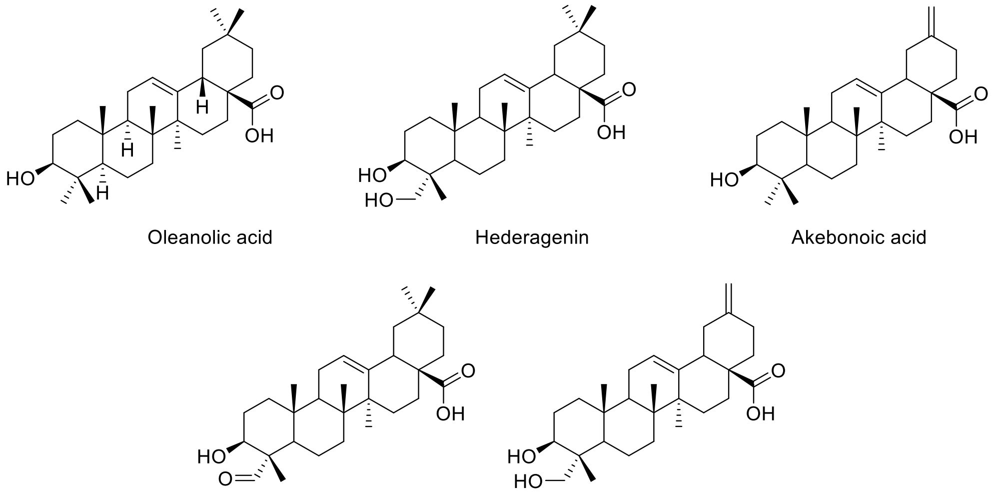
Saponins are a large and structurally diverse group of secondary metabolites that are widely found in flora, although not exclusively, since they also occur in lower marine animals (Lásztity et al. 1998; Oleszek 2002; Hostettmann and Marston 2005; Oleszek and Bialy 2006; Vincken et al. 2007). Nowadays they are defined on the basis of their molecular structure as triterpene, steroid or steroid alkaloid glycosides (Hostettmann and Marston 2005). An example of each aglycone type (the non-saccharide portion) is shown in Figure 5.



Oleanolic acid Nuatigenin Solanidine

Figure 5: *Three different saponin aglycones from three different substance classes: Oleanolic acid (triterpene class), nuatigenin (steroid class) and solanidine (steroid alkaloid class). Structures according to Fu et al., Pecio et al., and Zhang et al. (Fu et al. 2005; Pecio et al. 2012; Zhang et al. 2016b).*

A huge variety of aglycones exists, especially because certain methyl groups are frequently oxidized to hydroxymethyl, aldehyde or carboxyl functionalities (Hostettmann and Marston 2005). Aglycones which are isolated or proposed within sugar beet are shown in Figure 6 (Massiot et al. 1994; Yoshikawa et al. 1996b; Yoshikawa et al. 1997b; Yoshikawa et al. 1998; Mikołajczyk-Bator et al. 2016b).



Oleanolic acid Hederagenin Akebonoic acid

Gypsogenin Norhederagenin

Figure 6: *Aglycones which are isolated or proposed in sugar beet. Structures according to Fu et al. and Mikołajczyk-Bator et al. (Fu et al. 2005; Mikołajczyk-Bator et al. 2016b).*

Gypsogenin and Norhederagenin were proposed after mass spectrometry experiments (Mikołajczyk-Bator et al. 2016b). Oleanolic acid, hederagenin and akebonoic acid are constituents of isolated saponins (Massiot et al. 1994; Yoshikawa et al. 1996b; Yoshikawa et al. 1997b; Yoshikawa et al. 1998).

In saponins, the aglycone is always attached to one, two or (rarely) three sugar chains. Saponins with one sugar chain are designated as monodesmosidic, while those with two sugar chains are designated as bidesmosidic (Hostettmann and Marston 2005). The difference is illustrated in Figure 7.

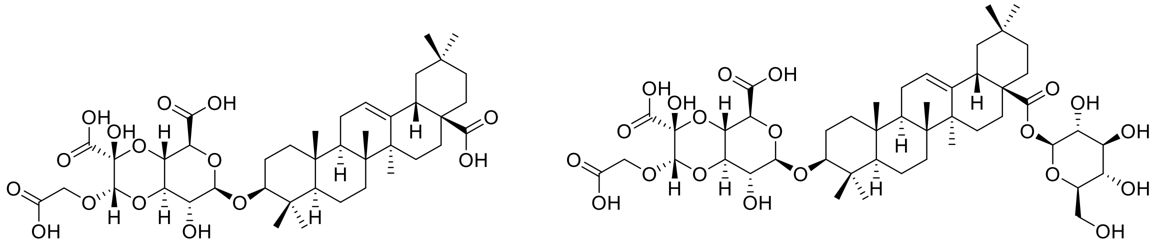


Figure 7: *Betavulgaroside II (left) as an example of monodesmosidic saponins and betavulgaroside I (right) as an example for bidesmosidic saponins. Structures according to Yoshikawa et al. (Yoshikawa et al. 1996b).*

Typical saccharide chains are linear or branched, whereas the two sugar-beet saponins shown in Figure 7 possess a unique acid substituent (Yoshikawa et al. 1996b; Hostettmann and Marston 2005). Although the largest number of monosaccharides found in a saponin is eleven, most isolated saponins contain sugar chains with between two and five monosaccharides. The most common monosaccharides found in plant saponins are D-glucose (Glc), D-galactose (Gal), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), L-rhamnose (Rha), L-arabinose (Ara), D-xylose (Xyl) and D-fucose (Fuc). In rare cases, acylated sugar units are also found (Hostettmann and Marston 2005).

It is known that saponin contents in plants depend on cultivars (Kapusta et al. 2005; Mroczek et al. 2012), plant age and environmental conditions, such as ambient temperature, and access to water and light (Jochum et al. 2007; Martínez et al. 2009). Additionally, contents can vary quite considerably between different parts of the plants (Hostettmann and Marston 2005).

Biosynthesis of saponins

The biosynthesis of any saponin skeleton starts with the synthesis of oxidosqualene (Vincken et al. 2007), which is formed by a complex biochemical process. The process begins with the formation of isopentenyl pyrophosphate from acetyl-CoA and acetoacetyl-CoA. Further details of this can be found elsewhere (Berg et al. 2017). Squalene is subsequently formed from six isopentenyl pyrophosphate molecules through the following reaction sequence:

$$C_5 \rightarrow C_{10} \rightarrow C_{15} \rightarrow C_{30}$$

The step from C_{15} to C_{30} can be regarded as a dimerization of the C_{15} molecule. Again, further details can be found elsewhere (Berg et al. 2017). The final step in the formation of the precursor (oxidosqualene) is the oxidation of squalene, as shown in Figure 8 (Berg et al. 2017).

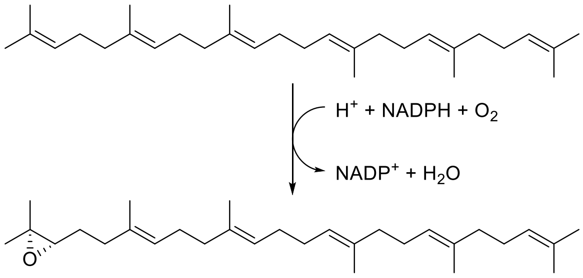


Figure 8: *Oxidation of squalene to oxidosqualene according to Berg at al. (Berg et al. 2017).*

It is quite easy to identify the isoprene subunits within squalene. Further reactions of oxidosqualene are manifold, and Figure 9 only shows the pathway to the formation of oleananes (Vincken et al. 2007).

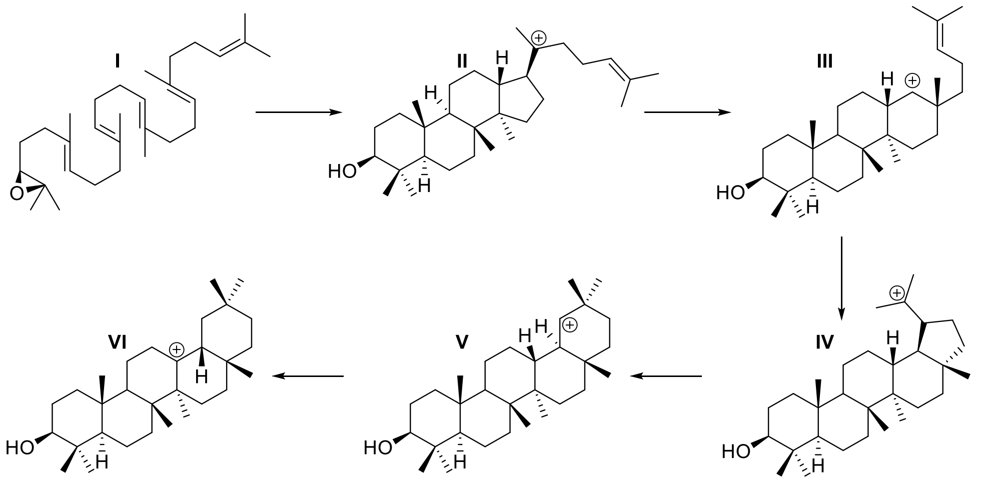


Figure 9: *The formation pathway of oleananes according to Vincken et al. (Vincken et al. 2007).*

The cyclization of oxidosqualene (I) starts with protonation and epoxide-ring opening, and proceeds through the ‘chair–chair–chair’ conformation. The cation formed (II) can undergo numerous reactions. In this case, a shift of the C16–C17 bond takes place (III), followed by a cyclization that forms a new five-membered ring (IV). After further rearrangement, another carbocation is formed (V) that undergoes a series of hydride shifts to oleanyl carbocation (VI), which is finally neutralized by proton elimination and the formation of a double bond. Additionally, some modifications are possible that mostly comprise oxidation reactions, leading to the following functional groups: –OH, =O, –CH_2OH, –CHO, and –COOH (Vincken et al. 2007). Finally, the aglycones are glycosylated through reactions which are currently not well understood (Dutta et al. 2003; Vincken et al. 2007). Ultimately, saponins like those shown in Figure 7 are created.

Toxicity and Occurrence of Saponins in Food

There are at least 32 food plants that contain saponins. These include soybeans, spinach, licorice, asparagus, and oats (Oakenfull 1981; Fenwick and Oakenfull 1983; Ruales and

Nair 1993; Cheeke 2000; Mroczek et al. 2012). It has been shown for soy beans that saponins survive the rigors of cooking and food processing, and only fermentation lowers the saponin concentration significantly (Fenwick and Oakenfull 1983). The daily saponin intake for various consumer groups in the United Kingdom was published in 1988. The lowest determined intake was 10 mg for Caucasian males, while an average family member consumed 15 mg. This value was significantly higher, at 110 mg, for vegetarians, while the highest value was determined for vegetarian Asians, who consumed 214 mg per person per day (Ridout et al. 1988; Hostettmann and Marston 2005). It is evident that the increased intake of the average family member coincided with a significant increase in sales of soy-based foods, which are the main contributors (Ridout et al. 1988; Granato et al. 2010).

The oral toxicity of saponins to warm-blooded animals is relatively low, and the toxicity of glycoalkaloids, in particular, has largely been overestimated (George 1965; Hostettmann and Marston 2005). The lowest oral LD_{50} values are around 50 mg/kg, and they can reach levels of more than 5000 mg/kg, depending on the saponin (George 1965; Oakenfull 1981; Witthawaskul et al. 2003). Lethal intravenous doses are usually significantly lower, with a factor of between 3 and 1000 (George 1965). The reason for this great difference is their feeble absorption in the intestinal tract (Hostettmann and Marston 2005). Long-term toxicity studies conducted on mice and rats did not demonstrate any long-term toxicity (Phillips et al. 1979; Drake et al. 1982). However, it is known that a high consumption of licorice can lead to health problems, particularly hypokalemia and hypertension. A study was therefore carried out in humans by van Gelderen et al. to determine the no-effect level, which was found to be 0.2 mg of glycyrrhizic acid per kg body weight (van Gelderen et al. 2000). Whether these results are transferable to other saponins is not known. It could, however, be very important regarding the authorization of further saponins such as sugar-beet saponins as food additives (Waldron 2009).

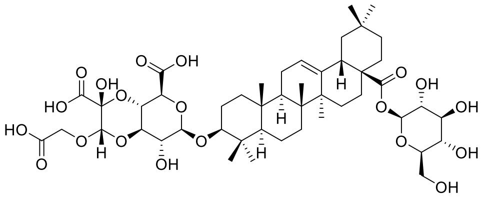
Sugar-Beet Saponins

The first sugar-beet saponin was probably isolated by Smolenski in 1911 (Smolenski 1911; Eis et al. 1952) but it took a long time for the exact structure to be fully elucidated by Ridout et al. in 1994 (Ridout et al. 1994). In the meantime, methods have been developed to quantify the total saponin content at different sugar-beet processing stages (Walker 1956; West and Gaddie 1956; Bauserman and Hanzas 1957; van der Poel and Schwartz 2000), which was an important step towards solving the problem of floc in refined beet sugar (Hanzas and Barr 1968). All measurement data depend on the validity of the test; it is known, for instance, that the antimony pentachloride test, the one that was used most frequently, cannot distinguish between saponin and oleanolic acid (Clarke et al. 1999) as well as several other substances (West and Gaddie 1956; Bauserman and Hanzas 1957). This traditional test must therefore either be re-evaluated (Clarke et al. 1999) or replaced by a modern one. Polarographic methods are also unsuitable as they are not specific to saponins and other surface active substances (van der Poel and Schwartz 2000). Two tests that were only mentioned by Eis et al. for the quantification of sugar-beet saponins are the hemolysis test and a direct gravimetric method (Eis et al. 1952). Another test that was applied to the quantification of sugar-beet saponins is the Tollens naphthoresorcinol test (Eis et al. 1952; Bauserman and Hanzas 1957). However, none of these tests are state-of-the-art.

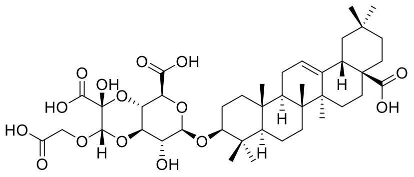
Nevertheless, a great deal of knowledge has been amassed about the occurrence of sugar-beet saponins. The most comprehensive overview was compiled by van der Poel et al. (van der Poel and Schwartz 2000). The (total) saponin mass fractions in sugar beet vary within a range of 0.01 to 0.4%, depending on the literature source (Clarke et al. 1999; van der Poel and Schwartz 2000; Brezhneva et al. 2001; Asadi 2006). They occur mainly in the periderm as well as the stems and leaves (Edwards et al. 1989; Clarke et al. 1999; van der Poel and Schwartz 2000). Particularly high amounts were recorded in damaged beets (Asadi 2006). Furthermore, sugar-rich varieties show higher saponin contents, while increased nitrogen fertilization leads to lower values (van der Poel and Schwartz 2000).

During sugar-beet extraction, between 20 and 40% of the saponins are transferred to the raw juice, and around 90% of these are precipitated during the liming process as poorly-soluble calcium salts (van der Poel and Schwartz 2000). The spent cake of the liming process is known to be a good source of saponins (Marsh and Levvy 1956; Brezhneva et al. 2001). Moreover, large quantities were found in beet-pulp water (1,200 mg/L) and molasses (Brezhneva et al. 2001). Finally, saponins are present at every stage of the sugar-beet conversion process and even find their way into granulated sugar (van der Poel and Schwartz 2000). Saponin concentration during the sugar-beet conversion process has been the subject of intensive research (Eis et al. 1952; Gaddie 1956; Walker 1956; Bauserman and Hanzas 1957; Gaddie and West 1958; Johnson 1960; Hanzas and Kohn 1961). However, concentrations of individual saponins are unknown.

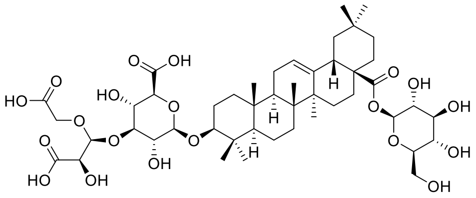
As mentioned above, the first structure elucidation of sugar-beet saponins was carried out by Ridout et al. in 1994 (Ridout et al. 1994). Further saponins were isolated and also structurally elucidated by Yoshikawa et al. and Massiot et al., as shown in Figure 10.



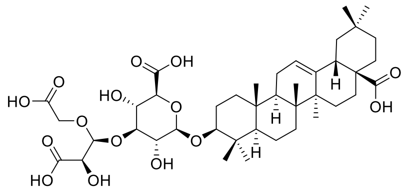
Betavulgaroside I



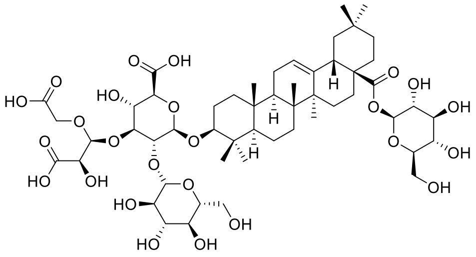
Betavulgaroside II



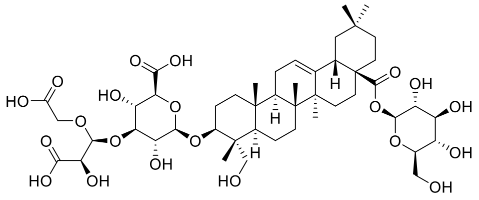
Betavulgaroside III



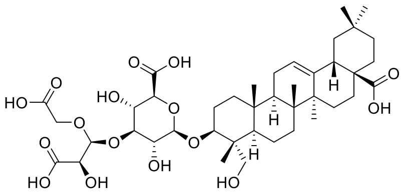
Betavulgaroside IV



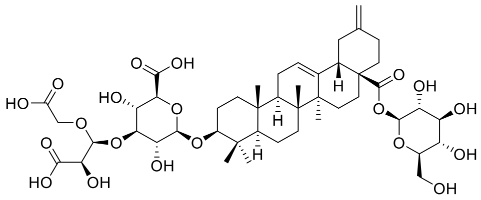
Betavulgaroside V



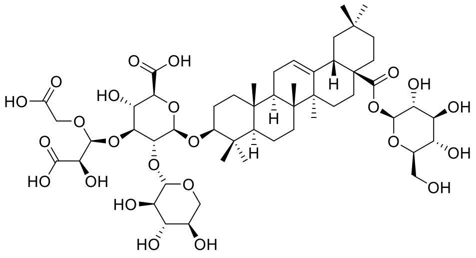
Betavulgaroside VI



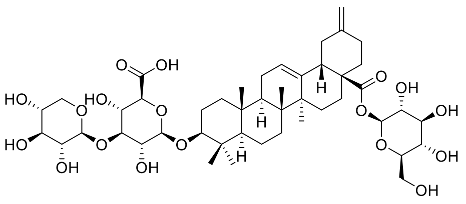
Betavulgaroside VII



Betavulgaroside VIII

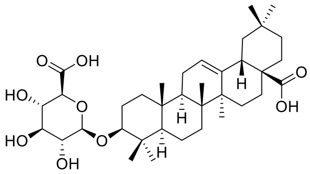


Betavulgaroside IX

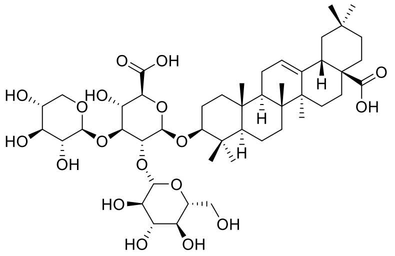


Betavulgaroside X

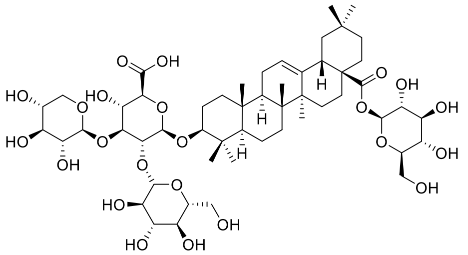
Figure 10: *Structures of sugar-beet saponins mentioned in the literature, according to Massiot et al., Ridout et al. and Yoshikawa et al. (Massiot et al. 1994; Ridout et al. 1994; Yoshikawa et al. 1996b; Yoshikawa et al. 1998).*



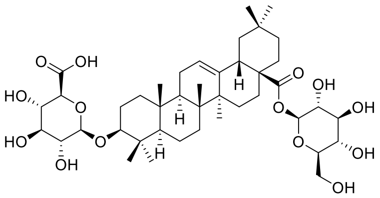
3-*O*-*β*-D-glucuronopyranosyl-3*β*-hydroxy-olean-12-en-28-oic acid



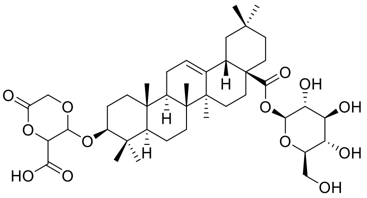
3-*O*-*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid



3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid



Chikusetsu saponin IVa



Without published name

Figure 10 (continuation): *Structures of sugar-beet saponins mentioned in the literature, according to Massiot et al., Ridout et al. and Yoshikawa et al. (Massiot et al. 1994; Ridout et al. 1994; Yoshikawa et al. 1996b; Yoshikawa et al. 1998).*

Betavulgarosides I, II, III, IV, VI, VII, VIII and chikusetsu saponin IVa were isolated from sugar-beet roots, and betavulgarosides V, IX and X from sugar-beet leaves (Yoshikawa et al. 1996b; Yoshikawa et al. 1998). 3-*O*-*β*-D-glucuronopyranosyl-3*β*- hydroxy-olean-12-en-28-oic acid, 3-*O*-*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid and 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid were isolated from sugar-beet molasses (Ridout et al. 1994). The final saponin shown in Figure 10 (which does not have a published name) was isolated from sugar-beet roots and leaves (Massiot et al. 1994). It is possible that betavulgaroside III was first isolated by Massiot et al., since the basic framework of the structure is the same as the one of betavulgaroside III, but the stereogenic centers were not elucidated by them (Massiot et al. 1994; Yoshikawa et al. 1996b). The proposed structures of betavulgaroside II, III and IV were verified by synthesis (Yoshikawa et al. 1997b; Murakami et al. 1999; Zhu et al. 2008). Further structures have recently been proposed by Mikołajczyk-Bator et al. on the basis of low- and high-resolution HPLC-MS/MS measurements (Mikołajczyk-Bator et al. 2016b).

Very little is known about sugar-beet saponins. For instance, no information has been found in the literature regarding their taste. However, it is known that they possess a potent inhibitory effect on the increase in serum glucose levels in glucose-loaded rats (Yoshikawa et al. 1995; Yoshikawa et al. 1996b; Yoshikawa et al. 1997b; Yoshikawa et al. 1998; Murakami et al. 1999). Further biological tests with a crude saponin mixture were carried out by Brezhneva et al. (Brezhneva et al. 2001). It is possible that they might show hemolytic activity, which was tested by Eis et al. with a crude saponin mixture (Eis et al. 1952). Finally, a crude saponin mixture showed abnormal effects on eelworms (Bauserman and Olson 1957). Very little is known about the foaming activity of sugar beet saponins, although this is a typical property of saponins (Hostettmann and Marston 2005; Asadi 2006).

1.7 Foam Activity of Saponins

Typically, saponins produce soap-like foams when in aqueous solution. This is caused by their surfactant properties, which are also the reason for the name saponin, which is derived from *sapo,* the Latin word for soap (Dutta et al. 2003). Foam is defined as a dispersion of gas in a second, continuous phase, frequently a liquid-like, aqueous phase (Murray and Ettelaie 2004). Foam stability is influenced by very similar factors to those in emulsions (Moro et al. 2013). The major difference is that air is the nonpolar medium and surfactants concentrate at the air-water interface (Stauffer 1999). Foam formation caused by saponins is also a problem during the sugar-beet conversion process and is the reason why antifoaming agents are required (van der Poel and Schwartz 2000; Asadi 2006). Quantifying foaming behavior is complicated, as detailed below.

Foam-Activity Measurements

Foam-activity measurements are not easy to do, since saponins are not the only natural products capable of producing foams. Furthermore, not all saponins foam in aqueous solution (Hostettmann and Marston 2005). Additionally, the quality and volume of foam produced during foam-activity measurements are a function of many complex factors (Ross and Miles 1941). They depend greatly on the pH value of the aqueous solution (Mangan 1959) and of course on the molecular structure of the saponin. Oleszek et al. mentioned that monodesmosidic saponins are particularly foam-active (Kjellin and Johansson 2010). However, no experimental work could be found to confirm this claim.

Several systems for measuring foam-active substances and mixtures are described in the literature. One of the first was created for foam-number measurements, which is a classification figure. It corresponds to the dilution factor of a solution containing saponin (10 mL) that produces a foam height of one centimeter in a test tube (inner diameter 16 mm) after shaking for 15 seconds and leaving to rest for 15 minutes (Kofler 1922). A brief review of other, previously used, systems was provided by Ross and Miles. They developed a jacketed cylinder for foam measurements with 200 mL of sample solution (Ross and Miles 1941). Others later used a high-speed disperser, vibrator or even a household milk foamer (Naofumi and Etsushiro 1988; Gohtani et al. 1996; Noudeh et al. 2011; Konak et al. 2014). It seems to be difficult to reproduce these conditions. Therefore, some researchers have tried to standardize the measurement conditions by adding pH-stabilizing reagents, such as K_2HPO_4 (Shany et al. 1970), or buffers (Noudeh et al. 2011). Others also observed foam decay and considered it an indicator of foam stability (Mangan 1959; Shany et al. 1970; Gohtani et al. 1996; Martinez et al. 2013; Konak et al. 2014; Böttcher and Drusch 2016). One current publication used a foam-scan instrument (Martinez et al. 2013), which seems to be a more reliable method than the others. However, all the aforementioned methods need large amounts of sample material. In addition, hardly any literature exists on the foam activity of individual saponins (Böttcher and Drusch 2016).

Enrichment of Foam-Active Saponins by Foam Fractionation

Foam fractionation is one of many adsorptive bubble-separation technologies (Pinfold 1970; Burghoff 2012). It was invented by Ostwald in 1918 and offers great potential for enriching dilute solutions of diverse valuable products, mainly natural products (Burghoff 2012). A simple foam-fractionation system is shown in Figure 11.

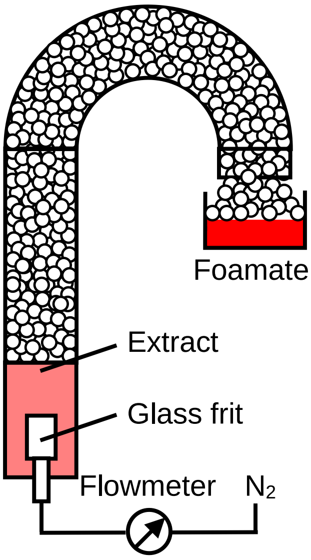


Figure 11: *Foam-fractionation apparatus. Based on Backleh-Sohrt et al. (Backleh-Sohrt et al. 2005).*

The most important prerequisite for foam fractionation is an extract that contains surface-active substances. Gas bubbles are introduced into the extract through a glass frit and regulated by a flowmeter. Simultaneously, surface-active molecules attach to the gas-liquid interface of the bubbles, which results in the formation of foam. The continuous formation of gas bubbles creates an emerging foam column above the liquid level. Ideally, most of the entrained liquid drains back into the extract, which increases the concentration of the surface-active substances. The emerging foam column is collected in a separate container. Collapsed foam that contains a higher concentration of foam-active substances than the extract is called foamate (Burghoff 2012).

Both Yan et al. and Li et al. have shown that this enrichment method is applicable to the natural product class of saponins (Yan et al. 2011; Li et al. 2013).

1.8 Bitter Taste

Besides foam activity, bitter taste is another typical characteristic of saponins (Hoffmann 2003; Hostettmann and Marston 2005). It is one of the five basic taste qualities that can be distinguished and are widely accepted. The other four are sweet, sour, umami and salty (Behrens and Meyerhof 2013). Bitterness of individual saponins measured on a quantitative basis is rarely mentioned in the literature. Thus, precise data only exist for a few saponins. Reported bitter taste thresholds are within the range of 4 to 9 μmol/kg in the case of oat saponins (Günther-Jordanland et al. 2016). The bitterness of gymnema saponins and asparagus saponins is not quite as intense, with values of 10 to 78 μmol/kg and 10.9 to 199.7 μmol/kg, respectively (Dawid and Hofmann 2012a; Pickrahn 2017). Two bitter tasting saponins obtained from licorice possess thresholds of 51.2 and 210.0 μmol/L

(Schmid 2018). More details are provided in Table 3 (see Section 4.6). Although ginseng is known for its bitter saponins, no thresholds for individual ginseng saponins were found (Court 2000).

The perception of bitter taste varies greatly between individuals, partly for hereditary reasons (Bartoshuk 1993; Drewnowski 2001; Kim and Drayna 2005). Taste-receptor cells are responsible for detecting (bitter) taste. They are aggregated into taste buds, which are distributed across the tongue and the palate epithelium (Chandrashekar et al. 2006). The taste receptor two family (gene symbol: TAS2R) is responsible for the mediation of bitter-taste perception (Behrens and Meyerhof 2013). In humans, ~25 functional TAS2Rs are known (Reichling et al. 2008; Behrens and Meyerhof 2013). Based on two bitter substances, it was found that unusually sensitive people have a very high density of receptors on the tongue (Bartoshuk et al. 1994; Drewnowski and Gomez-Carneros 2000).

Unusual bitterness is often a good indicator of dietary danger, since rancid fats, hydrolyzed proteins, plant-derived alkaloids, and toxins generally possess an unpleasant bitter taste. Bitter foods tend to be rejected by the consumer (Drewnowski and Gomez-Carneros 2000), however, a clear correlation between bitterness and toxicity has not been established (Glendinning 1994; Meyerhof et al. 2010). This might be explained by the fact that some bitter taste receptors exhibit a narrow agonist spectrum with, to date, only one to three known cognate bitter compounds activating them and, on the other hand, three receptors which together are able to recognize half of all known bitter compounds recognized by humans (Meyerhof et al. 2010; Behrens and Meyerhof 2013). Receptors of toxic compounds such as strychnine and aristolochic acid are particularly sensitive (Behrens and Meyerhof 2013). The interactions between bitter taste receptors and agonists are not yet fully understood. Corresponding agonists have not even been found for all receptors (Behrens and Ziegler 2020). Currently, agonists are known for 21 of the 25 human bitter taste receptors (Meyerhof et al. 2010; Behrens and Ziegler 2020). The search for proper agonists is challenging, especially because no experimental crystal structures are known, and current models of the tertiary structures are of low resolution. Moreover, bitter taste receptors share very limited homology with one another, which expands their selectivity range. Additionally, some receptors are known to exhibit multiple agonist binding sites, which further expands selectivity (Behrens and Ziegler 2020). The interaction between bitter tasting saponins from licorice and bitter taste receptors has, for example, been studied by Christian Schmid (Schmid 2018).

Bitterness is not always automatically rejected (Mattes 1994; Behrens and Meyerhof 2013) as is clear, for instance, from the example of beer (Intelmann et al. 2009). A certain degree of bitterness is expected in certain foods and beverages. The individual taste response to bitterness varies enormously, and so the threshold for what is or is not acceptable may vary from one person to another. Finally, it is difficult to blend concentrations such that bitterness levels are optimal for everyone (Behrens and Meyerhof 2013).

1.9 Activity-Guided Fractionation

Bitter tasting molecules in complex food matrices can be identified using activity-guided fractionation. This was established by Frank et al. for the study of non-volatile tastants (Frank et al. 2001).

Basically, activity-guided fractionation starts with the preparation of extracts or other fractionable products with the aim of obtaining appealing taste-active molecules. This is often done with common solvents such as water, methanol, ethanol, pentane or ethyl acetate. When using multiple solvents, the extraction process can already be polarity oriented, and the extracts might be collected separately. These can be examined by taste dilution analysis (TDA) or other assays. For extracts obtained with only one solvent, further separation is usually performed in advance of the TDA (Dawid 2012). More details regarding the TDA are given in Section 3.1. Separation can be performed by numerous methods, such as high pressure liquid chromatography (HPLC), gel permeation chromatography (GPC), or ultrafiltration. Based on activity and complexity, further subfractionation(s) might be necessary until the compounds of interest have been identified or isolated. Using this method, key taste-active components can be identified from a mixture of several compounds (Dawid 2012). Numerous examples are known from the literature, including asparagus (Dawid and Hofmann 2012b), black tea (Scharbert et al. 2004), and red wine (Hufnagel and Hofmann 2008).

Taste dilution analysis can not only be used to identify key tastants, but also to improve food products. For instance, the dominant bitter substances in potato fibers (Duggan et al. 2020), pea protein (Gläser et al. 2020) and canola protein (Hald et al. 2019) have all recently been identified. Manufacturing processes can thus be optimized to remove or deplete these compounds during food production.

2 Motivation and Objectives

Quillaja saponin extract was the first such extract to be used as a commercial food additive (George 1965). Although it is a promising emulsifier for food products and might be able to replace synthetical emulsifiers (Yang et al. 2013), the use of large quantities of quillaja saponins is not very sustainable (Oleszek and Marston 2013), and they impart a bitter off-taste (Waller and Yamasaki 2013).

There is only scant literature about other natural and sustainable alternatives. The only ones are yucca saponins and glycyrrhizin (Uematsu et al. 2000; Carocho et al. 2015). The latter is commonly used as a sweetener (Ahamed et al. 2001; Schmid et al. 2018). However, a large variety of different saponins exist in numerous plants (Hostettmann and Marston 2005), some of which could be suitable as ingredients for future food systems.

In the long term, it would be very useful to study a material containing saponins, which can be produced in large quantities for large-scale commercial use. Sugar beet is the best suited to this, followed by oat bran (FAOSTAT 2020). Other possible sources are beetroot, due to its similarities to sugar beet (Mroczek et al. 2012), and ginseng extracts, which are commercially available. This thesis therefore focuses on extracts taken from these natural products. One objective is the development of new extraction methods for these plant materials (with the exception of the ginseng extract and glycyrrhizin model systems) to enable their structural characterization and application potential.

The main objective of this work is to identify pleasant-tasting saponins from a plant material that is available in large amounts. As stated above, sugar beet is particularly suitable for this purpose. It is not only cultivated in large quantities, with an annual worldwide production of more than 275 million tons in 2019 (FAOSTAT 2020), but large amounts of by-product streams are created during sugar refining (Russ and Meyer-Pittroff 2004; Waldron 2009). It also offers a wide range of saponins, such as mono- and bidesmosidic, and those with a large number of acid groups (Massiot et al. 1994; Ridout et al. 1994; Yoshikawa et al. 1995; Yoshikawa et al. 1998). Most importantly, the amount of extractable saponins is comparatively high when the annual production is considered. The amount is still high even considering the small quantities of saponins (0.01 to 0.4%) contained in sugar beet material and mentioned in the literature (Clarke et al. 1999; van der Poel and Schwartz 2000; Brezhneva et al. 2001; Asadi 2006).

The wide variety of saponins in sugar beet increases the chances of finding pleasant tasting ones. Only very little is known about the taste profile of sugar beet saponins, and an indication of a bitter taste exists for only one sugar beet saponin (Mizui et al. 1990;

Woldemichael and Wink 2001). It is promising that these saponins might possess a pleasant taste, since others, like glycyrrhizin, do have a sweet taste (Mizutani et al. 1994).

Not only is there little knowledge of the taste of sugar beet saponins, but nothing is known about their foam and emulsifying ability on a molecular level, although it has been known for some time that they are foam active (Asadi 2006). Furthermore, little is known regarding saponins' foam activity on a molecular level (Böttcher and Drusch 2016). Consequently, foam activity measurements with pure saponins could provide interesting information about structure activity correlations and their emulsifying ability.

Finally, another objective of this work is to quantify those saponins in different plant materials that could support efficient large-scale extractions. Literature on the distribution of sugar beet saponins in sugar beet is scant (Yoshikawa et al. 1995; Yoshikawa et al. 1998). Knowledge about saponin distribution in other plants is also very limited (Shimoyamada et al. 1990; Hayashi et al. 1993; Huhman et al. 2005; Mostafa et al. 2013; Dawid and Hofmann 2014). In the end, sugar beet was found to be particularly well suited to studying saponin distribution, due to its large size and high production quantity.

3 Experimental Methods and Concepts

Despite the great importance of sugar beet in the food industry, very little is known about sugar beet saponins. There are a few older publications on the subject of sugar beet saponins but only one recent one, which focuses on the identification of saponins using low- and high-resolution mass spectrometry (Mikołajczyk-Bator et al. 2016b).

Numerous experimental methods were therefore conducted to close what was a wide knowledge gap. Extracts of oat bran and beetroot, and in particular sugar beet were produced and characterized in cooperation with the Department of Food Physics and Meat Science at the University of Hohenheim. To gain a deeper knowledge of sugar beet saponins on a molecular basis, they were isolated using activity-guided fractionation and then further characterized. Taste recognition thresholds were determined and foam activity curves obtained. The isolated saponins were also used for the purpose of quantification in different parts and varieties of sugar beet, as well as in products formed in the course of the sugar beet converting process. Several methods were used in these experiments, and these are described in the following.

3.1 Activity-Guided Fractionation

One of the most important methods employed for this work was activity-guided fractionation. As described in Section 1.9, it is useful for the isolation of taste-active molecules from complex matrices. It has been applied in prior research to identify taste-active molecules in asparagus, coffee, black pepper, oats and other foodstuffs (Somoza et al. 2003; Dawid et al. 2012; Dawid and Hofmann 2012a, 2012b; Günther-Jordanland et al. 2016). It is also possible to search for other activity characteristics (Spreng and Hofmann 2018). The basic principle behind the method was the separation of taste-active plant extracts or reaction mixtures into several fractions, followed by taste dilution analysis (TDA) or other assays. In most cases, separation was carried out using chromatographic methods, followed by the removal of the organic solvent and freeze drying. Subsequently, the fractions were dissolved in Evian water in accordance with their “natural” concentration and diluted one-to-one with Evian water. They were presented in order of increasing concentration to experienced sensory panelists, who were asked to evaluate the taste quality and to determine the detection threshold in a duo (or trio) test. Each concentration step was thus tested against pure Evian water. The taste dilution (TD) factor was defined as the dilution step at which a difference in taste could just be detected between the diluted fraction and the blank (Frank et al. 2001). For this work, the experiments were carried out in an air-conditioned room at 22 to 25 °C. Nose clips were employed by the panelists to prevent cross-model interactions with odorants (Dawid and Hofmann 2012b). Amber glass was used in combination with red light to exclude optical differentiation of the samples.

Quite in contrast to previous studies, activity-guided fractionation was employed inversely (i.e. it was used to screen for tasteless fractions or fractions with a low off-taste) to ensure that the isolated saponin fractions did not possess a strong bitter off-taste or any other unfavorable taste attributes. Finally, the human recognition threshold concentrations were measured with pure saponins.

3.2 Saponin Isolation Strategy

The isolation of pure saponins is a challenging procedure. Consequently, different methods for their extraction were used and improved. First, crushed sugar beet roots were extracted three times with a mixture of methanol and water, followed by a triplicate extraction with methanol in order to extract all the conceivable saponins and to gain knowledge about their abundance. This procedure was modified to a single extraction with methanol and water, which is much faster and produces only slightly lower amounts of saponins. The greatest optimization was achieved by changing the sugar beet material from root to pulp. This is an ideal source of sugar beet saponins (due to their high content) and was the main one used for isolation. All extractions were carried out at room temperature, since it is known that sugar beet saponins were derivatized into methyl esters during extraction with methanol under reflux (Yoshikawa et al. 1995).

3.2.1 Foam Fractionation

Both extracts obtained from sugar beet root material contained a relatively low proportion of saponins compared to sugar beet pulp extract, whereas they contained high amounts of sugar and other substances (van der Poel and Schwartz 2000). Foam fractionation is a useful tool for enriching foam active substances like saponins from complex matrixes (Backleh 2001). It was described above in Section 1.7 and is highly suited for extracts with relatively low amounts of saponins and large amounts of other substances with no foam activity.

As sugar beet extract has moderate foam activity, a small-diameter (10 mm) foam fractionation tube was chosen. Separation of larger quantities was enabled with a special device developed for a higher throughput. This device contained six foam fractionation tubes, as illustrated in Figure 12.

***Figure 12:** Self-made foam fractionation apparatus comprising six columns.*

It was planned together with and manufactured by Glasbläserei Bahr in Manching, Germany. It operates with nitrogen or compressed air with a pressure of approximately 1.0 bar and is regulated by a rotameter. The gas stream was introduced by a fine-pored frit (glass frit porosity 4 (P 16)). The resulting foam was directed to the foam tubes (with an inner diameter of 10 mm) through an inwardly curved top. The typical enrichment obtained by foam fractionation was roughly a factor of five, which could be increased by a second fractionation step using a traditional foam fractionation apparatus (cf. Figure 11), in particular, one with a longer foam tube.

3.2.2 Chromatographic Methods

Liquid chromatography plays a key role in this work. It was used not only to isolate the different saponins but also to quantify them. The basic principle remains the same, whether using column chromatography (in the form of solid phase extraction), preparative or semi-preparative HPLC as well as an analytical HPLC or U(H)PLC. There is always a mobile phase that flows through a column with solid particles (stationary phase) and carries different solutes through it. The time after which the solutes leave the stationary phase is called the retention time. It depends on the solute-solute, solute-mobile phase, solute-stationary phase, and stationary phase-mobile phase interactions, and is normally different for different molecules (Lough and Wainer 1995).

Solid phase extraction (SPE), a simple type of liquid chromatography, is especially suited to the crude separation of large amounts of extract. It was used for the separation of sugar beet root extract after foam fractionation and for the sugar beet pulp extract. Both extracts contained large amounts of polar substances, even though the sugar beet pulp was extracted with warm water during the production process (van der Poel and Schwartz 2000). A crude separation of individual sugar beet saponins was carried out with preparative HPLC, which displayed much better separation than with SPE. Additionally, a specific column material (Nucleodur C_{18} Pyramid) was used for separation. It consists of a silica phase with octadecyl modification and polar endcapping (Macherey-Nagel 2019) and resulted in significantly better separation than classic column materials, even if an isocratic gradient needed to be applied for sufficient separation. A semi-preparative column with the same column material was needed for the isolation of the crude substances as well as for their purification. Structure elucidation of the isolated sugar beet saponins was primarily carried out with NMR.

3.3 Structure Elucidation

This work also investigated the molecular structures of some saponins. These were determined by instrumental analysis. High-resolution mass spectrometry, as described in Section 3.6.2, is an important element of structure elucidation, providing both molecular formula candidates and fragment candidates (Kind and Fiehn 2010). Many different NMR experiments were performed to elucidate the molecular structure of the isolated saponins. These were ^{1}H, ^{13}C, COSY, HSQC, HMBC, DEPT-135, *J*-RESOLVED, and ROESY (Hesse et al. 2012; Friebolin 2013). Furthermore, the corresponding sugars of the saponins were identified using a recently developed LC-MS/MS method (Schmid et al. 2018), as described in Section 3.3.9.

NMR spectra for all compounds apart from betavulgaroside V were measured with a 500 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany) equipped with a cryo-TCI probe (300 K). Betavulgaroside V was analyzed with an 800 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany) equipped with a cryo-TCI probe (298 K). All saponins were dissolved with pyridine-D_5 and chemical shifts were referenced to the signals of pyridine.

3.3.1 ^{1}H-NMR

^{1}H-NMR spectra are the simplest types of NMR spectra. They consist of two dimensions: the abscissa corresponds to the frequency axis, which shows the chemical shifts of the proton signals from the measured substances, and the ordinate represents signal intensities. Signal intensities, chemical shifts and spin-spin coupling constants obtained from ^{1}H-NMR are very useful for structure elucidation (Friebolin 2013). Chemical shifts are given in ppm.

3.3.2 ^{13}C-NMR

Signal intensities cannot be obtained for ^{13}C-NMR spectra for several reasons, in particular the different relaxation times and the nuclear Overhauser effect. ^{13}C-NMR spectra therefore provide less information than ^{1}H-NMR spectra. They mainly provide chemical shifts. A long measurement time is needed due to the low abundance (1.1%) and low detection sensitivity, compared to ^{1}H-NMR spectra. Furthermore, broadband decoupling is necessary to suppress couplings between ^{1}H and ^{13}C nuclei. Chemical shifts are given in ppm and rounded to 0.1 ppm (Friebolin 2013).

3.3.3 COSY

One of the simple two-dimensional NMR-experiments is COrrelated SpectroscopY (COSY). Like the other two-dimensional experiments, it consists of two frequency axes. The intensities correspond to the third dimension. The projection of the spectrum onto one of the two axes corresponds to ^{1}H-NMR. COSY signals were divided into two groups: diagonal and cross signals. Cross-correlation signals were caused by scalar couplings between adjacent hydrogen atoms. This is very useful for identifying ^{1}H-resonances, as it shows the neighborhood relationships of all coupling nuclei (Friebolin 2013).

3.3.4 HSQC

Heteronuclear Single-Quantum Correlation (HSQC) is similar to Heteronuclear Multiple-Quantum Correlation (HMQC), a method that shows ^{1}J couplings between ^{1}H and ^{13}C, with the additional advantage that it offers a higher resolution than HMQC. With this method, it is possible to identify all hydrogen atoms bound to the corresponding carbon atom. Consequently, it is not possible to observe quaternary carbon atoms with this method. Another big advantage is that it enables the measurement of phase-sensitive spectra, in which cross peaks for CH_2 groups possess another color than CH and CH_3 groups within the spectra (Bienz et al. 2016).

3.3.5 HMBC

Heteronuclear Multiple-Bond Correlation (HMBC) is similar to HSQC. It shows cross-correlation signals between ^{1}H and ^{13}C atoms that are connected through two (^{2}J), three (^{3}J) and (rarely) four (^{4}J) bonds but not for direct neighbors (^{1}J). ^{1}J-couplings are normally suppressed and can be observed within HSQC or HMQC. A big advantage is that it enables the observation of couplings between protons and quaternary carbon atoms (Bienz et al. 2016). Frequently, protons show cross-correlation signals with numerous, adjacent carbon atoms and vice versa (Friebolin 2013).

3.3.6 DEPT-135

Distortionless Enhancement by Polarization Transfer (DEPT) uses polarization transfer to enhance absorptions and emissions of insensitive nuclei like ^{13}C. Different modifications of this experiment provide valuable spectra with clearly distinct characteristics. The so called DEPT-45 experiment results in spectra that show CH, CH_2 and CH_3 groups, whereas DEPT-90 only shows CH groups and DEPT-135 shows CH_2 and CH_3 groups. Quaternary carbon atoms cannot be measured with any DEPT experiment. By combining individual experiments, it is possible to show only CH, CH_2 or CH_3 groups in a spectrum (Bienz et al. 2016). DEPT-135 was sufficient for this work.

3.3.7 *J*-Resolved

J-Resolved spectra have one dimension more than ^{1}H-NMR spectra. The projection of the spectrum on the x-axis (chemical shift) corresponds to the ^{1}H-NMR (however, not for all versions of the experiment). The second axis shows the corresponding *J*-couplings. This means that it is possible to separate the effects of chemical shifts and *J*-couplings into two independent dimensions (Ludwig and Viant 2010). This technique is very useful, especially for structural elucidation of large molecules like saponins (Bienz et al. 2016).

3.3.8 ROESY

The physical fundamentals of Rotating frame Overhauser Enhancement (Effect) SpectroscopY (ROESY) are different to the previously mentioned NMR measurements. The experiments described so far are based on magnetization transfer between scalar coupled nuclei. Based on this principle, only interactions between nuclei that are connected by chemical bonds can be observed. This is quite in contrast to ROESY, which is based on dipole-dipole interactions (Friebolin 2013). It is possible to measure interactions between hydrogen nuclei that are not more than 0.5 nm away from each other (Wangsakan et al. 2004).

3.3.9 Identification of Corresponding Sugars with LC-MS/MS

Distinguishing between D- and L-enantiomers of sugars is not easy. Often, polarimetry, electrophoresis or HPLC with a chiral stationary phase are used to determine them (Noe and Freissmuth 1995; Ono et al. 2006; Tanaka et al. 2007). However, all of these techniques have disadvantages and were therefore not used in this work. Fortunately, Tanaka et al. have developed a derivatization method that results in derivatized molecules that can be measured with basic HPLC instruments. Only pyridine (anhydrous), L-cysteine methyl ester and an isothiocyanate are required for the reaction (Tanaka et al. 2007). This method was further adapted by Schmid et al. for the derivatization of sugars from saponins.

First, saponins were hydrolyzed with aqueous HCl and the resulting monosaccharides were separated by extraction with water and ethyl acetate. Derivatization was then carried out similar to Tanaka et al. and the derivatized monosaccharides were measured with LC-MS/MS (Schmid et al. 2018).

3.4 Foam Activity

Another method of characterizing isolated saponins is to measure foam activity. This is of particular interest, because a connection between foam activity and emulsifying ability has been reported (Moro et al. 2013). Due to the low occurrence of some saponins, a special measuring apparatus was developed. Like the foam fractionation apparatus (cf. Section 3.2.1), it was manufactured by Glasbläserei Bahr in Manching, Germany. An example is shown in Figure 13.

Figure 13: *Foam tube filled with a foam active saponin solution.*

The inner diameter is 10 mm, and nitrogen is introduced into it through a fine-pored frit (glass frit porosity 4 (P 16)). Flow is regulated by a rotameter and set to an overpressure of 1.0 bar. This is an advanced version of the one published by Ross et al. (Ross and Miles 1941). This device enabled the measurement of saponins' foam activity of using very small quantities (Edelmann et al. 2020a).

3.5 qNMR

As reference solutions with a known concentration of saponins were needed for quantification, quantitative NMR (qNMR) experiments were carried out as described by Frank et al. Their big advantage is that the response of the NMR signals is directly proportional to the number of nuclei generating a corresponding resonance signal in the spectrum. This means that response factors known from other techniques are not necessary (Frank et al. 2014).

The measurements were performed by dissolving isolated substances with deuterated solvents. Subsequently, 600 μL were given into NMR tubes. The instrument was calibrated in advance and the sample then measured. Evaluation was based on known and characteristic proton signals.

3.6 Mass Spectrometry

Mass spectrometry was an important part of this work, and two types were used, both coupled with UHPLC-systems. These are high-resolution mass spectrometry (LC-HRMS) and tandem mass spectrometry (LC-MS/MS).

3.6.1 LC-HRMS

High-resolution mass spectrometry coupled with liquid chromatography (LC-HRMS) is a useful tool for targeted and non-targeted screening within different matrices. This means that matrices were examined for known (targeted) and unknown (non-targeted) substances. This is possible with LC-HRMS, as its high resolution means it is able to derive the accurate molecular mass, resulting in values with up to four decimals (e.g. 43.9898 g/mol for CO_2). Based on the molecular mass, it is possible to compare these with known compounds or to determine molecular formula candidates for unknown compounds. The isotope pattern is often very helpful for identification purposes (Schymanski et al. 2015). Both methods (targeted and non-targeted) were applied in this work.

3.6.2 LC-MS/MS

To supplement LC-HRMS, tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) is mainly used for (targeted) quantifications. Compared to classic HPLC, it offers the big advantage that a complete chromatographical separation of target analytes is not necessary for selective detection. However, there should be sufficient separation, as matrix effects sometimes suppress or, less frequently, enhance analyte signals. This problem can be reduced or avoided by applying (isotopically labeled) internal standards (Gros et al. 2006). The special construction of the LC-MS/MS system ensures high selectivity and high sensitivity. This is achieved by using three quadrupole units. The first quadrupole analyzer separates the original precursor ion(s). The second one serves as a collision cell to fragment the ions sent into it. The third and final one separates the fragments produced by the second quadrupole before they are detected. The mode of operation described is one of four possible ones, and is known as the multiple reaction monitoring (MRM) mode. It is most suitable for the quantification of known molecules, and was therefore used in this work (McMaster 2005).

4 Results and Discussion

There are only two saponin-based emulsifiers that are currently in use in the food industry. These are yucca and quillaja saponin extracts (cf. Section 1.3.2.4), the use of which is meanwhile established in a few food applications (Güçlü-Üstündağ and Mazza 2007; Wojciechowski 2013; de Faria et al. 2017). Glycyrrhizin, the most abundant saponin in *Glycyrrhiza glabra*, is regarded as an interesting alternative, due to its relatively low cost compared to other saponins and the absence of any accompanying substances (Ralla et al. 2020a, 2020b). Despite its interesting physico-chemical characteristics, it is restricted to a small selection of applications, such as beverages and sweets, due to its intensely sweet taste and high price (Schmid 2018; Ralla et al. 2020a, 2020b). Ginseng extracts derived from *Panax ginseng* have also been used as a model system (Ralla et al. 2017a). However, they are not suitable for commercialization because of the known bitter-tasting saponins that ginseng contains (Court 2000). Further model system alternatives were produced in the course of this work, including extraction development and the determination of approximate saponin contents, which was carried out for all extracts and glycyrrhizin. Extracts of sugar beet, oat bran, and beetroot were produced, and their physico-chemical and techno-functional characterization, which was carried out by the Department of Food Physics and Meat Science at the University of Hohenheim, is described in four peer-reviewed publications and also briefly in Section 4.1 herein (Ralla et al. 2017b, 2017c, 2018a; Ralla et al. 2019). The results for ginseng extracts and glycyrrhizin, which were described in three publications, are also outlined in Section 4.1 (Ralla et al. 2017a; Ralla et al. 2020a, 2020b).

A more in-depth analysis of the saponins found in the most promising extract, identified as sugar beet extract (cf. Section 2) for utilization as a food additive, is described in Sections 4.2 to 4.12 and represents a major part of this work. The most important results are presented in the publications by Edelmann et al. (Edelmann et al. 2020a; Edelmann et al. 2020b). These are taken up and discussed in the following along with further findings.

4.1 Techno-Functionality of Plant-Based Saponins

The sugar beet, beetroot and oat bran extracts were self-made, while the ginseng extracts and glycyrrhizin were obtained commercially (Ralla et al. 2017a; Ralla et al. 2017b, 2017c, 2018a; Ralla et al. 2019; Ralla et al. 2020a, 2020b). The ginseng extracts were used as supplied, whereas the glycyrrhizin ammoniacal was treated twice with formic acid followed by freeze-drying to reduce its ammonium content (Ralla et al. 2017a; Ralla et al. 2020a, 2020b). The production of the sugar beet, beetroot, and oat bran extracts is described in the following subsection (Section 4.1.1). The semi-quantification of their saponins is then described (Section 4.1.2) and the results of the investigations by the Department of Food Physics and Meat Science are presented afterwards (Section 4.1.3).

4.1.1 Preparation of Plant-Based Saponin Extracts

For the investigation of the techno-functional properties, three plant-based saponin extracts (sugar beet, beetroot, and oat bran) were produced. The sugar beet and beetroot samples had to be prepared prior to extraction, while the oat bran could be extracted directly (Ralla et al. 2017b, 2017c, 2018a; Ralla et al. 2019). Both materials were washed, chopped, frozen in liquid nitrogen, and ground in a blender prior to extraction (Ralla et al. 2017b, 2017c; Ralla et al. 2019). Rapid processing was required, as the sample material underwent visible degradation if not stored frozen. Sugar beet extract was subsequently obtained by triplicate extraction using methanol, followed by triplicate extraction using a methanol/water mixture (Ralla et al. 2017b, 2017c). The extraction sequence was reversed for beetroot to first extract polar substances from the starting material, which might allow better extraction of the saponins (Ralla et al. 2019). In contrast to the other two extracts, the oat bran extract was first defatted with distilled *n*-pentane and then extracted three times using methanol, followed by triplicate extraction with a methanol/water mixture (Ralla et al. 2018a). All technical details for these three extracts are presented in the respective publications (Ralla et al. 2017b, 2017c, 2018a; Ralla et al. 2019). It was found during the sugar beet saponin isolation that a single extraction with methanol/water should be most economical for the extraction of saponins (cf. Section 4.2). However, oat defatting could not be avoided because of the interfering properties of the lipids.

4.1.2 Semi-Quantification of Saponins

To support the characterization of all extracts, their approximate saponin content was determined. This was done by separating the extracts using medium-pressure liquid chromatography (MPLC) and weighing the fractions obtained that contained saponins. The fractions were identified using high-resolution mass spectrometry (LC-HRMS) and comparison with saponins known from the literature. All details are provided in the corresponding publications (Ralla et al. 2017a; Ralla et al. 2017b, 2017c, 2018a; Ralla et al. 2019). It should be noted that the approach used is not a quantitative but a semi-quantitative method, because other substances may elute at the same time as the saponins and increase the weight of the individual fractions. Another reason is that not yet known saponins may be found in other fractions that were not deemed to contain saponins. This is a particular problem with less studied plants, such as beetroot. Fortunately, beetroot and sugar beet both belong to the same species which is *Beta vulagaris* (Mroczek et al. 2012). There are no other alternatives that are better suited for quantification that can easily be used, apart from the method developed here for the quantification of sugar beet saponins, which is described in Section 4.9 (Edelmann et al. 2020b). The lack of suitable quantification methods is also due to many saponins not being UV-active (Hostettmann and Marston 2005). Mass spectrometry is no alternative, since molecular masses should be known for most saponins, and molecules with the same or similar masses could be present. Furthermore, ionization is substance-specific (McMaster 2005). Nevertheless, the semi-quantitative method used was proved to be helpful for expanding the level of knowledge.

4.1.3 Techno-Functional Properties of Extracts and Glycyrrhizin

The most saponin-rich material was glycyhrrizin, with a content of 86% measured by qNMR. The largest impurity was water, with a content of 15.3% measured by Karl Fischer titration (Ralla et al. 2020a). In the course of the investigations, new insights were gained regarding the behavior of saponins as emulsifiers. Despite the low surface activity, glycyrrhizin formed stable oil-in-water emulsions at very low molecule-to-oil ratios. Surface activity was determined by measuring surface and interfacial tension, which are classic interfacial techniques used to predict the performance of emulsifiers. The results showed that predictions for glycyrrhizin are not possible based on these traditional methods and that other techniques may be better suited (Ralla et al. 2020a). In addition to interfacial and emulsifying properties, emulsion stability was also investigated. As performed for some of the aforementioned experiments, 10% oil-in-water emulsions at pH 7 were prepared using a high-shear blender and subsequent high-pressure homogenization (Ralla et al. 2020a, 2020b). The emulsions were exposed to external environmental stresses comprising extreme pH, ionic strength and temperature. Formed emulsion droplets were stable against a broad range of pH values (pH 5 to 9). Lower pH values led to a reduction in electrostatic repulsion, which may have been caused by protonation of negatively charged $-COO^-$ groups, leading to aggregation of the emulsion droplets. The addition of cations influenced the electrostatic repulsion through the screening of the charges by mono- and bivalent ions. Thus, the prepared emulsions were stable up to the addition of 200 mM NaCl or 5 mM $CaCl_2$. Stability at elevated temperatures was also investigated. Emulsions were stable up to temperatures of 55 °C but showed complete phase separation when stored over 7 days (Ralla et al. 2020a). Furthermore, emulsions were unstable after one freeze-thaw cycle, and displayed phase separation (Ralla et al. 2020b). Nevertheless, the results showed that glycyrrhizin could form and stabilize oil-in-water emulsions and may therefore be suitable for different applications (Ralla et al. 2020a, 2020b).

In addition to glycyrrhizin, two ginseng extracts were purchased and used as model systems. These do not only have different compositions but also different characteristics with regard to their performance as emulsifiers. One extract was obtained from Finzelberg GmbH & Co. KG (abbreviated as extract FB) and the other from CheilJedang (abbreviated as extract CJ). Extract FB contained ~5.6% saponins, while extract CJ had a slightly lower content of ~4.6%. In contrast to glycyrrhizin, both extracts significantly reduced interfacial tension by up to 80% and formed stable oil-in-water emulsions at a low emulsifier to-oil-ratio. Extract FB was superior to extract CJ in terms of its emulsion formation performance. Mean particle sizes for extract FB were in the sub-micrometer range, whereas larger values (up to 25 μm) were measured for extract CJ. Exposure to external stress showed that the emulsions for both extracts were stable within a pH range of 4 to 9 and at high ionic strength (≤100 mM NaCl). However, the addition of $CaCl_2$ (10 mM) caused flocculation. Storage for four weeks at an elevated temperature (≥ 55 °C) resulted in phase separation. Based on the results obtained, the emulsifier performance might be explained via the formation of a saponin-protein complex layer, which led to a stronger interfacial network (Ralla et al. 2017a). Ginseng extract FB showed remarkable emulsifying properties, similar to the highly effective quillaja saponaria extract that is already established as an emulsifier (Yang et al. 2013; Ralla et al. 2017a).

The extract derived from oat bran, a common by-product of oat refinement, showed itself to be a conceivable alternative to quillaja extract (Ralla et al. 2018a). It was therefore examined more closely. With an approximate saponin content of 4.6%, oat bran extract displayed a high surface activity, similar to both ginseng extracts (Ralla et al. 2017a; Ralla et al. 2018a). Even at low emulsifier-to-oil ratios, emulsion droplets were formed in the sub-micrometer range. Stability measurements showed that these emulsions are stable against different external stress factors, such as a pH range of 4 to 9 and temperatures of up to 50 °C. Longer storage times of up to 42 days could only be achieved at 25 °C and not at higher temperatures. The emulsions did not tolerate high ionic strengths and freeze thawing (Ralla et al. 2018a). Finally, the measurements indicated that emulsion stability and formation were associated with interfaces containing oat saponins or oat saponin-protein complexes, which led to a comparable emulsifying ability as quillaja saponin extract (Yang et al. 2013; Ralla et al. 2018a). Unfortunately, oat bran extract is known to contain bitter tasting saponins, which limits the range of applications (Günther-Jordanland et al. 2016).

Beetroot extract also showed promise. It was obtained from red beet, although it could also be obtained from red beet peel, a typical industrial by-product. The saponin content of around 0.9% in the extract was lower than that of the other extracts. As expected, its performance as an emulsifier was significantly reduced. This is most obvious if the particle size is considered which was within the micrometer range at a low emulsifier-to-oil ratio of 0.75:10 (Ralla et al. 2019). Similar to ginseng and oat bran extract, an emulsion stability was observed within a pH range of 4 to 9 (Ralla et al. 2017a; Ralla et al. 2018a; Ralla et al. 2019). Moreover, the emulsions were unstable after one freeze-thaw cycle and at higher temperatures. Unlike the other extracts, resistance against ionic strength was not determined, due to the rather low emulsifying properties (Ralla et al. 2019).

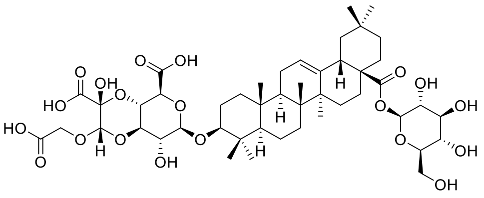
Sugar beet extract was identified as the most promising one in the investigation. Although it has a saponin content of around 0.50%, which is even lower than beetroot, sugar beet grows in very large quantities, as mentioned in Section 1.4 above (Ralla et al. 2017c; FAOSTAT 2020). In addition, only a weak indication was found in the literature to the effect that one sugar beet saponins possesses a bitter taste, as discussed in Section 4.6 (Koziol 1991). Together with the fact that remarkable emulsifying properties were observed, it thus represents a promising material, especially considering that sugar beet extract, and indeed the other extracts, are only crude saponin extracts (Ralla et al. 2017a; Ralla et al. 2017b, 2017c, 2018a; Ralla et al. 2019). It was found that the behavior of sugar beet extract was comparable to that of beetroot extract as it formed emulsions with a particle size of 1.3 μm at a low emulsifier-to-oil ratio of 0.75:10 (Ralla et al. 2017b; Ralla et al. 2019). As observed for the other extracts, interfacial tension and surface tension were significantly reduced, indicating a high surface activity (Ralla et al. 2017a; Ralla et al. 2017b, 2018a; Ralla et al. 2019). The emulsions were stable within a pH range of 5 to 8 and against thermal treatment up to 60 °C. However, they were unstable after the addition of salt (> 0.1 M NaCl) and upon freeze thawing. Nevertheless, crude sugar beet extract displayed a high potential for use as an emulsifier in a variety of food applications (Ralla et al. 2017b, 2017c).

More details about the techno-functionality of glycyrrhizin and the extracts investigated are provided by the publications (Ralla et al. 2017a; Ralla et al. 2017b, 2017c, 2018a; Ralla et al. 2019; Ralla et al. 2020a, 2020b). In contrast, the present work focuses on the molecular elucidation of the characteristic properties of sugar beet saponins, these being taste, foam activity, and chemical structure. Quantification of sugar beet saponins was also carried out. However, for all of these experiments, it was first necessary to isolate them.

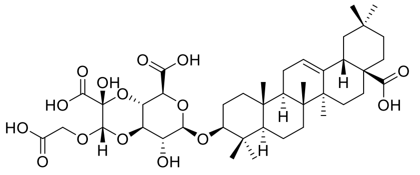
4.2 Saponin Isolation Strategy

Isolation of sugar beet saponins is not an easy task. Saponin content in sugar beet root varies between 0.086 and 0.25% (Edelmann et al. 2020b). Additionally, various saponins exist, with very different mass proportions (cf. Section 1.6 and Section 4.9). Isolation seems to be more promising from sugar beet leaves than from sugar beet roots, since the quantity of saponins found in the leaves was generally higher, with values of up to 0.54% (Edelmann et al. 2020b). A later authorization as a food additive appears to be easier for sugar beet root than for leaves or other sugar beet materials (European Union 2008). Therefore, saponin isolation began with roots and was adjusted over time.

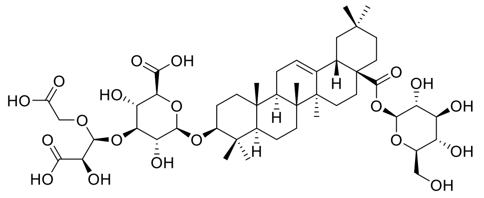
Freeze-dried and pulverized sugar beet roots were first of all extracted three times using a mixture of methanol and water (70:30; v/v); this is reported to be appropriate for the extraction of saponins in the literature (Dawid and Hofmann 2012a; Günther-Jordanland et al. 2016; Edelmann et al. 2020a). The mixture not only extracts saponins but also saccharides and other substances. Because Massiot et al. and Yoshikawa et al. only used methanol for the extraction, the residue was additionally extracted three times with methanol to isolate all conceivable saponins (Massiot et al. 1994; Yoshikawa et al. 1995; Edelmann et al. 2020a). The obtained extract was screened for both known and unknown saponins according to the literature, using LC-HRMS. All saponins unequivocally identified in the sugar beet extract and corresponding subfractions are shown in Figure 14 together with their exact masses (Edelmann et al. 2020a). All of the presented structures were confirmed by this work (cf. Section 4.4).



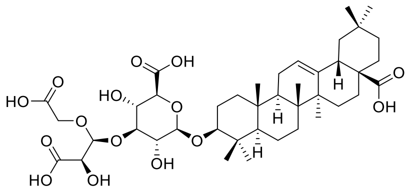
1 (954.4460 g/mol)



2 (792.3932 g/mol)

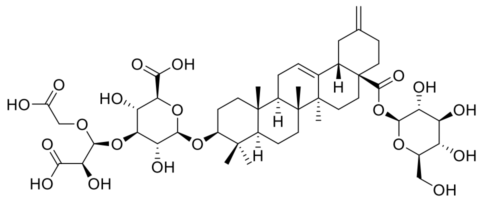


3 (956.4617 g/mol)

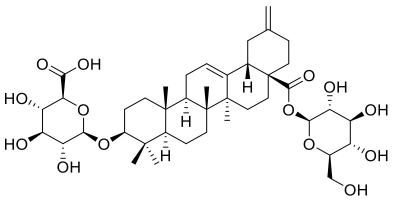


4 (794.4089 g/mol)

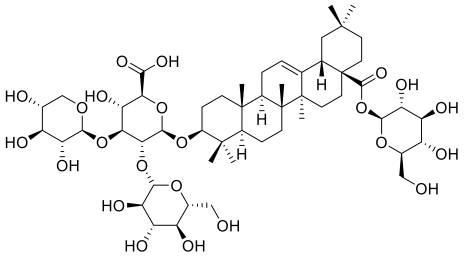
Figure 14: *Sugar beet saponins unequivocally identified in sugar beet extract and their corresponding subfractions. Structures and exact molecular masses (given in brackets) were derived from Massiot et al., Marouf et al., Ridout et al. and Yoshikawa et al. (Massiot et al. 1994; Ridout et al. 1994; Yoshikawa et al. 1996b; Yoshikawa et al. 1998; Marouf et al. 2001). Betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-O-[β-D-glucopyranosyl-(1→2)-(β-D-xylopyranosyl-(1→3))-β-D-glucuronopyranosyl]-28-O-β-D-glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**).*



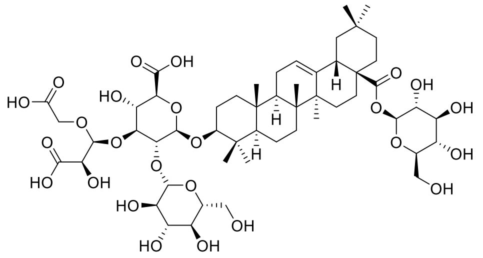
5 (940.4304 g/mol)



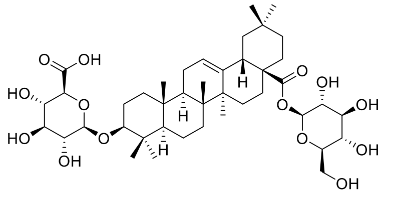
6 (778.4140 g/mol)



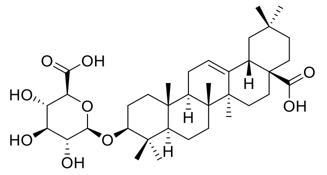
7 (1088.5403 g/mol)



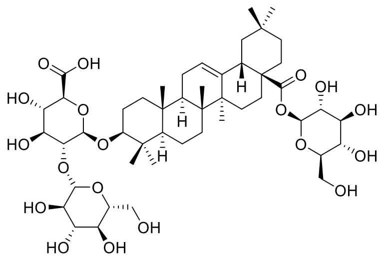
8 (1118.5145 g/mol)



9 (794.4453 g/mol)



10 (632.3924 g/mol)



11 (956.4981 g/mol)

Figure 14 (continuation): *Sugar beet saponins unequivocally identified in sugar beet extract and their corresponding subfractions. Structures and exact molecular masses (given in brackets) were derived from Massiot et al., Marouf et al., Ridout et al. and Yoshikawa et al. (Massiot et al. 1994; Ridout et al. 1994; Yoshikawa et al. 1996b; Yoshikawa et al. 1998; Marouf et al. 2001). Betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-O-[β-D-glucopyranosyl-(1→2)-(β-D-xylopyranosyl-(1→3))-β-D-glucuronopyranosyl]-28-O-β-D-glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**).*

The isolation process itself was extensive, since sugar beet roots contain a lot of saccharides (12 to 22%) which have to be separated from the saponins (Block, 1920). In the initial experiments, sugar beet extract was separated by MPLC, which divides saccharides and saponins into different fractions without possibly losing or discriminating large amounts of them. These fractions were then used in foam activity measurements (Section 4.7) and taste dilution analysis (Section 4.6). Both experiments found that the fractions containing saponins (identified with LC-HRMS) possess a slight off-taste (which was tested by taste dilution analysis) and high foam activity, which makes them particularly interesting (Edelmann et al. 2020a). The results are illustrated in Figure 15.

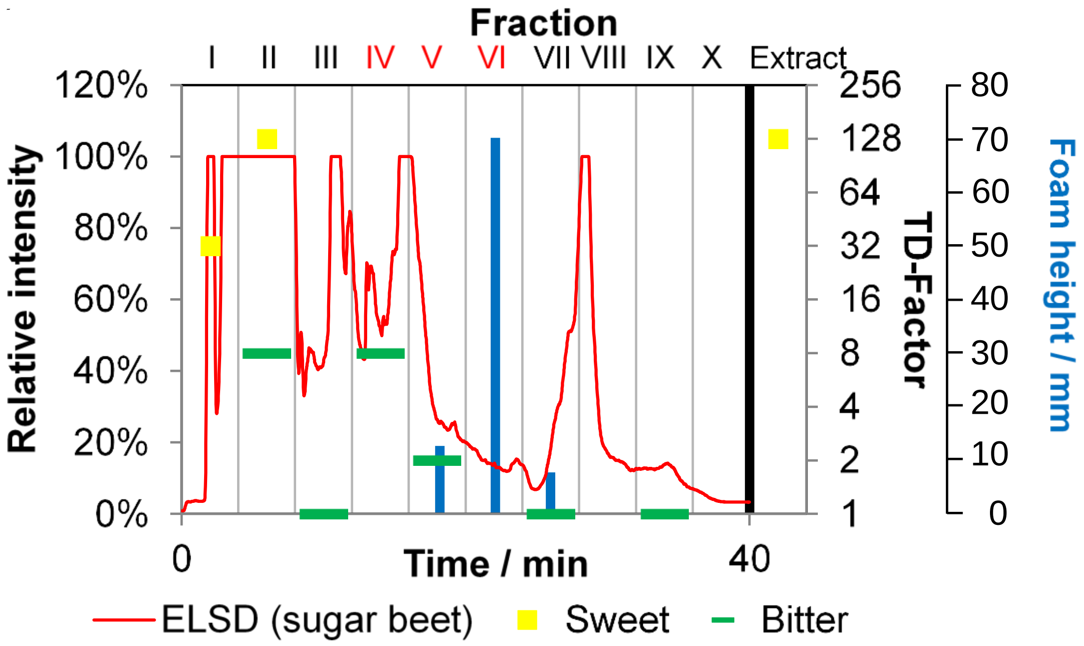


Figure 15: *Taste dilution analysis and foam activity measurements of MPLC fractions taken from sugar beet extract and crude sugar beet extract. Taste dilution (TD) factors are labelled for bitter taste in green and for sweet taste in yellow. Fractions containing saponins are highlighted in red. This figure is from the publication by Edelmann et al. (Edelmann et al. 2020a).*

A simplified extraction method for sugar beet root that only involves triplicate extraction with methanol and water (70:30; v/v) revealed a similar saponin composition. In this case, only methanol was added during the first extraction step. When combined with the water in the pulp (75-80%), a methanol and water mixture (70:30; v/v) was formed (Block 1920). The extracts obtained with these two methods were used to determine saponin abundance and only saponins of significant abundance were considered for further isolation.

An alternative to performing extract separation with MPLC, which is, however, less commonly used, is foam fractionation; this will be discussed in Section 4.8. It works without organic solvents and has occasionally been applied in the enrichment of saponins from sugar beet root extract. The method shows some advantages over MPLC, not least its use of water rather than organic solvents. Nevertheless, this interesting method was substituted by another procedure that uses an alternative saponin source.

A more suitable source of sugar beet saponins was found in the course of this work. This source, rarely mentioned in the literature, is sugar beet pulp (Yapo et al. 2007; Edelmann et al. 2020a), a by-product stream that occurs in large quantities during sugar beet processing (Russ and Meyer-Pittroff 2004). It has the second highest saponin content of all materials studied in this work (Edelmann et al. 2020b). This is not particularly surprising, since saponins were typically extracted using organic solvents, and sugar beet cossettes that yield sugar beet pulp were only extracted with warm water (Asadi 2006; Cheok et al. 2014). Large amounts of saponins remain within the sugar beet pulp when the majority of saccharides have been removed. This greatly simplifies extraction, and quantification showed that even saponins that are probably highly soluble in water were left in large amounts in the sugar beet pulp (Edelmann et al. 2020b).

Since this material was available at any time and in large quantities, only a single extraction was performed (Edelmann et al. 2020a). Multiple extractions were not considered worthwhile, although the yield might have been increased a little, because additional organic solvent would have needed to be removed, which would not have been easy due to the foam activity of the extracts. The extract obtained contained significantly smaller amounts of saccharides compared to sugar beet root extract. While this extract was only used for isolation and not for further experiments, it was separated into four fractions by solid phase extraction (SPE). Although this extract contained significantly less saccharides, the first fraction, which was eluted with water, was the dominant one. This made it possible to separate large quantities of extract (up to 5 g) with relatively small amounts of column material (10 g). The fact that it was possible to use up to twelve SPE columns with one SPE chamber at the same time illustrates its superiority over MPLC. Furthermore, less organic solvent was needed, which also has to be removed afterwards (Edelmann et al. 2020a).

SPE fraction number three, which contained the highest quantities of saponins, was used for further isolation with HPLC (Edelmann et al. 2020a). It possesses a much better chromatographic resolution compared to SPE and was able to separate all saponins occurring in significant amounts. Different column materials were tested for separation, and the most suitable column was found to be a Nucleodur C_{18} Pyramid. As stated in Section 3.2.2, it consists of a silica phase with octadecyl modification and polar endcapping (Macherey-Nagel 2019). Due to the complexity of the fraction, coarse separation was carried out with preparative HPLC (column dimensions 250 × 21 mm), followed by separation and purification of the individual saponins with semi-preparative HPLC (column dimensions 250 × 10 mm). An isocratic gradient was required, which took far longer than usual to separate these saponins (30 to 34 minutes for separation and up to 55 minutes for purification). All details are given in the publication by Edelmann et al. (Edelmann et al. 2020a). The pure saponins ultimately obtained may differ in water content. These are betavulgaroside I (**1**), betavulgaroside III (**3**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), and betavulgaroside V (**8**). Three saponins (chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**)) were not isolated, as they are commercially available. Two more (betavulgaroside II (**2**) and betavulgaroside IV (**4**)) were synthesized, as described in the following section (Edelmann et al. 2020a).

4.3 Synthesis of Monodesmosidic Saponins from Bidesmosidic Saponins

It proved virtually impossible to isolate the monodesmosidic saponins (cf. Figure 14) betavulgaroside II (**2**) and betavulgaroside IV (**4**) from the sugar beet pulp extract, due to the large number of substances which simultaneously elute during the HPLC separation. As it turned out during the development of the quantification method, it might have been easier if 1% formic acid had been used instead of the 0.1% formic acid, because the peaks of these two substances both become much sharper at lower pH values. Formic acid concentrations above 0.1% were avoided, as it is known that some saponins decompose at too low pH values (Dawid 2012). Since the discovery of sharper peaks occurred quite late during this work, the saponins were obtained by synthesis (Edelmann et al. 2020a).

The high mass fraction of betavulgaroside I (**1**) and betavulgaroside III (**3**) in sugar beet pulp combined with the fact that it is much simpler to isolate them from a mixture of these two substances made the synthesis of betavulgaroside II (**2**) and betavulgaroside IV (**4**) out of these compounds easier compared to the isolation. Alkaline hydrolysis with 5% aqueous sodium hydroxide under reflux as published by Yoshikawa et al. was not successful (Yoshikawa et al. 1995). Therefore, a working synthesis was established based on the method of Ohtani et al. with slight changes (Ohtani et al. 1984; Edelmann et al. 2020a). The method was originally developed to quantitatively cleave ester bonds between sugars and aglycones without cleaving glycosidic bonds (Ohtani et al. 1984).

Ester cleavage of sterically hindered carboxylic acid groups requires relatively strong alkaline conditions, which can lead to side reactions. The reaction published by Ohtani et al. is selective and aims to cleave ester bonds quantitatively. Anhydrous LiI, 2,6-lutidine and anhydrous methanol were used for synthesis (Ohtani et al. 1984). The mixture of betavulgaroside I (**1**) and betavulgaroside III (**3**) was first dried over silica gel (Edelmann et al. 2020a). Cleavage of the ester bond is caused by nucleophilic substitution of the carboxylic oxygen atom by iodine. The resulting sugar iodide reacts further with methanol to form a methyl glycoside (Ohtani et al. 1984). Fortunately, the remaining parts of betavulgaroside I (**1**) and betavulgaroside III (**3**) remained intact and both betavulgaroside II (**2**) and betavulgaroside IV (**4**) were successfully synthesized. The reaction is illustrated in Figure 16. However, the yield of the reaction was only 15%, and further separation of the two components was required (Edelmann et al. 2020a).

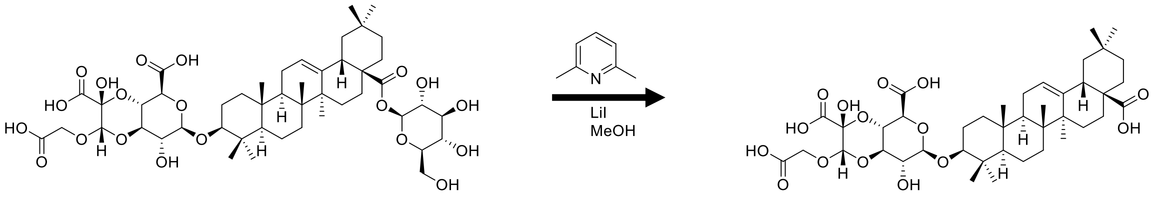


Figure 16: *Synthesis of Betavulgaroside II from Betavulgaroside I.*

4.4 Structure Elucidation

Detailed structural elucidation of compounds **1** to **11,** which were either isolated, synthesized or obtained commercially, was given in the publication by Edelmann et al., where the complete assignment of individual hydrogen and carbon atoms was also described (Edelmann et al. 2020a). The aglycon structure was determined by NMR spectroscopy with the aid of the literature (Seebacher et al. 2003). The identification of sugars was much more complex due to the complexity of the NMR spectra. NMR experiments provide the positions of the individual protons relative to one another (vicinal couplings) as well as the bonds to the corresponding carbon atoms and their next neighbors, in the event that they are carbon atoms. However, it was not possible to identify the absolute structure of the corresponding sugars using traditional NMR experiments (Hesse et al. 2012). Other methods were therefore required. A method that was recently published by Schmid et al. was modified and employed for unequivocal identification of the absolute sugar structure (Schmid et al. 2018; Edelmann et al. 2020a). This is a modified version of the method published by Tanaka et al. based on the fact that D- and L-sugars show different retention times during separation by HPLC after derivatization (Tanaka et al. 2007). After identification of the aglycones and sugars, their relative positions and linkages were determined by NMR. The relative positions were identified with ROESY, which shows couplings between atoms that are spatially close to each other (Hesse et al. 2012). Linkages between sugars were identified using HMBC and ROESY. Despite the numerous possibilities, it was not possible to determine the stereo centers shown in Figure 17 by traditional NMR experiments (Edelmann et al. 2020a).

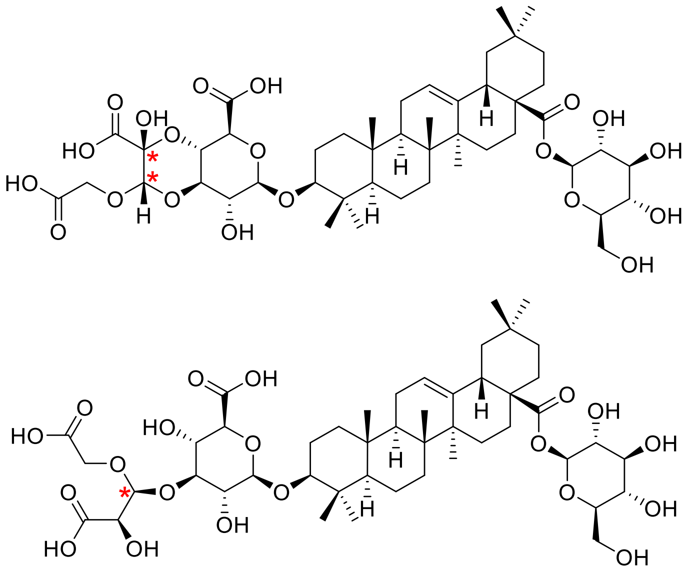


Figure 17: *Betavulgaroside I (above) and betavulgaroside III (below) with labeled stereogenic centers that cannot be ascertained by NMR spectroscopy.*

The stereocenters shown in Figure 17 were determined with the aid of the literature. The structure of betavulgaroside I (**1**) was obtained from Yoshikawa et al. and agrees with the NMR data of this thesis (Yoshikawa et al. 1996b; Edelmann et al. 2020a). They compared literature data from Ida et al. and discovered that the substituent from achyranthoside A trimethyl ester, which was identified by X-ray crystallography, is almost identical to the one from betavulgaroside I (**1**), and that the stereogenic centers can be obtained from this analysis (Ida et al. 1994). Betavulgaroside II (**2**) possesses the same substituent that was demonstrated by comparison of NMR data (Edelmann et al. 2020a). This was also supported by Yoshikawa et al. (Yoshikawa et al. 1996b).

Betavugaroside III (**3**) has a stereogenic center that cannot be determined by NMR spectroscopy (cf. Figure 17). Zhu et al. carried out a stereogenic synthesis that demonstrated the absolute structures of all stereogenic centers and agreed with the NMR data of this thesis (Zhu et al. 2008; Edelmann et al. 2020a). A few years earlier, it was synthesized from momordin I through stereoselective synthesis by Yoshikawa et al. (Yoshikawa et al. 1997b). Comparison of the NMR data showed that the substituent from betavulgaroside III (**3**) has the same stereogenic center as the substituents in betavulgaroside IV (**4**) and V (**8**), which was supported by Yoshikawa et al. (Yoshikawa et al. 1998; Edelmann et al. 2020a).

4.5 CCS Values

Collision cross-section (CCS) values of compounds **1** to **11** were measured using a Vion IMS QTof (Edelmann et al. 2020a). They represent an orthogonal physicochemical parameter which allows the separation of isobaric substances (Kromidas 2017; Griffiths et al. 2020). CCS values are influenced by chemical structure and three-dimensional conformation. They depend on the characteristic time of the substance needed to cross the ion mobility separation cell. They can also be used as a molecular descriptor for improving the accuracy of analyte identifications (Griffiths et al. 2020). Substances with more than one CCS value have rarely been identified and have only been reported by Stark et al. (Stark et al. 2019). It is not known why individual saponins have multiple CCS values. The fact that all monodesmosidic saponins analyzed in this work possess only one CCS value was quite in contrast to the bidesmosidic ones, which all possess three CCS values. Additionally, most of the CCS values measured differ significantly from each other, as shown in Table 2 (Edelmann et al. 2020a).

Table 2: *Collision cross-section (CCS) values for unequivocally identified saponins. Values obtained from Edelmann et al. (Edelmann et al. 2020a).*

	Collision cross-section (CCS) values / Å^2		
Compound	**CCS No. 1**	**CCS No. 2**	**CCS No. 3**
Betavulgaroside I (**1**)	198.4	239.3	330.3
Betavulgaroside II (**2**)	306.3	-	-
Betavulgaroside III (**3**)	198.3	241.6	330.0
Betavulgaroside IV (**4**)	309.0	-	-
Betavulgaroside VIII (**5**)	195.8	254.2	314.5
Boussingoside A2 (**6**)	181.6	222.9	282.7
3-*O*-[*β*-D-glucopyranosyl… (**7**)	212.4	272.8	367.1
Betavulgaroside V (**8**)	209.8	249.5	260.9
Chikusetsu saponin IVa (**9**)	183.5	220.8	287.0
Calenduloside E (**10**)	271.5	-	-
Ginsenoside R0 (**11**)	197.5	249.5	324.1

4.6 Taste Activity of Sugar Beet Saponins

Taste activity is another characteristic of saponins (Hostettmann and Marston 2005). Literature on bitter taste thresholds of individual saponins is scant, despite the large variety of saponins. Table 3 presents the bitter taste thresholds found in the literature. Some of these compounds have additional taste attributes (Günther-Jordanland et al. 2016; Pickrahn 2017; Edelmann et al. 2020a).

Table 3: *Bitter taste threshold values of different saponins found in the literature.*

Saponin	**Bitter taste threshold**
3-(*O*-α-L-Rhamnopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→3)-*β*-D-glucopyranosyl(1→4)]-*β*-D-glucopyranosid)-26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-5-ene-3*β*,22,26-triol*	4 μmol/kg
Avenacoside A*	6 μmol/kg
Avenacoside B*	7 μmol/kg
3-(*O*-α-L-Rhamnopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→4)]-*β*-D-glucopyranosid)-26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-5-ene-3*β*,22,26-triol*	9 μmol/kg
Gymnemic acid III**	10 μmol/L
(25*R*)-Furost-5-ene-3*β*,22,26-triol-3-*O*-[α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside and (25*S*)-Furost-5-ene-3*β*,22,26-triol-3-*O*-[α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside (mixture)***	10.9 μmol/kg
Gymnemic acid I, II, IV, XV, XIX, XX, and XXI**	~20 µml/L
(25*R*)-Furostane-3*β*,22,26-triol-3-*O*-[α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside and (25*S*)-Furostane-3*β*,22,26-triol-3-*O*-[α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside]-26-*O*-*β*-D-glucopyranoside (mixture)***	25.5 μmol/kg
11-Deoxo-(20α)-Glycyrrhizin*4	51.2 μmol/L
Protodioscin and Neoprotodioscin (mixture)***	65.9 μmol/kg
3-*O*-[{α-L-Rhamnopyranosyl-(1→2)}{α-L-rhamnopyranosyl-(1→4)}-*β*-D-glucopyranosyl]-(25*S*)-spirost-5-ene-3*β*-ol and 3-*O*-[{α-L-Rhamnopyranosyl-(1→2)}{α-L-rhamnopyranosyl-(1→4)}-*β*-D-glucopyranosyl]-(25*R*)-spirost-5-ene-3*β*-ol (mixture)***	70.6 μmol/kg
Ginsenoside Rc *5	77 μmol/L
Gymnemic acid V**	78 μmol/L
Ginsenoside Rg1 *5	117 μmol/L
3-*O*-[{*β*-D-Glucopyranosyl-(1→2)}{*β*-D-xylopyranosyl-(1→4)}-*β*-D-glucopyranosyl] (25*S*),5*β*-spirostan-3*β*-ol***	199.7 μmol/kg
(20α)-Glycyrrhizin*4	210.0 μmol/L
Betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**) *5	> 1,000 μmol/L
Soyasaponin I *6	1,620 μmol/L

* Data obtained from Günther-Jordanland et al. (Günther-Jordanland et al. 2016)
** Data obtained from Pickrahn (Pickrahn 2017)
*** Data obtained from Dawid and Hofmann (Dawid and Hofmann 2012a)
*4 Data obtained from Schmid (Schmid 2018)
*5 Data obtained from Edelmann et al. (Edelmann et al. 2020a)
*6 Data obtained from Gläser et al. (Gläser et al. 2020)

Most of the taste thresholds in Table 3 are below 200 μmol/kg. This might be because high taste thresholds are less frequently explored and therefore most of them might not yet have been found or reported. Soyasaponin I is a different case, as it was described as a bitter-tasting compound in the literature (Price and Roger Fenwick 1984). A recent work shows that its bitter taste threshold is rather high, with a value of 1,620 μmol/L (Gläser et al. 2020). Additionally, several saponins with no perceived bitter taste were reported by Christian Schmid, who worked with licorice saponins (Schmid 2018).

It is assumed that the bitter taste of sugar beet saponins is in a similar range to that of soyasaponin I, which indicates that it is negligible. Since some of the panelists were not able to perceive a bitter taste even at the highest concentrations (1,000 μmol/kg), no bitter taste thresholds were obtained after applying a significance test with probability levels of 5% (Edelmann et al. 2020a). The difference in perception of the bitter taste was not surprising and varies greatly across individuals, as described in Section 1.8 (Bartoshuk 1993; Drewnowski 2001; Kim and Drayna 2005). Taste thresholds above 1,000 μmol/kg nevertheless appear relatively low for use as a food additive (Edelmann et al. 2020a). The current permission for quillaja saponins is significantly lower, with a concentration of 200 mg/L (Berlowska et al. 2017). When higher concentrations are required, it should be possible to mask their bitter taste using taste-active ingredients.

A few sugar beet saponins also occur in other plants, such as quinoa (Woldemichael and Wink 2001). The slight bitter taste of sugar beet saponins indicates that saponins are not alone responsible for the intense bitter taste that was reported for extracts from quinoa (Koziol 1991). It could also originate from other compounds such as polyphenols (Hufnagel and Hofmann 2008; Alvarez-Jubete et al. 2010).

4.7 Foam Activity

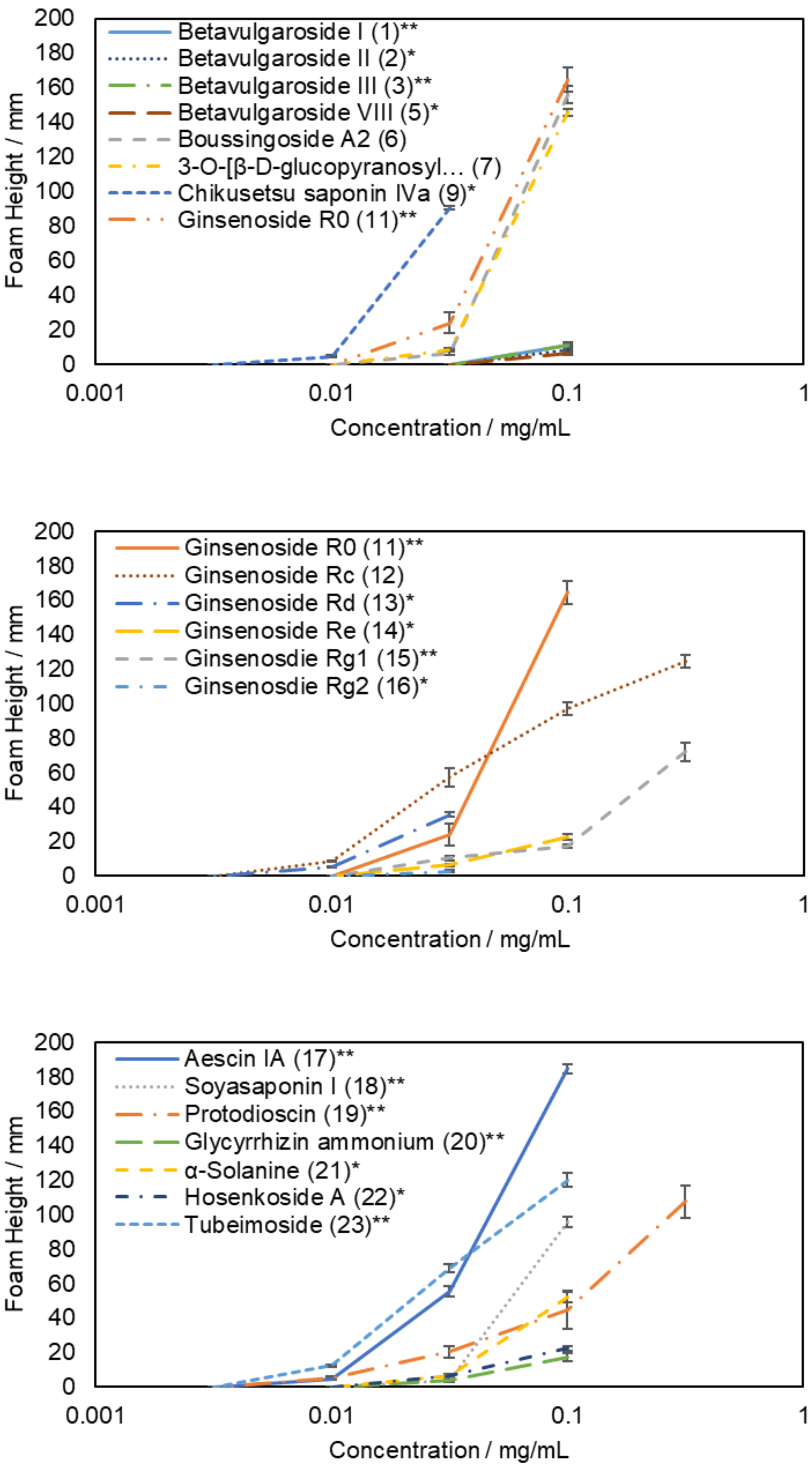
Foam activity is one of the most important characteristics of saponins (Hostettmann and Marston 2005). However, only very little information about foam activity measurements on a molecular basis was found within the literature (Böttcher and Drusch 2016). Since not only saponins are known to be foam active it was important for them to be as pure as possible in the foam activity measurements. Furthermore, it was expected that various saponins would show different foam activities, which was supported by the statement by Oleszek et al. that monodesmosidic saponins are particularly foam-active (Kjellin and Johansson 2010).

The first foam activity measurements were made about 100 years ago. As stated above, they were made with crude saponin mixtures and were not standardized. A common method was to determine the foam number. It was measured by shaking (crude) saponin solutions with known concentrations for 15 seconds. The one that gave a 1 cm foam column after 15 minutes of resting indicated the foam number, which corresponds to the dilution factor. Several differences between saponin-containing extracts were determined by this simple method (Kofler 1922). It took quite a while before better methods were developed. One system, which is still in use today, was developed by Ross and Miles

(Ross and Miles 1941; Chen et al. 2010). It can be viewed as an older version of the device used in this work, which was described in Section 3.4. The one used here was miniaturized as far as possible to enable it to operate on as small quantities of saponins as possible (Edelmann et al. 2020a). An example is shown in Figure 13. Two recent publications were based on more advanced methods (partially automated), but they both mainly used saponin blends and were not able to work with small amounts of saponins (Böttcher and Drusch 2016; Santini et al. 2019).

Although foam activity measurements have long been performed on saponins, the methods are not well standardized. The pH value, in particular, influences foam activity (Mangan 1959), and it is obvious that a number of other factors also influence it, including purity, water content and presumably salt content. To eliminate these factors, several aspects were modified in relation to the methods described in the literature. One of the most important ones was that a buffer system was used for dissolving all saponins. Furthermore, foam activity measurements were carried out by introducing a defined flow of nitrogen into the measurement system through a fine-pored frit. All sugar beet saponins measured with this system were freeze-dried together to ensure they had similar water contents (Edelmann et al. 2020a). The water content could not be measured, due to the small amount of substance material. Water and salt contents of commercially obtained substances were not known, but they could have affected these measurements.

Various criteria were considered for the selection of saponins used in the foam activity measurements. First, all saponins that were not isolated from sugar beet had to be commercially available. They had to be inexpensive, a common problem in relation to saponins. Furthermore, a wide variety of structures had to be included, and the substances had to be of high purity. Some of the saponins selected were not soluble at suitable concentrations or had a limited solubility range. The remaining ones were suitable for foam activity measurements. However, the only structure-activity relationships found were contradictory to the literature, since the foam activity of monodesmosidic saponins was mostly lower than that of bidesmosidic ones (Kjellin and Johansson 2010; Edelmann et al. 2020a). Bearing in mind their structural similarity, an unexpectedly high difference in foam activity, which cannot be explained, was found between chikusetsu saponin IVa and boussingoside A2 (Edelmann et al. 2020a). The foam activity measurements are shown in Figure 18 for illustration. All details can be found in the publication by Edelmann et al. (Edelmann et al. 2020a).



* Measurement points within the solubility.

** At higher concentrations, the liquid completely turns into foam.

Figure 18: *Foam activity measurements with pure saponins. Figures taken from the publication by Edelmann et al. (Edelmann et al. 2020a).*

4.8 Foam Fractionation

Foam fractionation is a very useful tool, which takes advantage of the foam activities of several different substances. It was described in Section 1.7 and is highly suitable for the enrichment of saponins from sugar beet extract. The enrichment of tea saponins has already been reported in the literature (Yan et al. 2011). With less foam-active solutions, enrichment does not work so well, because a small tube diameter is needed, otherwise the foam will burst because of the large diameter. However, a small diameter reduces the amount of substance that can be enriched in a given period of time. It is for this reason that the apparatus described in Section 3.2.1 was developed. It has little resemblance to the classic system shown in Figure 11 and was made especially for the enrichment of extracts with low foam activity. To compensate for the smaller diameter, it has six foam columns. All of the columns are approached evenly by the gas flow through the inwardly curved top. Bigger systems with more foam columns and/or a larger volume are also possible. Another system for solving this problem consist of parallel inclined channels, invented by Dickinson et al. (Dickinson et al. 2010), while another has a circular section in the foam columns, and was invented by Stevenson (Yang et al. 2011, 2012). Both systems were developed for different purposes but can also be used for the separation of sugar beet extract (Dickinson et al. 2010; Yang et al. 2011, 2012).

Numerous influencing factors, including pH, temperature, gas flow, and column dimensions, influence the efficiency of the foam fractionation (Backleh 2001). This makes it challenging to specify an exact enrichment factor. For the enrichment of sugar beet extract it is normally in the range of about five, but it can differ significantly, since many parameters change from time to time. The extract used is not always the same and the saponin concentration in the solution is therefore always different. Temperature and gas flow differ from time to time, and the gas flow during the fractionation is not constant but increases. The increase in gas flow is necessary since foam activity decreases over time. Another disadvantage is that foam fractionation is not able to remove the last remaining foam active compounds from the extract, as a minimum quantity is required for foam formation (Yan et al. 2011). This is one of the reasons why sensory experiments for activity-guided fractionation were carried out with fractions obtained by MPLC.

Saponin enrichment can be further increased with the aid of a second foam fractionation step. Foamate, obtained during the first foam fractionation, can be used as a starting material for the second foam fractionation step, using an apparatus like the one shown in Figure 11. After the first enrichment, foam activity was significantly higher, and a countercurrent could be used to wash out the non-foaming compounds. This method greatly increased the degree of separation compared to enrichment without countercurrent (Lemlich and Lavi 1961). Unfortunately, saponin content was not high enough to enable efficient separation by HPLC. Consequently, separation by solid phase extraction was still needed to isolate the key compounds efficiently. As described in Section 4.2, this process was rarely used, due to the high saponin content found in dried sugar beet pulp.

4.9 Quantitative Analysis of Saponins

There are no state-of-the-art methods for the quantification of sugar beet saponins in sugar beet pulp or other materials. However, as stated in Section 1.6, some older methods exist (Eis et al. 1952; Walker 1956; West and Gaddie 1956; Bauserman and Hanzas 1957; van der Poel and Schwartz 2000). These have been very helpful for determining the total saponin content of various products formed during sugar beet processing, as well as for process optimization (van der Poel and Schwartz 2000). Besides having low specificity, these methods quantify the sum of all saponins and not individual ones. This is the most important difference between existing methods and the recently developed one (Edelmann et al. 2020b). Furthermore, the new method is expected to be much more sensitive, which, however, cannot be proven, since no limits of quantification have been reported for the other methods.

The newly developed quantification method is based on tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS). It ensures high selectivity by the fact that the individual saponins (**1** to **11**) are first separated chromatographically and then analyzed by mass spectrometry. Very high sensitivity was achieved with this method, particularly due to the tandem mass spectrometry system used. Sample preparation is far simpler than with established methods (Bauserman and Hanzas 1957; Edelmann et al. 2020a). It consists of a single extraction step, which includes the addition of internal standards. For this purpose, two different substances were selected as internal standards. These are hederacoside C and alpha-hederin that are shown in Figure 19 (Edelmann et al. 2020a).

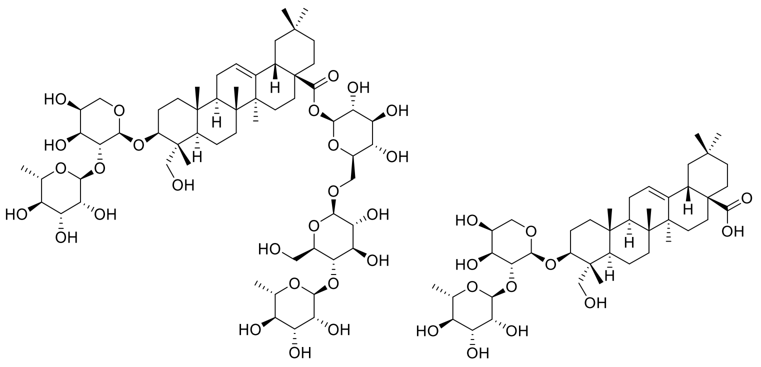


Figure 19: *Illustration of the internal standards hederacoside C (left) and alpha-hederin (right).*

These two internal standards are quite similar to sugar beet saponins. Even more similar standards were available commercially, but they were much more expensive. The best internal standards could be obtained by hydrogenation of the double bond within the corresponding aglycones. The corresponding double bond in Figure 20 is marked in red.

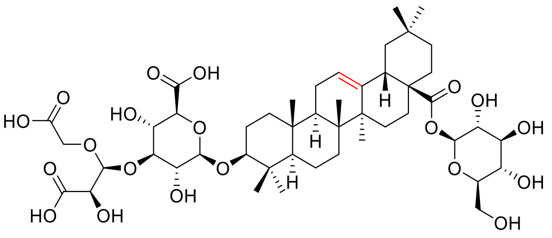


Figure 20: *Betavulgaroside III with red labeled double bond.*

The addition of internal standards not only ensures a much more precise quantification but also makes sample preparation much easier. Not only do they correct mistakes during sample preparation and variations during the ionization process, but matrix effects too (Kromidas and Kuss 2009). This was especially important for the quantification of sugar beet saponins, which cannot be easily separated from the sample matrix (Edelmann et al. 2020a). In the case of sugar beet saponins, the internal standards were added, and an equilibrium was established by shaking for 60 minutes (Edelmann et al. 2020b). This ensured that the distribution of the added internal standard between matrix and solvent was comparable to that of the analytes and ensured that further extraction steps become superfluous. Despite the addition of internal standards, systematic errors are possible. The most likely error is the formation of derivatives from analyte molecules.

4.9.1 Formation of Derivatives

Sugar beet saponins offer a few possibilities for the formation of derivatives. The most likely one is the formation of methyl esters, as reported by Yoshikawa et al. According to them, it occurs during the treatment of betavulgaroside I (**1**) with methanol under reflux or standing for several days (Yoshikawa et al. 1996b). Methyl esters were not observed by

Christian Schmid after the extraction of licorice with methanolic solutions (Schmid 2018). Also, they were not observed after sample preparation using UPLC-ToF-MS.

There are two other ways of forming derivatives. These are reactions with the ester or ether bonds of the saponin molecules. Not only is it then possible to cleave esters or to induce transesterification, but cleavage of an ether bond is also feasible. However, both reactions are unlikely, as they require strong reaction conditions and do not normally take place at room temperature. The high amount of calenduloside E in sugar beet fiber is an indication that cleaving occurs during the sugar beet conversion process under harsher conditions (Edelmann et al. 2020b).

4.9.2 Quantification of Different Plant Materials

Besides sugar beet fiber, numerous other materials were also analyzed. These are sugar beet roots and leaves, raw juice, thick juice, molasses, dried sugar beet pulp, flume water, process water, Goldsaft, and beetroot. In total, twelve different sugar beet varieties (root and leaves) were analyzed. Furthermore, different compartments of the Hannibal variety were also examined. To ensure the validity of the results, the method was extensively validated as described in the publication by Edelmann et al. (Edelmann et al. 2020b). This work thus represents the largest overview of the occurrence of sugar beet saponins found in the literature. Moreover, it provides quantitative data for eleven different sugar beet saponins (betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**)) and describes their abundance in the aforementioned materials. The results are discussed in the publication by Edelmann et al. and are shown in Table 4. All technical details can also be found in the publication (Edelmann et al. 2020b).

***Table 4:** Quantitative data relating to sugar beet roots, leaves, sugar beet compartments, side streams, commercial products, and beetroot. Values below the limit of quantification are given in brackets. The standard deviation is also given. Data are based on the publication by Edelmann et al. (Edelmann et al. 2020b).*

	The value indicated is the mass fraction in mg·kg^{-1}										
	Betavulgaroside I (1)	**Betavulgaroside II (2)**	**Betavulgaroside III (3)**	**Betavulgaroside IV (4)**	**Betavulgaroside VIII (5)**	**Boussingoside A2 (6)**	**3-*O*-[*β*-D-gluco… (7)**	**Betavulgaroside V (8)**	**Chikusetsu saponin IVa (9)**	**Calenduloside E (10)**	**Ginsenoside R0 (11)**
Annarosa leaves	545.9 ± 5.3	13.0 ± 0.3	1724.1 ± 23.1	114.2 ± 1.9	464.4 ± 5.9	3.5 ± 0.1	1602.8 ± 22.5	538.5 ± 6.7	19.8 ± 0.2	-	15.3 ± 0.2
Annarosa root	317.3 ± 3.5	135.2 ± 1.9	360.3 ± 6.0	189.6 ± 1.7	102.6 ± 3.1	1.0 ± 0.0	25.8 ± 0.7	18.3 ± 0.4	-	-	-
Annemaria leaves*	274.1 ± 1.9	271.2 ± 1.0	1454.9 ± 9.7	1959.7 ± 15.6	75.2 ± 0.9	1.8 ± 0.0	391.7 ± 3.3	309.6 ± 1.7	(8.4 ± 0.5)	18.5 ± 0.1	-
Annemaria root*	538.0 ± 6.3	515.0 ± 1.6	534.0 ± 7.2	689.5 ± 3.1	105.7 ± 1.7	3.3 ± 0.0	39.4 ± 0.3	27.5 ± 0.3	-	-	-
Artus leaves	88.0 ± 0.9	132.3 ± 0.7	190.3 ± 1.0	457.6 ± 1.4	55.2 ± 1.9	2.0 ± 0.1	275.0 ± 0.8	64.6 ± 0.3	-	-	-
Artus root	287.1 ± 3.6	747.9 ± 2.5	112.1 ± 1.4	455.0 ± 3.1	36.3 ± 0.3	1.3 ± 0.0	19.7 ± 0.6	3.8 ± 0.1	-	-	-
Beretta leaves	139.9 ± 0.5	5.4 ± 0.3	764.0 ± 1.8	95.8 ± 2.1	644.4 ± 6.4	4.1 ± 0.1	2343.7 ± 14.8	363.2 ± 1.3	(6.1 ± 0.1)	-	37.4 ± 0.5
Beretta root	402.2 ± 9.9	187.0 ± 3.6	285.6 ± 3.2	176.6 ± 4.9	89.8 ± 0.6	0.7 ± 0.0	20.8 ± 0.2	6.5 ± 0.1	-	-	-
BTS leaves	73.4 ± 0.9	14.0 ± 0.1	208.6 ± 2.6	78.0 ± 0.2	104.5 ± 2.1	0.9 ± 0.0	286.4 ± 4.7	141.5 ± 2.1	-	-	-
BTS root	476.7 ± 4.5	58.3 ± 1.5	374.1 ± 3.3	90.5 ± 1.4	69.0 ± 0.7	0.5 ± 0.0	35.3 ± 0.3	18.0 ± 0.2	-	-	-
Daphna leaves*	369.5 ± 1.2	19.0 ± 0.5	1209.6 ± 6.4	157.2 ± 3.0	873.7 ± 12.2	5.1 ± 0.1	2183.9 ± 6.6	550.3 ± 1.3	(7.1 ± 0.2)	-	22.2 ± 0.2
Daphna root*	445.7 ± 9.3	159.5 ± 0.6	812.0 ± 14.5	384.4 ± 2.5	141.6 ± 1.3	0.9 ± 0.0	13.8 ± 0.3	9.9 ± 0.1	-	-	-
Eva Maria leaves	562.1 ± 6.8	21.1 ± 1.5	1426.1 ± 16.7	120.2 ± 6.4	434.1 ± 2.4	2.9 ± 0.0	1850.5 ± 29.7	365.2 ± 4.8	17.8 ± 0.5	-	(11.2 ± 0.3)
Eva Maria root	600.3 ± 9.8	104.3 ± 1.8	553.2 ± 13.2	130.3 ± 1.6	155.7 ± 3.9	1.2 ± 0.0	14.5 ± 0.3	6.8 ± 0.2	-	-	-
Kleist leaves	326.0 ± 3.0	14.4 ± 0.3	967.2 ± 11.1	104.8 ± 1.4	447.1 ± 4.9	2.9 ± 0.0	2903.2 ± 35.2	459.2 ± 3.5	12.9 ± 0.4	-	53.7 ± 0.8
Kleist root	344.8 ± 4.7	58.6 ± 0.6	277.4 ± 5.6	69.7 ± 0.8	87.3 ± 1.6	0.6 ± 0.0	17.5 ± 0.0	6.3 ± 0.2	-	-	-
Lisanna leaves	97.8 ± 0.5	26.3 ± 0.5	119.0 ± 3.0	54.6 ± 0.5	85.9 ± 2.8	0.5 ± 0.0	628.8 ± 2.1	103.3 ± 0.3	-	-	-
Lisanna root	460.2 ± 5.6	75.8 ± 0.8	536.1 ± 3.4	147.0 ± 0.9	97.2 ± 0.5	0.7 ± 0.0	38.8 ± 0.4	23.1 ± 0.2	-	-	-
Marley leaves	44.4 ± 1.0	6.3 ± 0.2	154.5 ± 4.8	54.4 ± 0.3	73.9 ± 3.0	0.5 ± 0.0	531.0 ± 14.8	137.7 ± 3.9	-	-	-
Marley root	367.6 ± 5.5	46.7 ± 0.5	373.0 ± 6.8	85.2 ± 0.7	64.3 ± 0.6	0.4 ± 0.0	40.5 ± 0.6	26.7 ± 0.4	-	-	-
Rhinema leaves	372.3 ± 2.1	23.1 ± 0.5	1348.9 ± 4.6	166.7 ± 2.3	322.7 ± 3.9	2.2 ± 0.0	965.6 ± 1.4	394.5 ± 2.2	12.9 ± 0.1	-	(5.5 ± 0.1)
Rhinema root	401.0 ± 3.6	105.5 ± 4.3	301.0 ± 4.6	113.6 ± 3.6	79.8 ± 2.2	0.7 ± 0.0	16.4 ± 0.2	13.8 ± 0.3	-	-	-

Table 4 (continuation): *Quantitative data relating to sugar beet roots, leaves, sugar beet compartments, side streams, commercial products, and beetroot. Values below the limit of quantification are given in brackets. The standard deviation is also given. Data are based on the publication by Edelmann et al. (Edelmann et al. 2020b).*

	The value indicated is the mass fraction in mg·kg^{-1}										
	Betavulgaroside I (1)	**Betavulgaroside II (2)**	**Betavulgaroside III (3)**	**Betavulgaroside IV (4)**	**Betavulgaroside VIII (5)**	**Boussingoside A2 (6)**	**3-O-[β-D-gluco… (7)**	**Betavulgaroside V (8)**	**Chikusetsu saponin IVa (9)**	**Calenduloside E (10)**	**Ginsenoside R0 (11)**
Hannibal leaves	8.8 ± 0.4	8.2 ± 0.1	91.7 ± 3.3	177.7 ± 2.0	195.5 ± 11.5	1.2 ± 0.1	309.6 ± 8.3	52.6 ± 1.7	-	-	-
Hannibal stalk	85.8 ± 2.4	37.2 ± 0.4	161.0 ± 4.6	120.9 ± 0.9	213.6 ± 6.0	1.3 ± 0.0	95.6 ± 2.8	58.7 ± 1.7	-	-	-
Hannibal inner top	186.1 ± 3.5	49.2 ± 0.6	75.2 ± 1.7	38.1 ± 0.3	26.8 ± 0.7	0.1 ± 0.0	14.4 ± 0.3	9.6 ± 0.2	-	-	-
Hannibal inner middle	314.9 ± 8.6	137.8 ± 1.0	107.6 ± 0.7	71.8 ± 0.7	17.2 ± 0.1	(0.1 ± 0.0)	11.0 ± 0.0	4.4 ± 0.0	-	-	-
Hannibal inner bottom	195.4 ± 1.4	418.4 ± 2.9	59.7 ± 0.7	188.6 ± 1.1	7.6 ± 0.1	(0.1 ± 0.0)	4.7 ± 0.1	0.8 ± 0.0	-	-	-
Hannibal pod top	152.3 ± 5.6	147.5 ± 2.0	182.6 ± 5.1	280.0 ± 5.1	67.9 ± 2.4	0.5 ± 0.0	28.6 ± 1.0	22.4 ± 0.7	-	-	-
Hannibal pod middle	436.9 ± 8.6	323.4 ± 4.8	352.2 ± 4.0	364.8 ± 4.3	69.8 ± 1.4	0.5 ± 0.0	45.0 ± 0.2	24.9 ± 0.4	-	-	-
Hannibal pod bottom	721.0 ± 4.8	347.8 ± 5.4	513.5 ± 5.8	353.2 ± 6.9	94.0 ± 1.4	0.6 ± 0.0	45.1 ± 0.5	22.0 ± 0.3	-	-	-
Raw juice	3.3 ± 0.9	482.9 ± 5.9	2.9 ± 1.0	525.8 ± 6.5	-	-	-	-	-	-	-
Thick juice	0.3 ± 0.1	-	0.5 ± 0.1	23.2 ± 0.7	-	-	-	-	-	-	-
Molasse	-	-	2.2 ± 0.1	135.0 ± 1.7	-	-	(0.9 ± 0.1)	-	-	165.2 ± 1.7	-
Dried sugar beet pulp	3871.2 ± 56.8	767.9 ± 34.7	3028.3 ± 55.1	1187.1 ± 71.5	646.3 ± 13.5	68.7 ± 1.7	243.3 ± 3.4	118.6 ± 3.1	391.6 ± 10.7	-	-
Flume water	434.4 ± 7.4	133.2 ± 1.2	916.6 ± 12.5	371.9 ± 6.5	135.7 ± 1.8	2.0 ± 0.0	56.5 ± 0.6	5.6 ± 0.4	-	-	-
Process water	51.6 ± 0.9	-	98.6 ± 0.5	231.4 ± 1.4	32.3 ± 0.4	(1.0 ± 0.1)	(2.7 ± 0.1)	-	-	-	-
Sugar beet fiber	5246.4 ± 27.9	1113.6 ± 33.8	1006.5 ± 4.2	622.5 ± 11.9	188.4 ± 3.9	351.4 ± 8.4	329.3 ± 4.0	45.7 ± 0.9	2823.7 ± 19.9	943.9 ± 25.8	12.5 ± 0.9
Goldsaft	629.2 ± 10.8	29.3 ± 1.0	-	3.1 ± 0.9	-	78.8 ± 1.8	53.1 ± 1.5	-	592.9 ± 13.1	18.6 ± 0.9	-
Beetroot	205.4 ± 0.1	4.6 ± 0.1	442.3 ± 2.0	33.6 ± 0.2	2.7 ± 0.1	0.0 ± 0.0	65.3 ± 0.7	40.7 ± 0.3	-	-	-

* Obtained from freeze dried-material.

4.10 Saponins with a Unique Substituent

The diversity of the sugar beet saponins quantified in this work is not only due to the different glycosylation patterns but also due to the different substituents. The pathway of their formation from aglycones and sugars is not yet known. Glycosyltransferases most likely play an important role. Normally, they represent the final step in the synthesis of glycosylated natural compounds (Vogt and Jones 2000). This is probably not the case for many sugar beet saponins, since most of them contain substituents that might be formed through oxidation from sugars. This has already been proposed by Yoshikawa et al. (Yoshikawa et al. 1998). A possible precursor not mentioned by them could be 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), which was isolated during this work. The first isolation was carried out by Ridout et al. (Ridout et al. 1994). As shown in Figure 21, this substance could be a precursor of betavulgroside V (**8**).

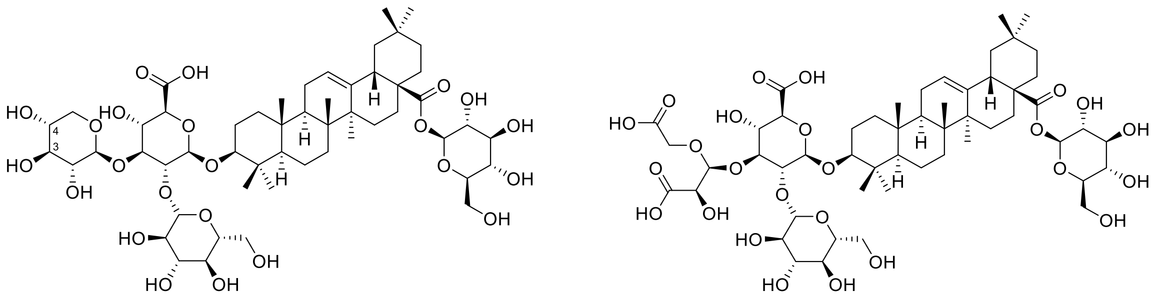


Figure 21: *Comparison of 3-O-[β-D-glucopyranosyl-(1→2)-(β-D-xylopyranosyl-(1→3))-β-D-glucuronopyranosyl]-28-O-β-D-glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid (left) with betavulgaroside V (right).*

The substituent in betavulgaroside V (**8**) might be formed through enzymatic oxidation of the terminal xylose unit that might be cleaved between carbon atoms 3 and 4 (the two carbon atoms on the far left in Figure 21). Further oxidation of the resulting substituent might explain the formation of other sugar beet saponins, as shown by comparison of betavulgaroside III (**3**) and betavulgaroside I (**1**) in Figure 22.

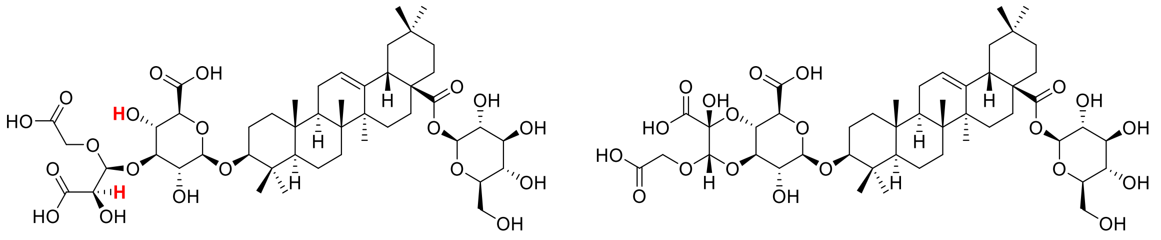


Figure 22: *Betavulgaroside III (left) and betavulgaroside I (right). The hydrogen atoms labelled in red indicate the major differences between these two substances.*

The removal of the two hydrogen atoms marked in red in Figure 22 indicates the possible formation of betavulgaroside I (**1**) out of betavulgaroside III (**3**). These two hypothetical reaction pathways might also explain the formation of all other sugar beet saponins with unique substituents and create a greater variety of structures.

4.11 Occurrence of the Isolated Saponins in other Plants

Due to their unique molecular structure, betavulgaroside II (**2**), IV (**4**), and VIII (**5**) were previously only known to occur in sugar beet (Yoshikawa et al. 1995; Yoshikawa et al. 1998). This work shows that they also occur in beetroot (Edelmann et al. 2020b). There is also a good chance that other plants contain these substances.

This is supported by the fact that betavulgaroside III is not only known from sugar beet (Yoshikawa et al. 1995) but also from *Achyranthes fauriei* (Zhu et al. 2008). Moreover, betavulgaroside V (**8**) is also known from *Achyranthes bidentata* (Hoshino et al. 2013). Both of these also occur in beetroot (Edelmann et al. 2020b).

Two sugar beet saponins are reported to occur in two other plants apart from sugar beet. The first is betavulgaroside I (**1**) which also occurs in *Basella rubra* L. (Murakami et al. 2001) and *Achyranthes bidentata* (Hoshino et al. 2013), and the second is ginsenoside R0 (**11**), which was found in Panacis Japonici Rhizoma (Chen et al. 2019) and *Panacis japonica* (Zheng et al. 2019). Of these two, only betavulgaroside I (**1**) was found in beetroot (Edelmann et al. 2020b). This might be because no leaves were analyzed for beetroot, since ginsenoside R0 (**11**) was only found within sugar beet leaves (Edelmann et al. 2020b).

3-*O*-[*β*-D-Glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**) was found in *Kochia scoparia* (L.) Schrad (Wen et al. 1995; Yoshikawa et al. 1997a; Yoshikawa et al. 1997d) and *Pisonia umbellifera* (Lavaud et al. 1996). It was found in beetroot for the first time in this work (Edelmann et al. 2020b).

One of the smallest saponins in this work, boussingoside A2 (**6**), was found in very few plants. These are *Salicornia bigelovii* Torr. (Wang et al. 2012b), *Salsola imbricata* (Hamed et al. 2011), *Pfaffia glomerata* (Nakamura et al. 2010), and *Boussingaultia baselloides* (Espada et al. 1990). This is surprising, as it consists of an aglycon and only two sugars. It is similar to chikusetsu saponin IVa (**9**), which was found in numerous plants.

One of the most widespread saponins in this work is chikusetsu saponin IVa (**9**). It was found in *Acanthopanax senticosus* (Li et al. 2007), *Achyrantes aspera* (Kunert et al. 2000), *Achyranthes bidentata* (Li et al. 2005), *Achyranthes fauriei* (Yoo et al. 2006), *Achyranthes japonica* (Zhang et al. 2019), American ginseng (Lin et al. 2019), *Aralia armata* (Hu et al. 1995), *Aralia mandshurica* (Sinitsyn et al. 2019), *Chenopodium quinoa* (Woldemichael and

Wink 2001), *Cynara cardunculus* L. (Shimizu et al. 1988), *Dolichos lablab* (Yokosuka et al. 2019), *Galium rivale* (de Rosa et al. 2000), *Gardenia jasminoides* (Wang et al. 2012a), *Ilex dumosa* (Pires et al. 1997), *Panax stipuleanatus* (Liang et al. 2010), *Swartzia simplex* (Borel et al. 1987), *Pfaffia iresinoides* (Nishimoto et al. 1987), *Pisonia umbellifera* (Lavaud et al. 1996), *Salicornia bigelovii* (Shan et al. 2015), *Schefflera sessiliflora* (Phat et al. 2015), and *Tetrapanax papyriferum* (Strigunov et al. 2004).

The second of the two most widespread saponins in this work is calenduloside E (**10**) which is a component of many different plants. These are *Acanthopanax henryi* (Han et al. 2016), *Acanthopanax senticosus* (Li et al. 2007), *Aralia armata* (Hu et al. 1995), *Aralia elata* (Yoshikawa et al. 1996a; Song et al. 2000), *Boussingaultia baselloides* (Espada et al. 1991), *Chenopodiaceae* (Yoshikawa et al. 1997a), *Chenopodium album* (Chakraborty et al. 2016), *Chenopodium quinoa* (Zhu et al. 2002), *Cussonia paniculata* (Dovgii et al. 2006), *Dizygotheca kerchoveana* (Melek et al. 2004), *Gymnema sylvestre* (Yoshikawa et al. 1997c), *Hedera colchica* (Mshvildadze et al. 2001), *Hedera nepalensis* (Kizu et al. 1985), *Ilex pubescens* (Cao et al. 2018), *Kochia scoparia* (Wen et al. 1995), *Ligulariopsis shichuana* (Wu et al. 2017), *Melanthera elliptica* (Tagousop et al. 2018), *Pisonia umbellifera* (Lavaud et al. 1996), *Salicornia bigelovii* (Shan et al. 2015), *Salicornia europaea* (Yin et al. 2012), and *Tetrapanax papyriferum* (Strigunov et al. 2004).

4.12 Further Known Properties of the Isolated Saponins

Many saponins exhibit noteworthy (biological) properties (Hostettmann and Marston 2005). However, no further characteristics of betavulgaroside I (**1**), V (**8**), VIII (**5**) and boussingoside A2 (**6**) were found in the literature since they were only found in very few plants (cf. Section 4.11). Betavulgaroside II (**2**), III (**3**), and IV (**4**), which were also only found in very few plants (cf. Section 4.11), displayed hypoglycemic activity in a bioassay (Yoshikawa et al. 1996b). 3-*O*-[*β*-D-Glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**) even showed an inhibitory effect on the increase of serum glucose in glucose-loaded rats (Yoshikawa et al. 1997d). Very little is known about the saponins mentioned so far. On the one hand, these saponins may show little biological activity. On the other hand, they are not very widespread and therefore not widely available.

Much more information was found about saponins that are commercially available. Ginsenoside R0 (**11**) shows inhibitory effects on the growth of B16F10 melanoma (Zheng et al. 2019), inhibition of pancreatic lipase (Yu et al. 2017), and numerous other effects (Hosono-Nishiyama et al. 2006; Shin et al. 2016; Yuan et al. 2018). Chikusetsu saponin IVa (**9**) is also known for several biological activities, such as hypoglycemic activity (Zhang et al. 2019), anti-obesity (Yin et al. 2018), anti-inflammatory (Wang et al. 2015), and neuroprotective effects (Duan et al. 2016). Furthermore, it also shows inhibitory effects on the growth of B16F10 melanoma (Zheng et al. 2019). Finally, calenduloside E (**10**) displays inhibitory effects on the growth of B16F10 melanoma (Zheng et al. 2019), acts as a topoisomerase inhibitor, and induces apoptosis in the MCF7 cell line (Chakraborty et al. 2016). Additionally, it influences hypoglycemic activity (Matsuda et al. 1998). Since these three saponins have several properties, this list does not include all of them.

Whether any of these properties might impair the possible use of saponins as food ingredients cannot be estimated and further research is necessary. The reported use of sugar beet pulp, which contains the second-highest quantities of sugar beet saponins, as cattle feed indicates that any negative effects are negligible (Anal 2017; Edelmann et al. 2020b).

5 Conclusions and Perspectives

Natural and sustainable food ingredients are becoming more and more important to consumers. Plant-based ingredients in particular are an interesting alternative not only to synthetic but also to animal products. Entirely vegetable-based products are not yet fully established, often due to their limited scope of application. In the context of vegetable emulsifiers, this work not only presents new extracts suitable for emulsification, but also provides new insights into their molecular composition, characteristics like taste and foam activity, and the distribution of saponins in sugar beet extract.

First of all, several plant extracts containing saponins were produced and tested to determine their foam activity. This was found to be a good indicator of their emulsifying ability. Approximate saponin amounts were determined from these extracts, and they were further characterized in terms of their techno-functionality by the Department of Food Physics and Meat Science at the University of Hohenheim. With some limitations, all extracts studied are suitable for use as food additives. Sugar beet extract was found to be a particularly promising extract and was subjected to further investigation. Although sugar beet root does not have a particularly high saponin content (862 mg/kg to 2,452 mg/kg), it is processed in large quantities. This makes further investigation of its taste and other properties particularly valuable.

The investigation made extensive use of taste dilution analysis, which was applied inversely during this work. Sugar beet root extract obtained was separated and the resulting fractions were analyzed to determine their taste and other properties, such as foam activity and saponin abundance. It was shown that saponin-containing fractions possess a slightly bitter taste, but the greatest proportion of foam activity. Consequently, sugar beet saponins occurring in relevant amounts (i.e. betavulgaroside I (**1**), betavulgaroside III (**3**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), and betavulgaroside V (**8**)) were isolated (using different techniques, such as SPE and HPLC as well as a newly developed large-scale foam fractionation system) or synthesized (betavulgaroside II (**2**) and betavulgaroside IV (**4**)) in order to investigate the individual substances more closely. In addition, three commercially available saponins (chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**)) found in sugar beet were purchased.

Each of the saponins was examined for off-tastes with the aid of a sensory panel. For all sugar beet saponins analyzed, only a slight bitter taste (> 1000 *µ*mol/kg) was detected, which is less than that of many of the bitter-tasting saponins known from the literature (cf. Table 3). In addition, the foam activity of the substances was measured and compared with other, commercially available saponins. The measurements were carried out using a newly developed system, which showed significant foam activities even for concentrations of 0.01 mg/mL and required a volume of only 2.0 mL for each measurement. The most important finding from these measurements is that the foam activity of monodesmosidic saponins is generally not higher but indeed somewhat lower than that of bidesmosidic saponins. This was the first time that foam activity data were obtained using a highly standardized measurement system.

The characterizations performed also included structure elucidations. These were carried out on both the isolated and the synthesized saponins as well as on the three commercially obtained sugar beet saponins. The molecular structure was mainly elucidated with NMR, whereby all protons could be completely assigned for the first time except in betavulgaroside V (**8**), calenduloside E (**10**), and ginsenoside R0 (**11**). The absolute structure of the corresponding sugars was confirmed for the first time using a recently published LC-MS/MS method. The stereogenic centers of the unusual substituents linked to the sugar molecules were determined on the basis of the literature.

The sugar beet saponins obtained were used for characterization and quantification. A completely new method for the quantification of eleven sugar beet saponins was developed. Sample preparation began by crushing the sugar beet material. Aliquots were given into homogenization tubes and mixed with solvent and an internal standard. After homogenization, the samples were filtrated and directly measured with the help of an LC-MS/MS system. The method developed was validated intensively, and several completely new results were obtained.

The amount of different sugar beet saponins in different sugar beet varieties (beets and leaves) was determined, and different compartments of one variety were analyzed. Furthermore, several side streams arising during sugar beet processing were investigated and sugar beet pulp was identified as the best source for large-scale extraction of sugar beet saponins (cf. Table 4). Beetroot and household sugar originating from sugar beet were also analyzed. Unlike previous methods, this method is able to determine the content of individual saponins in numerous matrices and advances research on sugar beet. It could facilitate further utilization of sugar beet saponins by searching for further, particularly suitable sugar beet varieties.

This could be done by selecting individual varieties with a particularly high saponin content. The higher the content, the more economical large-scale isolation would be. Moreover, the large-scale isolation of individual saponins would not be effective. The production of sugar beet saponin extracts can only be reasonably implemented on an industrial scale if saponin extracts consist of saponin mixtures and associated substances, as is the case with quillaja extract. The extraction of pure substances would be far too complex and far too expensive. This work has shown that all key substances (sugar beet saponins) have only an insignificant bitter taste. One possible next step could therefore be to develop and test large-scale extraction processes. It would be advantageous to determine the techno-functional properties and taste attributes of these extracts. If they contain substances with an off taste, they need to be removed or extraction methods need to be modified. In addition, toxicological studies should be carried out to exclude any risk to consumers. Ultimately, the consumer will decide whether sugar beet or other saponin extracts will establish themselves on the market.

Apart from their possible use as a food ingredient, this work provides numerous starting points for future research. Most promising is the further use of the quantification method for optimized breeding experiments. This would require further investigations of known (and ideally unknown) sugar beet saponins in biological tests and could simplify targeted breeding of plants with high contents of biologically active saponins. Finally, it could lead to more robust varieties against pests.

Furthermore, a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer could be used to determine the distribution of individual saponins in the plant matrix more precisely (cf. Figure 3). It is also possible to examine many other sugar beet varieties as well as other plants to determine the presence of saponins. Finally, it would be valuable to determine the influence of cultivation conditions.

To sum up, the number of saponin-containing extracts that can be used as food additives was increased significantly. Extracts were produced from oat bran, beetroot and sugar beet, and their emulsifying properties determined by measuring foam activity. Finally, sugar beet saponins were investigated in more detail and several new insights were obtained.

6 List of Figures

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8 Literature

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9 Publications

Peer-reviewed first author publications are provided in this section.

Section 9.1:

Edelmann, M., Dawid, C., Hochreiter, K., Ralla, T., Stark, T., Salminen, H., Weiss, J., Hofmann, T., Molecularization of Foam-Active Saponins from Sugar Beet Side Streams (*Beta vulgaris* ssp. *vulgaris* var. *altissima*). *Journal of Agricultural and Food Chemistry* 2020*, 68* (39), 10962-10974.

Section 9.2:

Edelmann, M., Dawid, C., Ralla, T., Stark, T., Salminen, H., Weiss, J., Hofmann, T., Fast and Sensitive LC-MS/MS Method for the Quantitation of Saponins in Various Sugar Beet Materials. *Journal of Agricultural and Food Chemistry* 2020*, 68* (50), 15027-15035.

9.1 Molecularization of Foam-Active Saponins from Sugar Beet Side Streams (*Beta vulgaris* ssp. *vulgaris* var. *altissima*)

Summary:

In this work, the applicability of sugar beet saponins as emulsifiers in foodstuffs was investigated on a molecular level. For this purpose, individual saponins were obtained and examined in more detail. Individual taste threshold values were determined, and foam activities measured using a novel small-scale foam activity measurement system. The foam activities obtained provide indications about their emulsifying ability. Sugar beet saponins were shown to possess both low bitter taste activity (thresholds > 1000 *µ*mol/l) and high foaming activity, which makes them particularly attractive. To supplement studies relevant to food applications, the structure of the individual compounds was elucidated. Furthermore, foam activity measurements were carried out on both sugar beet saponins and a wide variety of commercially available saponins. All results are relevant to the utilization of saponins from other plant materials as well as to the use of sugar beet saponins as food additives.

The individual contribution of Matthias Edelmann is specified below:

Matthias Edelmann refined the investigation concept, carried out the majority of the laboratory work, collected data, analyzed all the data, discussed the results, designed the figures, developed the story of the paper, wrote the first draft of the manuscript, and partially revised the paper.

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Molecularization of Foam-Active Saponins from Sugar Beet Side Streams (*Beta vulgaris* ssp. *vulgaris* var. *altissima*)

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ABSTRACT: This work focuses on the isolation and characterization of saponins with a very low bitter intensity originating from sustainable plant materials, in particular the sugar beet pulp by-product stream. Via a concise foam activity screening of saponin-containing materials, which gives indications for their emulsifying ability, sugar beet root extract was selected and examined for low bitter saponins by means of activity guided fractionation. Individual saponins were isolated from sugar beet pulp, which was identified as the most convenient sugar beet saponin source. Liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis and one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy led to the unequivocal identification of the major, slightly bitter tasting compounds as a series of eight saponins. The complete assignment of ^{1}H and ^{13}C NMR signals for several saponins was carried out for the first time. A small-scale foam activity assay was established and applied to a broad spectrum of the isolated and commercially available saponins. Additionally, orosensory recognition thresholds were determined. Not only high recognition thresholds were determined (thresholds >1000 μmol/L) but also fundamental information about the foaming behavior of mono- and bidesmosidic saponins was collected. The obtained results are relevant to the utilization of saponins from other plant materials or by-product streams and for the use of sugar beet saponins as food additives.

KEYWORDS: *sugar beet, taste, taste dilution analysis, saponin, foam activity*

■ INTRODUCTION

An increasing consumer interest in "natural" and sustainable food products motivates the food industry to develop products without synthetically derived food additives. In the group of emulsifiers, which are surface-active molecules that adsorb to interfaces of immiscible phases and stabilize these by reducing interfacial tension, several "natural" representatives are already established, such as amphiphilic proteins, phospholipids, and polysaccharides.[1,2] However, most of them have limitations in terms of techno-functional properties. For example, the emulsifying properties of proteins strongly depend on pH, temperature, and ionic strength, whereas phospholipids typically build up only a thin membrane, making the emulsions prone to coalescence during storage. Polysaccharides usually exhibit a low surface activity and, therefore, require a high emulsifier-to-oil ratio.[3−6] Another promising biopolymer-based emulsifier group comprises the group of amphiphilic saponins, which can be obtained from a variety of by-products of local western plants such as oat, asparagus, red beet, or sugar beet.[7−14]

Nowadays, saponin-enriched extracts obtained from oat, ginseng, yucca, or red beets are known to be promising emulsifiers,[13−16] but the food industry mainly uses one food-grade saponin extract (E999), which is obtained from the South American soapbark tree (*Quillaja saponaria* Molina) and is not sustainable as it is native to the Andes region in South America.[14] Also, pure saponins, such as glycyrrhizin, the licorice tasting key component in *Glycyrrhiza glabra*, are known to have a promising emulsifying activity.[17,18] Although saponins are promising surfactants for future applications in the food industry, the use of saponins, such as quillaja and licorice saponins, is still limited, as they often possess a bitter or licorice taste, which leads to consumer rejection.[7−10,18,19] Fortunately, the class of saponins comprises a huge variety of structures and offers enormous potential in the search for pleasant tasting saponins with techno-functional properties.[20] One potential plant-based, natural saponin source, which is available especially in Europe and contains large quantities of saponin-rich side streams, is sugar beet (*Beta vulgaris* L.).[21,22] The saponin content of the sugar beet root is around 0.2% (w/w), and they are known to be enriched in sugar beet side streams, such as washing water, accruing during sucrose production.[23,24] Although some sugar beet saponins have been already isolated (e.g., betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsu saponin IVa (**9**), and calenduloside E (**10**), Figure 1), nothing is known about their taste, foam, or emulsifying activity.[25−28]

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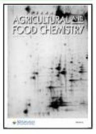

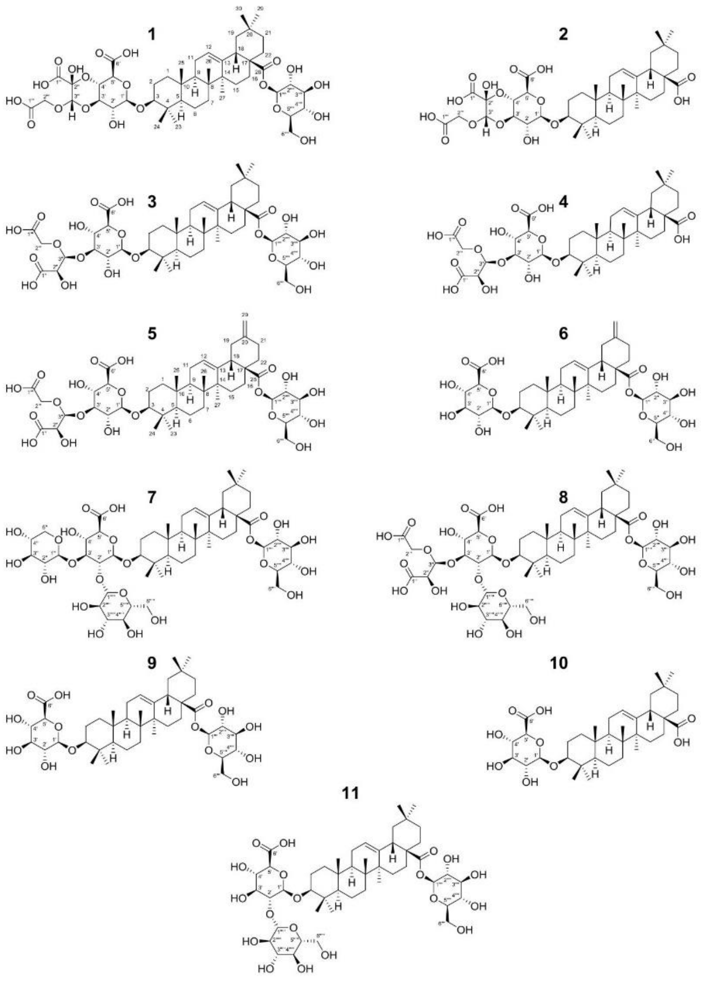

Figure 1. Isolated (**1**, **3**, **5**−**8**), synthesized (**2** and **4**), and commercially obtained (**9**−**11**) sugar beet saponins: betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**).

Traditional extraction processes established for the isolation of sugar beet saponins are not suitable for natural emulsifier isolation, especially for large-scale applications since all of them include extraction with methanol under reflux, which is known to partially produce saponins with methyl esters.[25−27] Due to the fact that the formation of stable foams is the most characteristic saponin property,[20] saponin enrichment by means of foaming seems to be a promising way to increase the concentrations from sugar beet and its by-products for technical applications. The foaming activity of saponins has been known for a long time and has also led to their name, which is derived from sapo, the Latin word for soap, which can produce foam in aqueous solution.[29] Foam activity measurements with saponin mixtures have already been described for more than 100 years.[30] Over time, various methods for the measurement of foam activity have been developed but none of them is particularly well standardized. It started with the shaking of saponin-containing solutions and ended with temperature-controlled gas stream piped devices.[30−34] Although research on the foam activity of saponins has been carried out for some time, and it is known that the addition of K_2HPO_4 helps us to standardize foam measurements,[35] there is hardly any literature on the foam activity of pure substances, including the latest measuring method by Böttcher et al.[36]

The objective of the present investigation was, therefore, to screen for (ideally) taste-inactive and at the same time foam-active saponins in sugar beet by application of a taste dilution (TD) approach,[37] which was already successfully used for the discovery of taste-active saponins from oat and asparagus.[7−10] In addition, a new versatile foam assay was developed and used to characterize the foam activity of saponin-enriched extracts, fractions, and pure reference compounds. Finally, taste thresholds of the most abundant saponins were obtained.

■ MATERIALS AND METHODS

Chemicals and Reagents. Ultrapure water was prepared with a Milli-Q Integral 3 system (Millipore, Molsheim, France). The following chemicals were obtained commercially: acetonitrile and methanol were of high-performance liquid chromatography (HPLC) grade (J.T. Baker, Deventer, Netherlands), acetonitrile used for liquid chromatography−mass spectrometry (LC−MS) analysis was of LC−MS grade (Honeywell, Seelze, Germany), ethyl acetate and ethanol (VWR, Ismaning, Germany), formic acid (98−100%), hydrochloric acid (37%), and monosodium phosphate monohydrate (Merck, Darmstadt, Germany), and liquid nitrogen (Westfalen, Münster, Germany). Quillaja extract "Andean QDP Ultra Organic" was obtained from Desert King, San Diego. Korean red ginseng high hydrostatic pressure extract was obtained from Dongwon F&B Co., Ltd., Seoul, South Korea. Acetonitrile-d_3, CD_3OD, D_2O, pyridine-d_5, anhydrous lithium iodide, anhydrous methanol, anhydrous pyridine, L-cysteine methyl ester hydrochloride, D-glucose, L-glucose, phenylethyl isothiocyanate, 2,6-lutidine, and D-xylose (Sigma-Aldrich, Seelze, Germany), D-glucuronic acid (Acros Organics, Geel, Belgium), calenduloside E (**10**) and timosaponin A-III (Carbosynth, Compton, U.K.), chikusetsu saponin IVa (**9**) and ginsenoside R0 (**11**) (PhytoLab, Vestenbergsgreuth, Germany), ginsenoside CK, ginsenoside Rc (**12**), ginsenoside Rd (**13**), ginsenoside Re (**14**), ginsenoside Rf, ginsenoside Rg1 (**15**), and ginsenoside Rg2 (**16**) (Extrasynthese, Lyon, France), aescin IA (**17**) and soyasaponin I (**18**) (PhytoLab), and protodioscin (**19**) (Sigma-Aldrich). Glycyrrhizin (**20**) was generously provided by Christian Schmid (Chair of Food Chemistry and Molecular Sensory Science, Technical University of Munich).[38] α-Solanine (**21**), diosgenin, hosenkoside A (**22**), trillin, and tubeimoside I (**23**) were supplied by PhytoLab. Finally, freeze-dried sugar beet root powder (*B. vulgaris* ssp. *vulgaris* var. *altissima*) cultivar Annemaria and sugar beet pulp were obtained from Pfeifer & Langen GmbH Co. KG (Köln, Germany).

Solvent Extraction of Freeze-Dried Sugar Beet Root (Extract A). An aliquot of freeze-dried sugar beet root powder (*B. vulgaris* ssp. *vulgaris* var. *altissima*) cultivar Annemaria (100 g) was extracted three times with a mixture of methanol/water (70:30, v/v; 500 mL) followed by three times with methanol (500 mL) for 30 min at room temperature. Methanol was removed from the combined extracts under vacuum at 40 °C, followed by lyophilization. The obtained extract A was used for taste dilution analysis (TDA) and kept at −25 °C until further use.

Separation of Extract A by Means of Medium Pressure Liquid Chromatography (MPLC). An aliquot (4.0 g) of extract A was dissolved in water (5.0 mL), filtered with syringe filters (Minisart NML Plus, 0.45 μm, Sartorius, Göttingen, Germany), and separated via MPLC using a self-packed polypropylene cartridge filled with the LiChroprep RP-18 material (150 mm × 40 mm i.d., 25−40 μm, Merck). The separation was performed on a Sepacore chromatography system (Buechi, Flawil, Switzerland) consisting of a manual injection port (20 mL loop), two C-605 pumps, a C-620 control unit, a C-635 UV/vis detector, and a C-660 fraction collector. An aliquot (1 mL/min) of the eluent was split into an evaporative light scattering detector (ELSD) (Sedex LT 80, Sedere, Alfortville, France) before the eluent was directed into the fraction collector. Water (A) and methanol (B) were used as solvents with a flow rate of 40 mL/min, and the following gradient was used: 0 min, 100% A kept constant for 5 min, and subsequently increased to 100% B within 20 min, kept constant for 15 min. In sum, 10 fractions (A-I to A-X) were collected, the organic solvent was removed under vacuum at 40 °C, and the fractions were freeze-dried. The obtained fractions were used for taste dilution analysis, foam activity measurements, and ultra-performance liquid chromatography−time-of-flight mass spectrometry (UPLC−ToF-MS) screenings (Figure 2).

Solvent Extraction of Sugar Beet Pulp (Extract B). An aliquot of sugar beet pulp (500 g) was extracted with a mixture of methanol/water (70:30; v/v; 2.5 L) for 30 min. The organic solvent was removed under vacuum at 40 °C, followed by lyophilization. The obtained extract B was kept at −25 °C until further use.

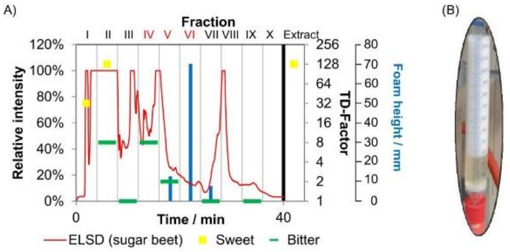

Figure 2. (A) Taste dilution analysis as well as foam activity measurements of the MPLC fractions A-I to A-X from sugar beet extract and sugar beet extract measured with foam assay method A. Saponin-containing fractions are labeled in red. Taste dilution (TD) factors for sweet are labeled in yellow, while the bitter taste is highlighted in green. (B) Corresponding foam tube, used for foam assay method A, with an inner diameter of 18 mm.

Fractionation of Extract B by Means of Solid-Phase Extraction (SPE). An amount of 5.0 g from extract B was dissolved in water, membrane filtered (Minisart NML Plus, Sartorius), and fractionated on a Chromabond C18 ec cartridge (10 g, Macherey-Nagel, Düren, Germany), which was conditioned with methanol (100 mL), followed by water (60 mL). The extract was separated into four fractions by elution with water (80 mL, fraction B-I), followed by methanol/water (80 mL, 30:70; v/v, fraction B-II), methanol/water (80 mL, 70:30; v/v, fraction B-III), and methanol (80 mL, fraction B-IV). Methanol was removed from all fractions (B-I to B-IV) under vacuum at 40 °C, and they were lyophilized and stored at −25 °C until further use.

Subfractionation of Fraction B-III. Aliquots of the lyophilized fraction B-III were dissolved in 30% acetonitrile (70 mg/5 mL) with ultrasonication. After membrane filtration (Minisart RC, 0.45 μm, Sartorius), the solution was further separated into seven fractions (B-III-1 to B-III-7; see Figure S1, Supporting Information) by reversed phase (RP)-HPLC using a Nucleodur C_{18} Pyramid column (250 × 21 mm^2, 5 μm, Macherey-Nagel) equipped with a guard column of the same type. Chromatography was performed using 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B with a flow rate of 20 mL/min. The effluent was monitored with an ELSD (Sedex LT 85, Sedere) at gain 8. The gradient was kept constant for 33 min with 34% B, increased to 100% B within 2 min, kept constant for 4 min, and readjusted to 34% B within 2 min. Finally, the gradient was kept constant for 4 min.

Isolation of Boussingoside A2 (6) and 3-*O*-[*β*-D-Glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic Acid (7) from Fraction B-III-2. Aliquots of the lyophilized fraction B-III-2 were fractionated into five subfractions (B-III-2-a to B-III-2-e; see Figure S1, Supporting Information) with a semi-preparative RP-HPLC and a Nucleodur C_{18} Pyramid column equipped with a guard column of the same type (250 × 10 mm^2, 5 μm, Macherey-Nagel). Chromatography was performed using 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B with a flow rate of 4.8 mL/min. The gradient was kept constant for 34 min with 31% B, increased to 100% B within 2 min, kept constant for 4 min, and readjusted to 31% B within 2 min. Boussingoside A2 (**6**) included in fraction B-III-2-b was further purified. Therefore, the gradient was kept constant for 32 min with 31% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 31% B within 2 min, and finally kept constant for 4 min. 3-*O*-[*β*-D-Glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**) included in fraction B-III-2-d was repurified: the gradient was kept constant for 36 min with 31% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 31% B within 2 min, and finally kept constant for 4 min.

Boussingoside A2 (**6**), Figure 1. LC−MS (negative electrospray ionization (ESI$^-$)): *m/z* 777.9 [M − H]$^-$. Liquid chromatography−tandem mass spectrometry (LC−MS/MS) [declustering potential (DP), −150 V; collision energy (CE), −72 V]: *m/z* 615.4 [M − Glc − H]$^-$ (100%), 597.3 [M − Glc − H_2O − H]$^-$ (10%), 553.3 [M − Glc − H_2O − CO_2 − H]$^-$ (88%), 439.2 [M − Glc − GlcA − H]$^-$ (29%). LC−ToF-MS: *m/z* 777.4072 (measured); *m/z* 777.4061 (calcd for [$C_{41}H_{61}O_{14}$]$^-$). Collision cross-sectional area (CCS): Å^2 181.6, 222.9, 282.7. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data are given in Tables S1 and S2 (Supporting Information).

3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), Figure 1. LC−MS (ESI$^-$): *m/z* 1087.9 [M − H]$^-$. LC−MS/MS [DP −285 V; CE −96 V]: *m/z* 925.4 [M − Glc − H]$^-$ (100%), 731.3 [M − Glc − Xyl − CO_2 − H_2O − H]$^-$ (59%), 569.3 [M − Glc − Glc − Xyl − CO_2 − H_2O − H]$^-$ (42%), 455.2 [M − Glc − Glc − GlcA − Xyl − H]$^-$ (57%). LC−ToF-MS: *m/z* 1087.5336 (measured); *m/z* 1087.5325 (calcd for [$C_{53}H_{83}O_{23}$]$^-$). CCS: Å^2 212.4, 272.8, 367.1. ^{1}H and ^{13}C NMR data are given in Tables S3 and S4 (Supporting Information).

Isolation of Betavulgaroside V (8) and Betavulgaroside VIII (5) from Fraction B-III-4. Fraction B-III-4 contained betavulgaroside V (**8**) as well as betavulgaroside VIII (**5**). Aliquots of the lyophilized fraction B-III-4 were prepared and fractionated into four fractions (B-III-4-a to B-III-4-d; see Figure S1, Supporting Information) with semipreparative RP-HPLC and a Nucleodur C_{18} Pyramid column equipped with a guard column of the same type (250 × 10 mm^2, 5 *μ*m, Macherey-Nagel). Chromatography was performed using 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B with a flow rate of 4.8 mL/min. The gradient was kept constant for 34 min with 32% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 32% B within 2 min, and finally kept constant for 4 min. Betavulgaroside VIII (**5**) coeluted in fraction B-III-4-b and was further purified: the gradient was kept constant for 40 min with 31% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 31% B within 2 min, and finally kept constant for 4 min. Betavulgaroside V (**8**), included in fraction B-III-4-c, was repurified with the following gradient: kept constant for 55 min with 30% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 30% B within 2 min, and finally kept constant for 4 min. The LC−MS/MS fragment abbreviations Diox = dioxolane and Act = acetal for the characterization of the isolated molecules were adopted from Mikołajczyk-Bator et al.[39]

Betavulgaroside V (**8**), Figure 1. LC−MS (ESI$^-$): *m/z* 1117.6 [M − H]$^-$. LC−MS/MS [DP −235 V; CE −90 V]: *m/z* 997.4 [M − $C_3H_4O_5$ − H]$^-$ (93%), 955.4 [M − Act − H]$^-$ and [M − Glc − H]$^-$ (100%), 793.4 [M − Act − Glc − H]$^-$ and [M − Glc − Glc − H]$^-$ (8%), 731.4 [M − Act − Glc − CO_2 − H_2O − H]$^-$ and [M − Glc − Glc − CO_2 − H_2O − H]$^-$ (2%). LC−ToF-MS: *m/z* 1117.5071 (measured); *m/z* 1117.5067 (calcd for [$C_{53}H_{81}O_{25}$]$^-$). CCS: Å^2 209.8, 249.5, 260.9. Obtained NMR data were not sufficient for a complete assignment due to a low amount of substance. However, ^{1}H and ^{13}C NMR as well as heteronuclear single quantum coherence (HSQC) data were in line with the literature.[28]

Betavulgaroside VIII (**5**), Figure 1. LC−MS (ESI$^-$): *m/z* 939.6 [M − H]$^-$. LC−MS/MS [DP −225 V; CE −82 V]: *m/z* 819.2 [M − $C_3H_4O_5$ − H]$^-$ (100%), 777.3 [M − Act − H]$^-$ and [M − Glc − H]$^-$ (39%), 595.4 [M − $C_3H_4O_5$ − Glc − CO_2 − H_2O − H]$^-$ (4%). LC−ToF-MS: *m/z* 939.4233 (measured); *m/z* 939.4226 (calcd for [$C_{46}H_{67}O_{20}$]$^-$). CCS: Å^2 195.8, 254.2, 314.5. ^{1}H and ^{13}C NMR data are given in Tables S1 and S2 (Supporting Information).

Isolation of Betavulgaroside I (1) and Betavulgaroside III (3) from Fraction B-III-6. Fraction B-III-6, which contained betavulgaroside I (**1**) as well as betavulgaroside III (**3**), was further separated into four fractions (B-III-6-a to B-III-6-d; see Figure S1, Supporting Information). Using a semipreparative RP-HPLC approach and a Nucleodur C_{18} Pyramid column equipped with a guard column of the same type (250 × 10 mm^2, 5 *μ*m, Macherey-Nagel), chromatography was performed using 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B with a flow rate of 4.8 mL/min. The gradient was kept constant for 30 min with 35% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 35% B within 2 min, and finally kept constant for 4 min. Betavulgaroside III (**3**) was included within fraction B-III-6-b that was further purified: the gradient was kept constant for 26 min with 37% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 37% B within 2 min, and finally kept constant for 4 min. Betavulgaroside I (**1**) coeluted within fraction B-III-6-c and was further purified via the following gradient: kept constant for 17 min with 39% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 39% B within 2 min, and finally kept constant for 4 min.

Betavulgaroside I (**1**), Figure 1. LC−MS (ESI$^-$): *m/z* 953.6 [M − H]$^-$. LC−MS/MS [DP −135 V; CE −38 V]: *m/z* 953.6 [M − H]$^-$ (100%), 909.4 [M − CO_2 − H]$^-$ (5%), 793.6 [M − Diox − H]$^-$ (8%), 631.4 [M − Diox − Glc − H]$^-$ (3%), 569.3 [M − Diox − Glc − CO_2 − H_2O − H]$^-$ (44%), 455.4 [M − Diox − Glc − GlcA − H]$^-$ (1%). LC−ToF-MS: *m/z* 953.4389 (measured); *m/z* 953.4382 (calcd for [$C_{47}H_{69}O_{20}$]$^-$). CCS: Å^2 198.4, 239.3, 330.3. ^{1}H and ^{13}C NMR data are given in Tables S5 and S6 (Supporting Information).

Betavulgaroside III (**3**), Figure 1. LC−MS (ESI$^-$): *m/z* 955.5 [M − H]$^-$. LC−MS/MS [DP −190 V; CE −90 V]: *m/z* 835.3 [M − $C_3H_4O_5$ − H]$^-$ (100%), 793.3 [M − Act − H]$^-$ and [M − Glc − H]$^-$ (60%), 673.3 [M − $C_3H_4O_5$ − Glc − H]$^-$ (6%), 569.3 [M − Act − Glc − CO_2 − H_2O − H]$^-$ (8%), 631.3 [M − Act − Glc − H]$^-$ (7%), 455.3 [M − Act − Glc − GlcA − H]$^-$ (6%). LC−ToF-MS: *m/z* 955.4543 (measured); *m/z* 955.4539 (calcd for [$C_{47}H_{71}O_{20}$]$^-$). CCS: Å^2 198.3, 241.6, 330.0. ^{1}H and ^{13}C NMR data are given in Tables S5 and S6 (Supporting Information).

Synthesis of Betavulgaroside II (2) and Betavulgaroside IV (4) from Fraction B-III-6. The synthesis of betavulgaroside II (**2**) and betavulgaroside IV (**4**) was performed with slight modifications according to a protocol reported in the literature for the selective cleavage of ester-type glycoside linkages (Figure S2, Supporting Information).[40] Thereby, fraction B-III-6, which contained a mixture of compounds **1** and **3**, was used as the starting material and dried over silica gel in advance. Fraction B-III-6 (50 mg) and anhydrous lithium iodide (30 mg) were placed into a screw cap and 2,6-lutidine (2 mL), and anhydrous methanol (1 mL) were added. The mixture was heated to 135 °C while stirring for 16 h, and after cooling to room temperature, the solvents were removed in a stream of nitrogen. The remaining residue was dissolved in a mixture of acetonitrile/water (1:1; v/v) and filtered with syringe filters (Minisart RC, 0.45 *μ*m, Sartorius). The product was separated from the mixture by RP-HPLC using a HyperClone ODS (C_{18}) column (5 *μ*m, 250 × 21 mm^2, Phenomenex, Torrance) equipped with a guard column of the same type. Chromatography was performed using 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B with a flow rate of 20 mL/min. The effluent was monitored using an ELSD (Sedex LT 85, Sedere) at gain 8. The gradient was kept constant for 5 min with 100% A, increased to 50% B within 2 min, further increased to 77% B within 8 min, increased to 100% B within 1 min, kept constant for 2 min and readjusted to 100% A within 2 min, and finally kept constant for 4 min. The obtained product was further separated by means of semipreparative RP-HPLC on a Nucleodur C_{18} Pyramid column equipped with a guard column of the same type (250 × 10 mm^2, 5 *μ*m, Macherey-Nagel). Chromatography was performed using 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B with a flow rate of 4.8 mL/min. The effluent was monitored using an ELSD (Sedex LT 85, Sedere) at gain 8. The gradient was kept constant for 28 min with 50% B, increased to 100% B within 2 min, kept constant for 4 min and readjusted to 50% B within 2 min, and finally kept constant for 4 min. The isolated compound betavulgaroside II (**2**) was repurified via the following gradient: kept constant for 23 min with 50% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 50% B within 2 min, and finally kept constant for 4 min. Betavulgaroside IV (**4**) was also repurified. Therefore, the gradient was kept constant for

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21 min with 50% B, increased to 100% B within 2 min, kept constant for 4 min, readjusted to 50% B within 2 min, and finally kept constant for 4 min. Different HPLC solvents (1% formic acid as solvent A and acetonitrile containing 1% formic acid as solvent B) were used for the purification of compounds **2** and **4**.

Betavulgaroside II (**2**), Figure 1. LC−MS (ESI$^-$): *m/z* 791.2 [M − H]$^-$. LC−MS/MS [DP −95 V; CE −74 V]: *m/z* 747.3 [M − CO_2 − H]$^-$ (51%), 631.3 [M − Diox − H]$^-$ (100%), 455.3 [M − Diox − GlcA − H]$^-$ (14%). LC−ToF-MS: *m/z* 791.3859 (measured); *m/z* 791.3854 (calcd for [$C_{41}H_{49}O_{15}$]$^-$). CCS: Å^2 306.3. ^{1}H and ^{13}C NMR data are given in Tables S7 and S8 (Supporting Information).

Betavulgaroside IV (**4**), Figure 1. LC−MS (ESI$^-$): *m/z* 793.4 [M − H]$^-$. LC−MS/MS [DP −120 V; CE −86 V]: *m/z* 631.3 [M − Act − H]$^-$ (60%), 455.3 [M − Act − GlcA − H]$^-$ (6%). LC−ToF-MS: *m/z* 793.4020 (measured); *m/z* 793.4011 (calcd for [$C_{41}H_{51}O_{15}$]$^-$). CCS: Å^2 309.0. ^{1}H and ^{13}C NMR data are given in Tables S7 and S8 (Supporting Information).

Structure Elucidation of the Trace Compounds Chikusetsu Saponin IVa (9), Calenduloside E (10), and Ginsenoside R0 (11). Indications for the occurrence of chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**) within the sugar beet material were found with the help of UPLC−ToF-MS screenings and comparison of the retention times as well as CCS values when analyzing fractions A-I to A-X.

Chikusetsu saponin IVa (**9**), Figure 1. LC−MS (ESI$^-$): *m/z* 793.5 [M − H]$^-$. LC−MS/MS [DP −275 V; CE −80 V]: *m/z* 631.3 [M − Glc − H]$^-$ (95%), 569.2 [M − Glc − CO_2 − H_2O − H]$^-$ (100%), 455.3 [M − Glc − GlcA − H]$^-$ (22%). LC−ToF-MS: *m/z* 793.4392 (measured); *m/z* 793.4374 (calcd for [$C_{42}H_{65}O_{14}$]$^-$). CCS: Å^2 183.5, 220.8, 287.0. ^{1}H and ^{13}C NMR data are given in Tables S9 and S10 (Supporting Information).

Calenduloside E (**10**), Figure 1. LC−MS (ESI$^-$): *m/z* 631.6 [M − H]$^-$. LC−MS/MS [DP −195 V; CE −106 V]: *m/z* 455.3 [M − GlcA − H]$^-$ (100%). LC−ToF-MS: *m/z* 631.3851 (measured); *m/z* 631.3846 (calcd for [$C_{36}H_{55}O_9$]$^-$). CCS: Å^2 271.5. ^{1}H and ^{13}C NMR data are given in Tables S9 and S10 (Supporting Information).

Ginsenoside R0 (**11**), Figure 1. LC−MS (ESI$^-$): *m/z* 955.4 [M − H]$^-$. LC−MS/MS [DP −275 V; CE −86 V]: *m/z* 793.3 [M − Glc − H]$^-$ (97%), 731.3 [M − Glc − CO_2 − H_2O − H]$^-$ (100%), 631.3 [M − Glc − Glc − H]$^-$ (43%), 569.3 [M − Glc − Glc − CO_2 − H_2O − H]$^-$ (96%), 455.2 [M − Glc − Glc − GlcA − H]$^-$ (25%). LC−ToF-MS: *m/z* 955.4911 (measured); *m/z* 955.4903 (calcd for [$C_{48}H_{75}O_{19}$]$^-$). CCS: Å^2 197.5, 249.5, 324.1. ^{1}H and ^{13}C NMR data are given in Tables S3 and S4 (Supporting Information).

Identification of the Absolute Configuration of the Corresponding Monosaccharides. The absolute configuration of the corresponding monosaccharides was determined after acidic hydrolysis and derivatization, according to a protocol reported in the literature.[38] A solution of each saponin (**1−11**, 50 nmol, dissolved in CD_3OD or 80% CD_3CN) was dried in a stream of nitrogen. Afterward, HCl (1 mL, 2 M) was added and the mixture was shaken for 1 h at 100 °C with 300 rpm (Thermo-shaker PHMT-PSC24N, Grant Instruments, Cambridge, U.K.). Subsequently, the mixture was dried in a stream of nitrogen at room temperature. The residue was suspended in H_2O (750 μL) and extracted twice with ethyl acetate (750 μL). Next, the H_2O layer was evaporated to dryness at 60 °C under reduced pressure (Concentrator 5301, Eppendorf, Hamburg, Germany) to obtain the monosaccharide residue. Each residue was treated with a solution of L-cysteine methyl ester hydrochloride in anhydrous pyridine (50 μL; 2 mg/mL), shaken for 1 h at 60 °C before phenylethyl isothiocyanate (5 μL) was added, continuously shaken for 1 h at 60 °C, and dried under a stream of nitrogen. The residue was dissolved in a mixture of acetonitrile/water (1 mL, 1:1; v/v), filtrated with syringe filters (Minisart RC, 0.45 μm, Sartorius), and analyzed by means of UHPLC−MS/MS using a Kinetex F5 column (100 × 2.1 mm^2, 100 Å, 1.7 μm, Phenomenex, Aschaffenburg, Germany). Chromatography was performed using a flow rate of 0.4 mL/min and an injection volume of 1 μL. The mobile phase consisted of 1% formic acid (A) and acetonitrile with 1% formic acid (B), and the following gradient was applied: 0 min, 5% B; 2 min 5% B; 5 min, 20% B; 26 min, 25% B; 27 min, 100% B; 30 min, 100% B; 31 min, 5% B; 35 min, 5% B. Instrument settings were optimized with authentic samples (D-xylose, D-glucose, and L-glucose; D-glucuronic acid) treated the same way as described above. The derivatized monosaccharides were analyzed using the MRM transitions Q1/Q3 of *m/z* 461.0/298.1 (DP = 86 V, CE = 17 V, CXP = 6 V) for D-glucose, Q1/Q3 of *m/z* 461.1/298.2 (DP = 71 V, CE = 17 V, CXP = 6 V) for L-glucose, Q1/Q3 of *m/z* 475.0/312.1 (DP = 61 V, CE = 19 V, CXP = 8 V) for D-glucuronic acid, and Q1/Q3 of *m/z* 431.0/268.0 (DP = 76 V, CE = 17 V, CXP = 12 V) for D-xylose. The following retention times were observed for the corresponding sugar derivatives: D-glucose 11.11 min, D-glucuronic acid 11.27 min, L-glucose 11.68 min, and D-xylose 12.09 min.

Foaming Activity Measurements. Two different foaming activity measurement methods (method A and method B) containing two different foam tube versions were used during this work. All foam activity measurements were performed with custom-made foam tubes obtained from Glasbläserei Bahr (Manching, Germany). Method A with a foam tube inner diameter of 18 mm (Figure 2B) and method B with a foam tube inner diameter of 10 mm (Figure 3B). Both had an integrated glass filter disc (porosity 4, nominal max. pore size 10−16 μm). Foam height can be ascertained with the help of the annealed scale. All tubes were connected to a variable area flowmeter type FLQ-BSSS-CK-M (Vögtlin Instruments, Aesch, Switzerland), which are fed with nitrogen (1.0 bar overpressure) by a pressure regulator (ERC, Riemerling, Germany). Method A was used with a sample volume of 10 mL and a nitrogen flow of 5 mL/min. Water was used as a solvent, and samples were adjusted to a pH of 6.0 with aqueous formic acid. Method B, which is the final method of this work, used 2.0 mL of sodium phosphate buffer (10 mmol/L, pH 7.0) as the solvent for the foam-active substances or mixtures. Phosphate buffer was prepared by dissolving monosodium phosphate monohydrate in water and adjusting the pH with sodium hydroxide solution to 7.0. A nitrogen flow of 3 mL/min was used for method B. Furthermore, all solutions were filtered with syringe filters (Minisart RC15, 0.45 μm, Sartorius) prior to the measurements. After each measurement, the foam tubes were cleaned with water and methanol.

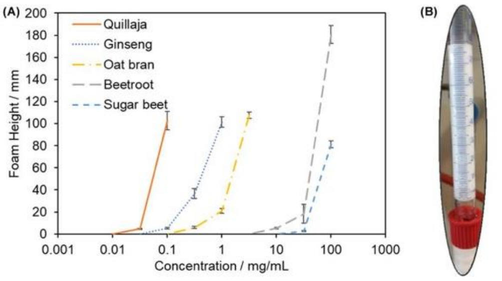

Figure 3. (A) Foam activity of different extracts prepared via foam assay method B. (B) Foam tube, used for foam assay method B, with an inner diameter of 10 mm.

Foam Activity of Different Plant Extracts. The foam activity of five different plant extracts was comparatively investigated using method B. While extracts from quillaja and ginseng were commercially purchased, oat bran as well as beetroot extract were prepared according to the literature.[13,14] Different concentrations of the extracts (0.010, 0.032, 0.10, 0.32, 1.0, 3.2, 10, 32, and 100 mg/mL) were prepared and measured in triplicate using foam activity measurement method B (Figure 3A, left).

Foam Activity of Fractions A-I to A-X. Fractions A-I to A-X were dissolved in water according to their natural concentration in the extract (summation of concentrations of fractions A-I to A-X, corresponding to 1.0 g/10 mL of the extract) (Figure 4). The measurements were carried out in triplicate using foam activity measurement method A (Figure 2A, left).

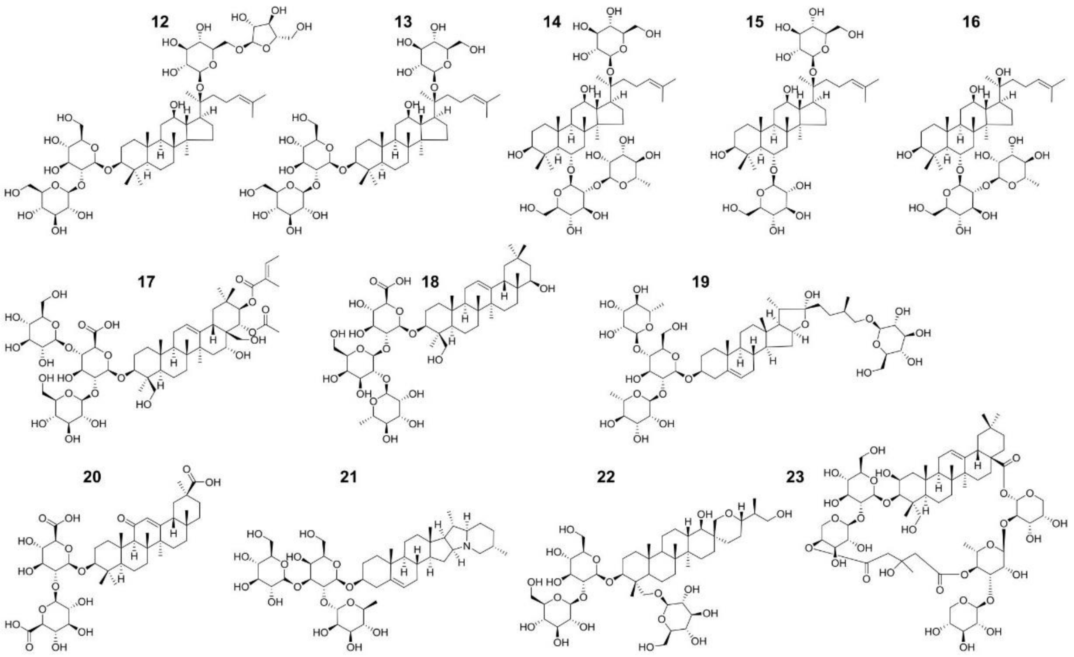

Figure 4. Commercially obtained saponins used for the foam activity measurements: ginsenoside Rc (**12**), ginsenoside Rd (**13**), ginsenoside Re (**14**), ginsenoside Rg1 (**15**), ginsenoside Rg2 (**16**), aescin IA (**17**), soyasaponin I (**18**), protodioscin (**19**), glycyrrhizic acid (**20**), α-solanine (**21**), hosenkoside A (**22**), and tubeimoside I (**23**).

Foam Activity Measurements of Saponins (1–23, Figures 1 and 4) by Means of Foam Assay Method B. Different concentrations (0.0032, 0.010, 0.032, 0.10, and 0.32 mg/mL) of these saponins were prepared and measured in triplicate (Figure 5).

Sensory Analysis. Sensory evaluations were performed in sensory booths at 22–25 °C by an experienced panel of 13 volunteers (six women and seven men, ages 23–32), who gave informed consent to participate in the sensory tests and had no history of known taste disorders. Nose clips were used to avoid cross-modal interactions with odorants. Light in the sensory booth was adjusted to red for masking of visual differences between the samples. All samples were adjusted to a pH of 6.0 using trace amounts of formic acid. The sip-and-spit method was used for every evaluation, which means that the test materials were not swallowed but expectorated.[41,42]

Taste Dilution Analysis (TDA). To determine the intrinsic taste quality of extract A and fractions A-I to A-X, a TDA was performed.[37] Therefore, the fractions A-I to A-X were dissolved according to their "natural" concentrations in Evian water and the pH was adjusted to 6.0 with diluted formic acid. Less polar fractions were solved in Evian water with the addition of 2% ethanol, and the pH was adjusted to 6.0 with formic acid. Each fraction and extract A were diluted 1:1 over seven steps with Evian water (pH 6.0) and then presented randomly in order of ascending concentrations to the panelists using a three-alternative forced-choice test (3-AFC). As previously reported, the dilution at which a taste difference between the diluted fractions or extract and the blanks (Evian water) could just be detected was defined as the taste dilution (TD) factor.[37] TD factors for each fraction were averaged (arithmetic mean).

Determination of Human Taste Recognition Thresholds. Human taste recognition thresholds of the saponins **1**–**7** and **9**–**11** were determined by means of two-alternative forced-choice tests that were carried out as a "half-tongue" test. Ginsenoside Rg1 (**15**) and ginsenoside Rc (**12**) were used as a control, dissolved in Evian water (pH 6.0), diluted 1:1 over seven steps (Evian water, pH 6.0), and provided to the panelists in ascending concentrations. The highest concentration for every saponin was 1000 μmol/L. Ethanol (2%) was added for betavulgaroside II (**2**), IV (**4**), VIII (**5**), and calenduloside E (**10**). The geometric mean of the first dilution at which a taste difference between the diluted saponins and the blank (Evian water) could just be detected and the last incorrect concentration was calculated and taken as the individual recognition threshold. Subsequently, the geometric mean of all individual thresholds was calculated as described by Dawid et al. and Lawless et al.[8,43] In addition, significance tests at probability levels of 5% were carried out for sugar beet saponins according to Lawless et al.[43]

High-Performance Liquid Chromatography (HPLC). Preparative and semipreparative separations were carried out on an HPLC apparatus (Jasco, Groß-Umstadt, Germany) consisting of two PU-2087 Plus pumps combined with an AS-2055 Plus autosampler and an MD-2010 Plus photodiode array detector. The system was equipped with a Degasys DG-1310 degasser (Uniflows, Tokyo, Japan), a 7725i injector (Rheodyne, Bensheim, Germany), and a Sedex LT-ELSD detector model 85 (Sedere). Chrompass 1.9 (Jasco) was used for data acquisition.

UPLC–ToF-MS. High-resolution mass spectra were measured on a Synapt G2-S HDMS coupled to an Acquity UPLC Core system as well as a Vion IMS QTof coupled to an Acquity UPLC I-Class System (Waters, Milford, MA). Both systems were operated in the negative electrospray mode and lock mass corrected using leucine enkephalin (Tyr-Gly-Gly-Phe-Leu, m/z 554.2615, $[M - H]^-$) as the reference. All chromatographic separations were performed with BEH C_{18} columns at 45 °C (2.1×150 mm^2, 1.7 μm, Waters). Chromatography was performed with a flow rate of 0.4 mL/min, and the following gradient was used. Starting with a mixture (95/5, v/v) of aqueous formic acid (0.1% in water) and acetonitrile containing 0.1% formic acid, the acetonitrile content was increased to 100% within 4 min, kept constant for 0.5 min, readjusted to 5% within 0.2 min, and finally kept constant for 0.3 min. MassLynx 4.1 (Waters) was used for

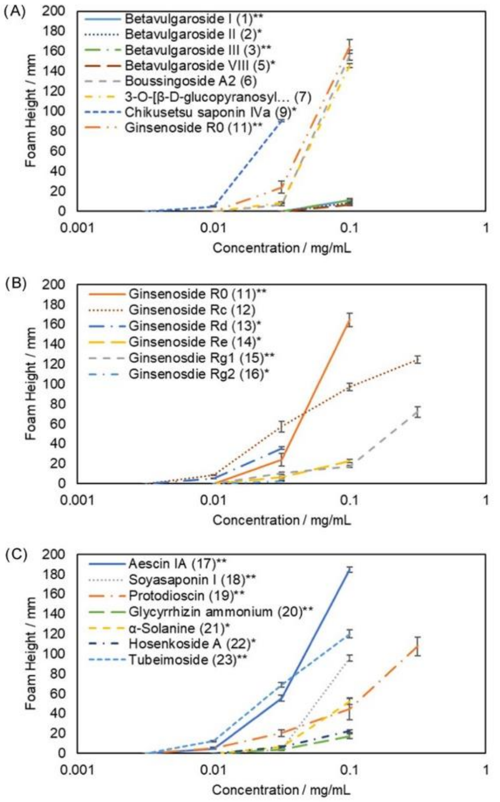

Figure 5. Foam activity measurements with foam assay method B of all suitable saponins (**1−3**, **5−7**, **9**, **11−23**). Divided in saponins from (A) sugar beet, (B) ginseng, and (C) other plants. *Measurement points within the solubility. **At higher concentrations, the liquid completely turns into foam.

operating the Synapt G2-S system with the following parameters: capillary voltage −2.5 kV, sampling cone voltage (−30 V), source temperature (150 °C), cone gas flow (50 L/h), desolvation gas flow (850 L/h), and desolvation temperature (450 °C). The system was calibrated in the range from m/z 50 to 1200 using a solution of sodium formate (5 mmol/L) in 2-propanol/H_2O (9/1, v/v). Unifi 1.9.4 (Waters) was used for operating the Vion system with the following parameters: capillary voltage −2.5 kV, source temperature (120 °C), cone gas flow (50 L/h), desolvation gas flow (850 L/h), and desolvation temperature (500 °C). The system was calibrated in the range from m/z 50 to 2000. The major mix calibration sample kit (Waters) was used for CCS calibration. Data interpretation was performed using MassLynx 4.1 (Synapt data) as well as Unifi 1.9.4 (Vion data).

Tandem Mass Spectrometry (MS/MS). For the structure elucidation of compounds **1−11**, mass and product ion spectra were acquired on a QTRAP 6500 System (AB Sciex, Darmstadt, Germany). The substances were dissolved in a mixture of acetonitrile/water (50/50, v/v) and directly introduced into the mass spectrometer. Electrospray ionization was used in the negative ion mode. The ion spray voltage was set to −4500 V, and nitrogen served as a curtain- and collision gas. Curtain gas, 35 psi; temperature, 450 °C; gas 1, 55 psi; gas 2, 65 psi; collision-activated dissociation, −2 V and entrance potential, −10 V. Data acquisition and instrumental control were performed using Analyst 1.6.2 (AB Sciex).

UHPLC−MS/MS. The absolute configuration of the monosaccharide released from saponins **1−11** was qualitatively determined via UHPLC−MS/MS. Therefore, the measurements were carried out with a QTRAP 6500$^+$ System (AB Sciex). The MS system was operated in multiple reaction monitoring modes. Electrospray ionization was used in the positive mode. The ion spray voltage was set to 5500 V, and nitrogen served as a curtain- and collision gas. Curtain gas, 35 psi; temperature, 500 °C; gas 1, 55 psi; gas 2, 65 psi; collision-activated dissociation, −2 V and entrance potential, −10 V. Furthermore, the MS system was coupled to an ExionLC system (consisting of an ExionLC AD Pump, ExionLC Degasser, ExionLC AD Autosampler, ExionLC AC Column Oven, ExionLC Controller, and ExionLC Solvent Valve; all AB Sciex Instruments). The oven temperature was set to 40 °C, and the autosampler temperature was set to 15 °C. Data acquisition and instrumental control were performed using Analyst 1.6.3 (AB Sciex, Darmstadt, Germany).

Nuclear Magnetic Resonance (NMR) Spectroscopy. One- and two-dimensional NMR spectra (^{1}H, ^{13}C, correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT)-135, *J*-RESOLVED (*J*-RES), rotating frame Overhauser enhancement spectroscopy (ROESY), HSQC, and heteronuclear multiple bond correlation (HMBC)) were recorded on a 500 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany) equipped with a cryo-TCI probe (300 K). ^{1}H, ^{13}C, and HSQC spectra of betavulgaroside V were recorded on an 800 MHz Avance III spectrometer (Bruker) equipped with a cryo-TCI probe (298 K). Pyridine-d_5 (600 μL) was used as a solvent, and chemical shifts are reported in parts per million (ppm) relative to the solvent signals in the ^{1}H NMR (7.58 ppm) and ^{13}C NMR (135.9 ppm) spectra. Data processing was performed using MestReNova 12.0.0 (Mestrelab Research, Santiago de Compostela, Spain) and TopSpin 3.2 (Bruker).

■ RESULTS AND DISCUSSION

Very recently, extracts with enriched saponin concentrations prepared from beetroot, oat bran, and sugar beet have been reported to be promising new natural emulsifiers.[11−14] Although all of them show promising techno-functional properties, nearly nothing is known about the taste or foam activity nor the molecular structure of the individual bioactive saponins.[11−14] Therefore, we screened for taste-inactive and at the same time foam-active saponins in sugar beets and ultimately isolated and characterized the chemical structures of the main saponins present in sugar beets and its side streams.

Foam Activity Measurement of Different Plant Extracts. To find the best source for the isolation of the saponins, first, five different plant-based extracts already reported to have emulsifying properties were screened for their foam activity (Figure 3A, left).[11,13,14,16] The foam activity of extracts prepared from quillaja, ginseng, oat bran, beetroot, and sugar beet are well in line with their emulsifying activity reported in the literature.[11,13,14,16] While the quillaja extract exhibited the highest foam activity, obviously related to a saponin concentration of 68.6%,[11] another extract exhibiting outstanding foam activity is the ginseng extract, with saponin concentrations of up to 5.6%.[16] In comparison, oat bran extract prepared according to Ralla et al. with a saponin content of around 4.6% showed a slightly lower foam activity.[13] Notwithstanding high saponin concentrations, as well as strong foam and emulsifying properties, "natural" emulsifiers in food applications are often limited by their bitter tasting off-flavor notes.[10,19,44] Therefore, it is reasonable to investigate the beetroot, as well as the sugar beet extract, although even lower foam activities were determined. Suspecting a direct correlation between the saponin concentration and the foam activity, one milestone is the production of pleasant tasting saponin-enriched extracts or purified subfractions showing high foam and emulsifying activity. As the quantity of the sugar beet material that is annually produced worldwide is much larger compared to beetroot, the sugar beet extract was selected for further investigations.[22]

Screening of Sugar Beet Extract. To isolate all conceivable saponins, freeze-dried sugar beet root was exhaustively extracted with a mixture of methanol/water, followed by methanol alone. Since the sweet taste of the extract suppressed all other taste impressions (Figure 2A), it was separated by means of MPLC into 10 fractions: A-I to A-X. Sensory experiments were combined with foam activity measurements to screen for ideally tasteless and foam-active saponins. Additionally, all MPLC fractions were investigated for literature-known sugar beet saponins and their substructures by UPLC−ToF-MS.[25−28]

Literature-known saponins were found in fractions A-IV to A-VI (Figure 2A). Only two of these fractions (fractions A-V and A-VI) exhibited foam activity (Figure 2A). The only other foam-active fraction is fraction A-VII. Generally, taste dilution analysis revealed that saponin-containing fractions evoked no or only a slightly bitter off-taste and simultaneously a high foam activity. Consequently, sugar beet saponins were isolated for further characterization.

Isolation of Sugar Beet Saponins. Saponins were isolated from dried sugar beet pulp (sugar-exhausted sugar beet root). As observed from UPLC−ToF-MS, as well as HPLC−ELSD (data not shown), the pulp contained low amounts of sugar and high amounts of saponins, which represented a perfect source of saponins. Surprisingly, the presence of saponins within sugar beet pulp has been rarely mentioned in the literature and even denied.[45,46] The pulp was extracted with a mixture of methanol and water. A preseparation by solid-phase extraction was performed with the extract, and four fractions (fractions B-I to B-IV) were obtained after the evaporation of the organic solvent and lyophilization. The obtained SPE fraction B-III contained the highest saponin amount and all saponins known from extract A and, therefore, was further fractionated with preparative HPLC into seven fractions. Saponin-containing fractions B-III-2, B-III-4, and B-III-6 (Figure S1, Supporting Information) were subfractionated with semipreparative HPLC, yielding the saponins **1**, **3**, and **5**−**8**.

Since the isolation of compounds **2** and **4** was hampered due to the complexity during HPLC separation, these two components were obtained via synthesis using **1** and **3** (Figure S2, Supporting Information).

Identification of Saponins (1−11) in Sugar Beet. Compound **1** was obtained from fraction B-III-6-c, and its structure was elucidated by MS/MS and NMR experiments. LC−MS (ESI$^-$) analysis revealed m/z 953.6 as the pseudomolecular ion ([M − H]$^-$), thus suggesting a molecular mass of 954.6 Da. This was confirmed by UPLC−ToF-MS, indicating an empirical formula of $C_{47}H_{70}O_{20}$, based on the measured m/z 953.4389. Further LC−ESI$^-$-MS/MS experiments led to the identification of the fragment ions m/z 909.4 [M − 44 − H]$^-$, 793.6 [M − 160 − H]$^-$, 631.4 [M − 160 − 162 − H]$^-$, 569.3 [M − 160 − 162 − 44 − 18 − H]$^-$, and 455.4 [M − 160 − 162 − 176 − H]$^-$, thus suggesting the presence of an acid group, one hexose, one uronic acid, and one dioxolane-type substituent, which is typical for betavulgaroside I.[39,47] The daughter ion m/z 455.4 indicated the presence of oleanolic acid or norhederagenin.[39] For unequivocal identification of the corresponding saccharides, an aliquot of **1** was hydrolyzed with aqueous hydrochloric acid and analyzed according to the modified method published by Schmid et al.[38] D-Glucuronic acid and D-glucose were identified as the corresponding saccharides, which is in line with the detected daughter ions in the MS experiments.

The ^{13}C NMR spectrum of **1** displayed a total of 47 signals resonating between 15.9 and 176.8 ppm (Tables S5 and S6, Supporting Information); 30 of these signals were assigned to the aglycone moiety that was identified as oleanolic acid by comparison with the literature.[48] Six signals (31.2 ppm (C-20); 37.3 ppm (C-10); 39.9 ppm (C-4); 40.3 ppm (C-8); 42.5 ppm (C-14); and 47.4 ppm (C-17)) were assigned as quaternary carbons by means of an HSQC experiment. Furthermore, 144.5 ppm (C-13) was assigned to be part of an alkene group and 176.8 ppm (C-28) was assigned as a carboxylic group. The ^1H NMR spectrum showed the presence of seven tertiary methyl groups (0.77-0.81 ppm (H-C(25)); 0.86−0.90 ppm (H-C(30)); 0.86−0.95 ppm (H-C(29)); 0.94−0.98 ppm (H-C(24)); 1.06−1.13 ppm (H-C(26)); 1.24−1.28 ppm (H-C(23)); and 1.25−1.30 ppm (H-C(27))). Furthermore, one olefinic proton was observed at 5.38−5.42 ppm. Moreover, two anomeric carbohydrate protons were observed and assigned as two β-configured glucopyranosyl protons (6.31−6.35 and 4.97−5.03 ppm) with coupling constants of 8.2 and 7.5 Hz, respectively. The bidesmosidic saponin structure was confirmed by means of an HMBC experiment. It showed long-range correlations between the anomeric proton H-C(1‴′) observed at 6.31−6.35 ppm and carbon C-28 resonating at 176.8 ppm as well as the anomeric proton H-C(1′) at 4.97−5.03 ppm and C-3 at 89.8 ppm. COSY, HMBC, and rotating frame Overhauser enhancement spectroscopy (ROESY) experiments were used for the assignment of the remaining carbohydrate atoms and the dioxolane-type substituent as well as their linkages. Especially, a combination of COSY and ROESY spectra was used for the unequivocal identification of the saccharide structure. The COSY revealed couplings of vicinal protons, whereas the ROESY shows couplings between protons that are spatially close to each other (on one level) like the coupling between H-C(2′) and H-C(4′). *J*-RES supported the structure elucidation through the separation of chemical shifts and *J*-couplings into two different spectral dimensions. However, it was not possible to elucidate the absolute structure of the carbon atom C-2″ of the dioxolane-type substituent using NMR experiments. The absolute structure was finally determined with the help of the literature comparison that used X-ray crystallographic analysis for the structure elucidation of a similar compound.[49] Yoshikawa et al. compared this substance with compound **1** and found that this component has the same substituent.[27,49] Although this substance is already known, the complete assignment of all protons was performed for the first time.[27] Based on these findings, compound **1** was identified as Betavulgaroside I.[27]

UHPLC−ESI-ToF-MS analysis of compound **2** revealed an m/z 791.3859 as the pseudomolecular ion ([M − H]$^-$), thus indicating a molecular mass of 792.2 Da and a molecular formula of $C_{41}H_{50}O_{15}$. MS/MS fragmentation exhibited daughter ions at m/z 747.3 [M − 44 − H]$^-$, 631.3 [M − 160 − H]$^-$, and 455.3 [M − 160 − 176 − H]$^-$, thus demonstrating the presence of an acid group, one uronic acid, and one dioxolane-type substituent, which is typical for betavulgaroside II.[27] Only D-glucuronic acid was identified as the corresponding saccharide, which is in line with the daughter ions. The NMR data matched those of compound **1**, with the difference of missing glucose signals. For the first time, the complete assignment of all proton signals was achieved even if this substance, which was identified as betavulgaroside II, is already published (Tables S7 and S8, Supporting Information).[27]

Compound **3** was obtained from fraction B-III-6-b. LC−MS and UPLC−ToF-MS analyses (m/z 955.4543) revealed a molecular mass of 956.5 Da and an empirical formula of $C_{47}H_{72}O_{20}$, suggesting betavulgaroside III, a known saponin from sugar beet.[27] MS/MS analysis of the ion m/z 955.5 revealed the following daughter ions m/z 835.3 $[M-120-H]^-$, 793.3 $[M-162-H]^-$, 673.3 $[M-120-162-H]^-$, 631.3 $[M-162-162-H]^-$, 569.3 $[M-162-162-44-18-H]^-$, and 455.3 $[M-176-162-162-H]^-$, thus demonstrating the presence of an acid group, one or two hexoses, one uronic acid and probably one acetal-type substituent, which is typical for betavulgaroside III.[27] The corresponding saccharides were identified as D-glucuronic acid and D-glucose with the strategy described above.

The NMR spectra of compound **3** showed some similarities to compound **1**, especially for the aglycone and glucose moieties (Tables S5 and S6, Supporting Information). Larger differences were observed for the remaining part of the molecule, which was not unexpected due to the very different structure that is emphasized in Figure 6. The substructure glucuronic acid as well as the acetal-type substituent were confirmed by a combination of COSY, HMBC, ROESY, and J-RES experiments and are consistent with the literature.[27] However, it was not possible to identify both the stereogenic centers of the acetal-type substituent. This knowledge was obtained from Zhu et al., who carried out the stereogenic synthesis of compound **3**. All ^{13}C NMR data match those provided by Zhu et al. and confirm the identification as betavulgaroside III.[50] For **3**, the complete assignment of the protons was carried out for the first time.[27,50,51]

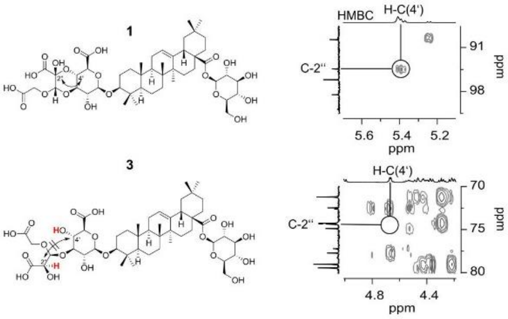

Figure 6. Excerpts of the HMBC spectra (500 MHz, pyridine-d_5) of betavulgaroside I (**1**) and betavulgaroside III (**3**).

Compound **4** was obtained via synthesis using **3** and revealed a molecular formula of $C_{41}H_{52}O_{15}$ (m/z 793.4011). All spectroscopic data were in line with betavulgaroside IV, and the proton NMR signals of **4** were completely assigned for the first time (Tables S7 and S8, Supporting Information).[27,51]

Compound **5** was isolated from fraction B-III-4-b. UPLC−ToF-MS analysis exhibited an m/z 939.4226 and suggested a molecular formula of $C_{46}H_{68}O_{20}$. MS/MS experiments showed daughter ions at m/z 819.2 $[M-120-H]^-$, 777.3 $[M-162-H]^-$, and 595.4 $[M-162-120-44-18-H]^-$, which demonstrated the presence of an acid group, and one hexose or one acetal-type substituent, which is expected for betavulgaroside VIII.[28,47]

The aglycone was identified with the help of NMR spectroscopy. One of the most obvious differences compared to compounds **1**−**4** was the fact that only five tertiary methyl groups (0.75−0.77 ppm (H-C(25)); 0.78−0.86 ppm (H-C(24)); 0.99−1.08 ppm (H-C(26)); 1.20−1.26 ppm (H-C(27)); and 1.22−1.26 ppm (H-C(23))) were found within the ^1H NMR spectrum. Another major difference is the HMBC correlation between H-C(29) and C-20, which indicated the involvement of C-20 to a double bond instead of two methyl groups (Figure 1). All other structural elements of the aglycone showed linkages like oleanolic acid and, therefore, the aglycone was identified as acebonoic acid.[39] Like compound **3**, the stereocenters of the acetal-type substituent could not be determined but assumed to be like in **3**. A complete assignment of all proton NMR signals was achieved, and **5** could be identified as betavulgaroside VIII (Tables S1 and S2, Supporting Information).[28]

Compound **6** was obtained from fraction B-III-2-b with an elemental composition of $C_{41}H_{62}O_{14}$ (UPLC−ToF-MS) based on the pseudomolecular ion m/z 777.4061 ($[M-H]^-$). MS/MS analysis revealed neutral losses of 176 amu and 162 amu, suggesting a uronic acid and a hexose moiety. Furthermore, the daughter ion at m/z 439.2 Da indicates the presence of an akebonoic acid aglycone. The corresponding saccharides determined after acidic hydrolysis and LC−MS/MS measurements are D-glucuronic acid and D-glucose.[39] NMR experiments clarified that chemical shifts of the aglycones from compounds **5** and **6** are comparable and differences could only be observed for the side chains. HMBC couplings between H-C(1″) and C-3 showed that D-glucuronic acid is bound to carbon atom C-3, whereas coupling between H-C(1‴) and C-28 indicates that D-glucose is bound to C-28. In summary, compound **6** was identified as boussingoside A2 and, for the first time, a complete assignment of all H-atoms was achieved (Tables S1 and S2, Supporting Information).[52]

Based on the measured accurate m/z 1087.5336 via UPLC−ToF-MS, an empirical formula $C_{53}H_{84}O_{23}$ could be assigned for compound **7** (fraction B-III-2-d). Further LC−MS/MS experiments led to the identification of the daughter ions m/z 925.4 $[M-162-H]^-$, 731.3 $[M-162-132-44-18-H]^-$, 569.3 $[M-162-162-132-44-18-H]^-$, and 455.2 $[M-176-162-162-132-H]^-$, thus demonstrating the presence of an acid group, two hexoses, one pentose, and one uronic acid. D-Glucuronic acid, D-glucose, and D-xylose were identified as the corresponding saccharides as described above. Daughter ion m/z 455.2 indicated oleanolic acid as the aglycone, which was confirmed by means of NMR experiments.[39] The most important linkages between the aglycone and sugars as well as linkages between sugars, which were observed via HMBC and ROESY experiments, are summarized in Figure S3 (Supporting Information). After complete assignment of all atoms, compound **7** was identified as 3-*O*-[β-D-glucopyranosyl-(1 → 2)-(β-D-xylopyranosyl-(1 → 3))-β-D-glucuronopyranosyl]-28-*O*-β-D-glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid. Although compound **7** was already postulated in the literature,[26] its full set of spectrometric and spectroscopic data was reported for the first time (Tables S3 and S4, Supporting Information).

Fraction B-III-4-c was the source of compound **8**. LC−MS and UPLC−ToF-MS (m/z 1117.5067) analyses revealed an elemental composition of $C_{53}H_{82}O_{25}$. MS/MS analysis led to the identification of the daughter ions m/z 997.4 $[M - 120 - H]^-$, 955.4 $[M - 162 - H]^-$, 793.4 $[M - 162 - 162 - H]^-$, 731.4 $[M - 162 - 162 - 44 - 18 - H]^-$, and 569.3 $[M - 162 - 162 - 162 - 44 - 18 - H]^-$, and indicated the presence of an acid group, two hexoses, one uronic acid, and one acetal-type substituent, which is typical for betavulgaroside V.[28] D-Glucuronic acid and D-glucose were identified as the corresponding saccharides with the method described above. ^{1}H and ^{13}C NMR data comparison with the literature confirmed betavulgaroside V (**8**).[28]

UPLC−ToF-MS screenings of fractions A-I to A-X potentially highlighted chikusetsu saponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**) within fractions A-V and A-VI.[26] The identity was confirmed by comparison of accurate mass fragments, retention time, and CCS values with commercially available references. Since a complete assignment of all protons of **9** via NMR experiments was not found in the literature,[53] the structures of compounds **9**−**11** were fully characterized with the same workflow as described above (Tables S3 + S9 and S4 + S10, Supporting Information).

Very recently, we highlighted by means of UPLC−ESI-TOF in the negative ion mode in combination with ion mobility separation for natural compounds isolated from *Garcinia* species more than one drift time species, which we assumed to be hydrogen bond stabilized conformers in the gas phase.[54] Interestingly, again for **1**, **3**, **5**−**9**, and **11**, more than one drift time species could be observed. Exemplified for **3**, the three drift time species (7.21, 8.92, and 12.12 ms) could be detected, which yielded a CCS of 198.3, 241.6, and 330.0 Å^2 (Figure S4, Supporting Information). It is notable that only monodesmosidic saponins like **2**, **4**, and **10** possess one CCS value, as shown for **4** in Figure S5 (11.42 ms; 309.0 Å^2, Supporting Information). These CCS values could be used as new and additional characteristic compound parameters in compound identification/screening/database applications to reduce dereplication and false positives and strengthen the identification. Already one or more CCS value(s) gives a hint of the chemical structure of this complex natural compound class of saponins.

Sensory Analysis of Pure Substances. Prior to the determination of human recognition thresholds, the purity of the sugar beet saponins was checked by NMR and LC−MS experiments. The two saponins ginsenoside Rc (**12**) and ginsenoside Rg1 (**15**) from ginseng were used as a bitter tasting control and confirmed that the sensory panel was sensitive to bitter taste.[44] A bitter taste threshold of 77 and 117 μmol/L was determined for ginsenoside Rc (**12**) (a few panelists mentioned a sweet taste in addition to the bitter taste of ginsenoside Rc) and ginsenoside Rg1 (**15**). As already implied during the activity guided fractionation, the bitter off-taste sensation for sugar beet saponins is much less intense compared to ginseng saponins. It was not even feasible to obtain individual taste thresholds for all panelists. Consequently, after applying a significance test, no bitter taste could be confirmed with a probability level of 5% for sugar beet saponins (compounds **1**−**11**) within the measured range (7.8−1000 μmol/L). Therefore, sugar beet saponins are proven to be saponins with a low bitter intensity.

These sensory data revealed that a later application in food is promising. The upper measured limit of 1000 μmol/L for all saponins corresponds to a mass concentration of 632−1119 mg/L if the smallest (632.8 g/mol) and the biggest (1089.2 g/mol) molecular mass of the observed sugar beet saponins are considered. This is even higher than the approved concentration for quillaja (200 mg/L).[55] In addition, only small quantities (≤7.5 g/L) of sugar beet saponins or saponin mixtures are expected to be deployed in food applications, since Ralla et al. used 0.75% crude sugar beet extract with a saponin amount of >0.5% for the emulsification of a mixture consisting of 10% lipid phase and 90% aqueous phase to achieve nanosized oil droplets.[11] Therefore, we do not expect that concentrations of sugar beet saponins are above their possibly perceived bitter taste threshold concentrations within real food systems. Furthermore, potential saponin extracts consist of different saponins, whereby the taste of possibly more potent saponins will be less dominant. An insignificant bitter taste of sugar beet saponins with values higher than 1000 μmol/L is not typical, especially compared to saponins from asparagus with very low bitter taste thresholds of 11−200 μmol/L or from oat with 4−9 μmol/L.[7,10]

Foam Activity Measurement of Purified Saponins 1−23. To investigate the foam activity of saponins, a small-scale foam assay was developed, which is an improved version of the one developed by Ross et al.[34] This assay could be used for measurements of foam-active solutions with volumes down to 2 mL and, therefore, extremely low amounts of foam-active substances. These measurements might give important insights into the interfacial and emulsifying ability of individual saponins since, as mentioned above, the foam activity of extracts is well in line with their emulsifying ability.

Consequently, several isolated, synthesized, and commercially obtained substances (**1**−**23**, Figures 1 and 4) were used for foam activity measurements. Some were not soluble in suitable concentrations (no foam activity was measured within the range of solubility), namely, compounds **4** and **10**, as well as ginsenoside CK, ginsenoside Rf, timosaponin A-III, and trillin. Others have a limited solubility range (foam activity was measured, but has not left the measurable range, that corresponded to a foam height of more than 250 mm until the solubility limit was reached), and some showed a particularly high activity, which quickly leaves the measurable range (Figure 5). Ginsenoside Rc (**12**) and 3-*O*-[β-D-glucopyranosyl-(1 → 2)-(β-D-xylopyranosyl-(1 → 3))-β-D-glucuronopyranosyl]-28-*O*-β-D-glucopyranosyl-3β-hydroxyolean-12-en-28-oic acid (**7**) were not measured at higher concentrations due to the low amount of available compound. Compound **9** revealed the highest foam activity, followed by compounds **23**, **17**, and **12**. All of them are bidesmosidic saponins, whereby compound **23** is a cyclic saponin. Less foaming active saponins were compounds **1**, **3**, **2**, and **5**, of which only one compound is monodesmosidic. The foam activities of all other saponins were somewhere in between the aforementioned ones. The foam formed by all saponins can be considered stable, starting from a foam height of around 50 mm. Smaller foam heights tended to degrade quickly. Higher foam heights, however, are very stable over several hours, hampering foam decay measurements.

A general observation is the lower foam activity of monodesmosidic saponins. This is quite in contrast to the statement from Oleszek et al., who mentioned that monodesmosidic saponins are particularly foam active.[56] The only noticeable monodesmosidic saponins are aescin IA (**17**), soyasaponin I (**18**), and α-solanine (**21**) that showed higher foam activity compared to a few bidesmosidic ones, but their foam activity is still low. Interestingly, all of these three saponins containing three saccharides within their side chain, which is the highest number for all measured monodesmosidic saponins, seem to increase foam activity.

Further foam activity research seems to be necessary to gain deeper insights into the structure−activity relationship. However, this work clearly disproved the claim that monodesmosidic saponins are particularly foam active.[56] It is noteworthy that chikusetsu saponin IVa (**9**) showed a much higher foam activity compared to boussingoside A2 (**6**) that contains a double bond instead of two methyl groups. As a result, even small molecular differences might play an important role.

In conclusion, this work shows that it is possible to obtain saponins with a very low bitter intensity from sustainable sources like sugar beet. Particularly noteworthy is the fact that they can be obtained easily from the by-product stream sugar beet pulp, which arises in large quantities during sugar production.[57] Additionally, it is the first time that foam activity measurements with a large number of pure saponins were carried out under standardized conditions and also clearly show that the common assumption that monodesmosidic saponins are particularly foam active is false. The obtained knowledge can be useful for the utilization of saponins from other plant materials or by-product streams and of course for the further utilization of sugar beet saponins as food additives. In the future, the emulsifying activity of the foam-active saponins **1**−**11** should be analyzed and tested in several real food applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c04603.

HPLC separation of fraction B-III as well as fractions B-III-2, -4, and -6, drift time distribution of betavulgaroside III (**3**) and betavulgaroside IV (**4**), structure elucidation of sugar beet saponins (**9**−**11**), NMR data (PDF)

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Notes

The authors declare no competing financial interest.

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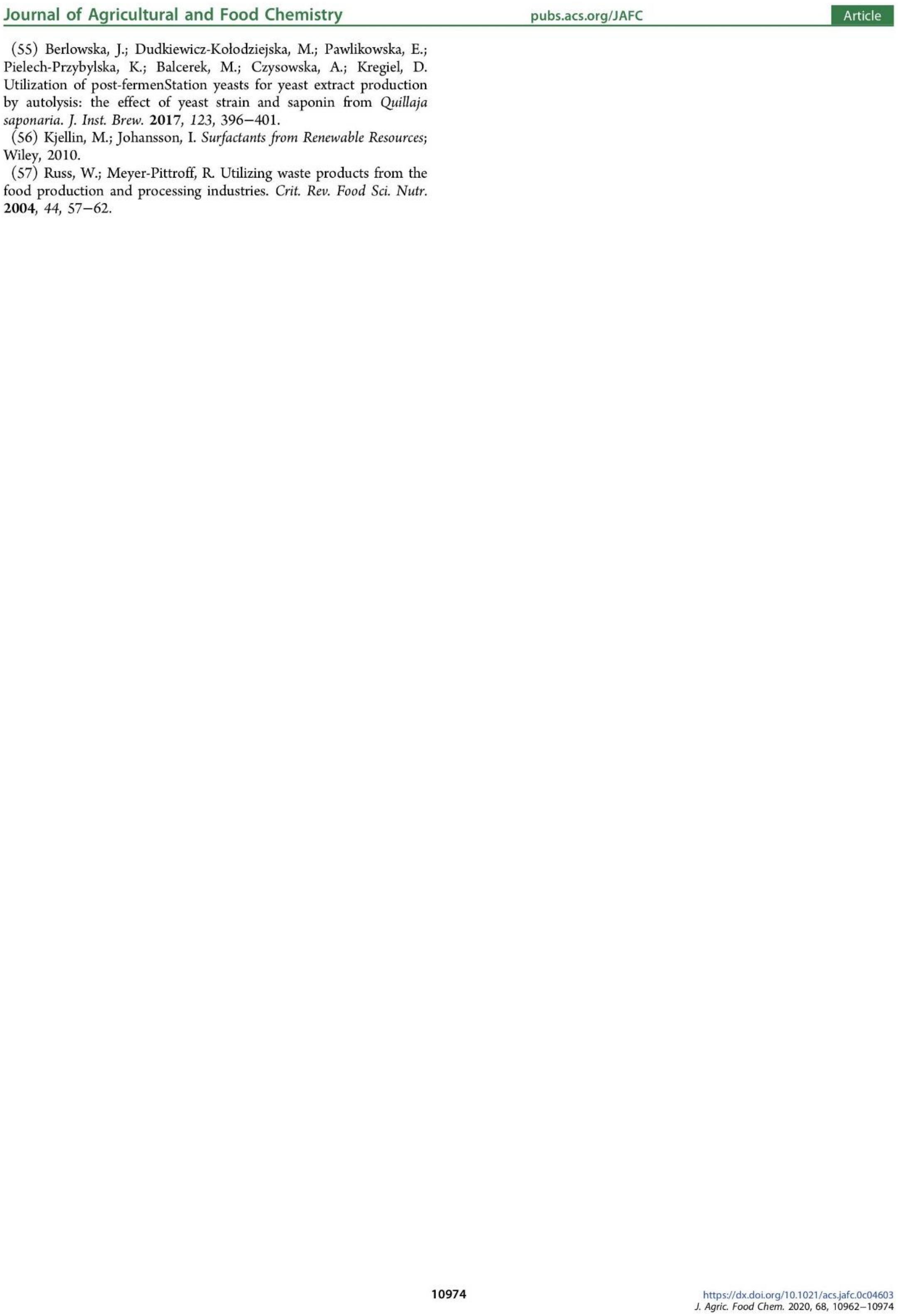

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Supporting Information:

Supporting Information

Molecularization of Foam Active Saponins in Sugar Beet Side Streams (*Beta vulgaris* ssp. *vulgaris* var. *altissima*)

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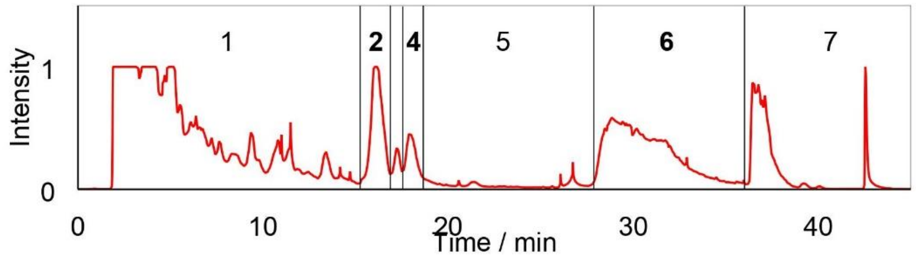
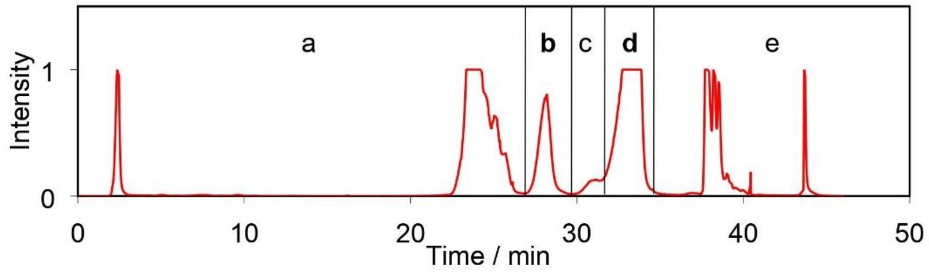
Edelmann et al. (Figure S1)

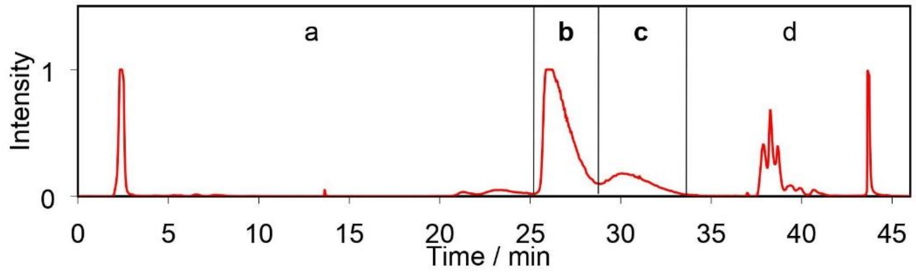
Fraction B-III



Fraction B-III-2



Fraction B-III-4



Fraction B-III-6

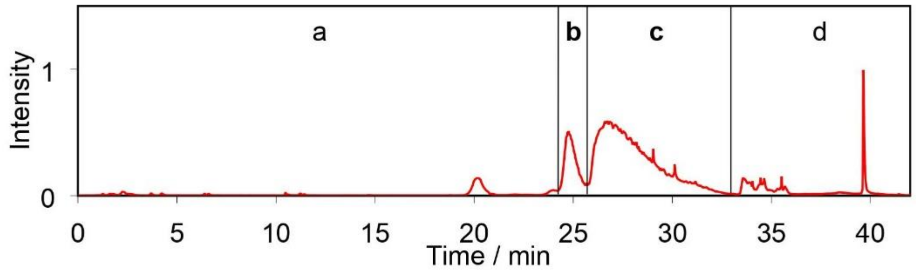


Figure S1. HPLC separation of fraction B-III as well as fractions B-III-2, -4, and -6.

Edelmann et al. (Figure S2)

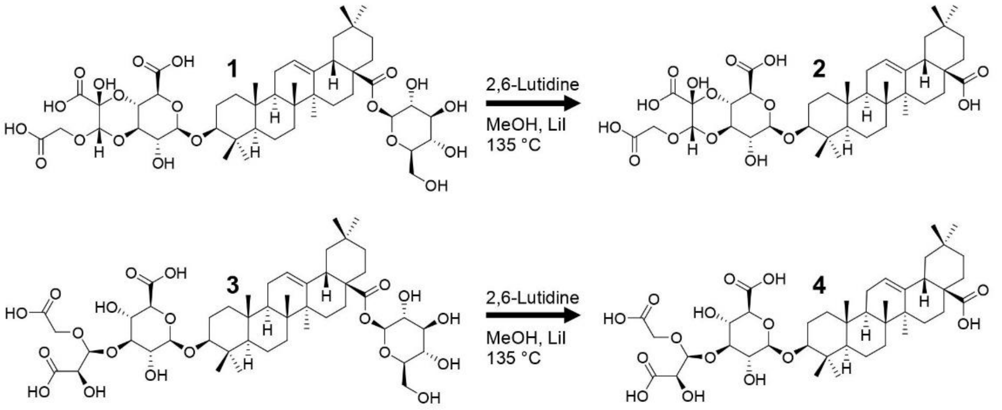
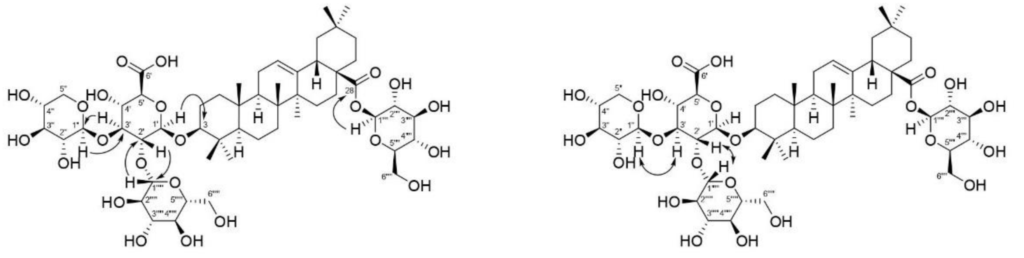


Figure S2. Synthesis of betavulgaroside II (**2**) and betavugaroside IV (**4**) out of betavugaroside I (**1**) and betavugaroside (**3**), synthezised according to Ohtani et al.[40]

Edelmann et al. (Figure S3)



HMBC

ROESY

Figure S3. Left: key HMBC couplings and right: key ROESY couplings used for structure elucidation of compound **7**.

Edelmann et al. (Figure S4)

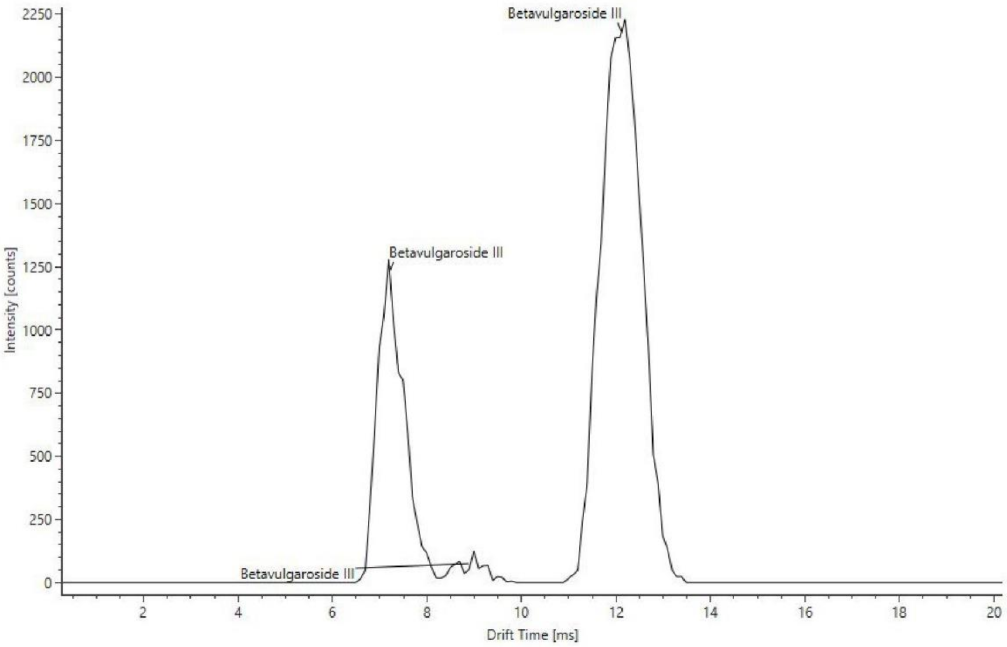


Figure S4. Drift time distribution of betavulgaroside III (**3**) showing three different drift time species (CCS values).

Edelmann et al. (Figure S5)

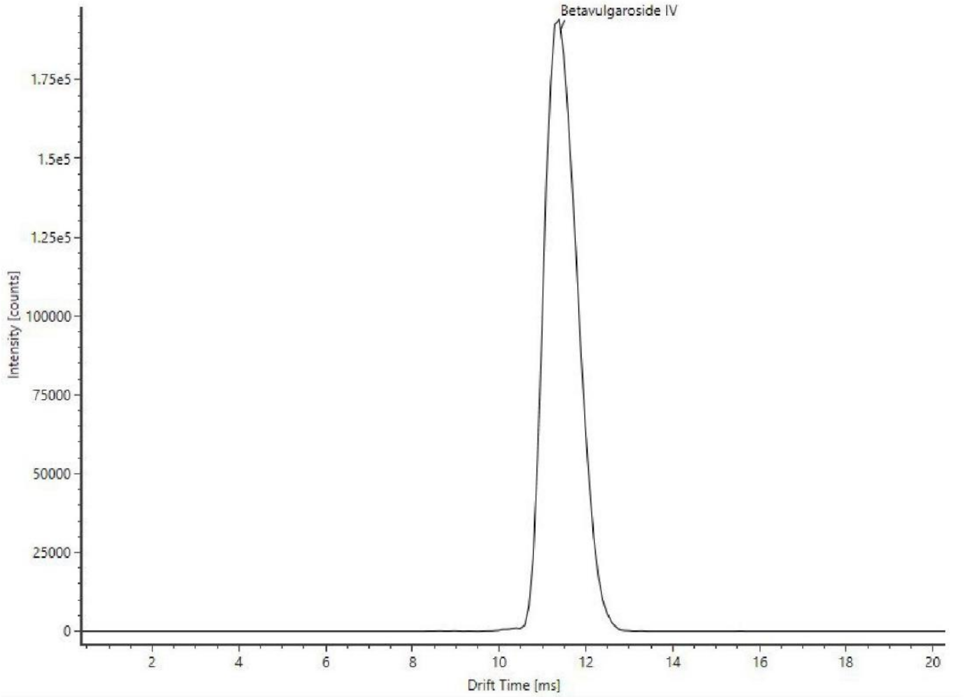


Figure S5. Drift time distribution of betavulgaroside IV (**4**) showing only one drift time species (CCS value).

Edelmann et al. (Table S1)

Table S1. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment (126 MHz, pyridine-d_5) of the aglycone moiety of betavulgaroside VIII (**5**) and boussingoside A2 (**6**).

	Betavulgaroside VIII				**Boussingoside A2**			
Position	**δC**	**HSQC**	**δH**	**M [*J*. Hz]**	**δC**	**HSQC**	**δH**	**M [*J*. Hz]**
C-1	38.9	[CH_2]	0.70-0.81	o	39.1	[CH_2]	0.81-0.87	o
			1.30-1.37	o			1.34-1.42	o
C-2	26.8	[CH_2]	1.70-1.80	o	26.9	[CH_2]	1.83-1.91	o
			2.08-2.20	o			2.30-2.37	m
C-3	89.2	[CH]	3.26-3.32	dd [*J* = 3.5; 11.2]	89.4	[CH]	3.41-3.45	dd [*J* = 4.8; 11.9]
C-4	39.7	[C]	-	-	39.9	[C]	-	-
C-5	56.0	[CH]	0.65-0.72	m	56.2	[CH]	0.75-0.81	o
C-6	18.6	[CH_2]	1.19-1.26	o	18.9	[CH_2]	1.25-1.32	o
			1.35-1.44	o			1.45-1.50	o
C-7	33.3	[CH_2]	1.24-1.35	o	33.5	[CH_2]	1.29-1.35	o
			1.35-1.46	o			1.43-1.50	o
C-8	40.1	[C]	-	-	40.3	[C]	-	-
C-9	48.2	[CH]	1.50-1.56	m	48.3	[CH]	1.56-1.61	m
C-10	37.1	[C]	-	-	37.3	[C]	-	-
C-11	23.8	[CH_2]	2.01-2.11	o	24.1	[CH_2]	1.79-1.91	o
			2.12-2.21	o				
C-12	123.6	[CH]	5.38-5.43	m	123.8	[CH]	5.41-5.46	m
C-13	143.8	[C]	-	-	143.8	[C]	-	-
C-14	42.3	[C]	-	-	42.5	[C]	-	-
C-15	28.4	[CH_2]	1.12-1.20	o	28.6	[CH_2]	1.16-1.26	o
			2.28-2.37	m			2.30-2.41	m
C-16	23.9	[CH_2]	1.21-1.26	o	24.0	[CH_2]	2.04-2.17	o
			1.77-1.85	o			2.17-2.25	o
C-17	47.6	[C]	-	-	47.7	[C]	-	-
C-18	47.9	[CH]	3.06-3.13	dd [*J* = 4.8; 13.2]	48.0	[C]	3.10-3.16	dd [*J* = 6.7; 14.0]
C-19	42.0	[CH_2]	2.14-2.19	o	42.1	[CH]	2.17-2.25	o
			2.56-2.60	m			2.58-2.65	m
C-20	148.8	[C]	-	-	148.9	[C]	-	-
C-21	30.4	[CH_2]	1.99-2.05	o	30.5	[CH_2]	2.04-2.10	o
			2.16-2.21	o			2.19-2.26	o
C-22	37.9	[CH_2]	1.62-1.70	o	38.0	[CH_2]	1.66-1.74	m
			1.97-2.02	o			2.00-2.05	o
C-23	28.4	[CH_3]	1.22-1.26	s	28.6	[CH_3]	1.30-1.37	s
C-24	17.1	[CH_3]	0.78-0.86	s	17.4	[CH_3]	0.97-1.02	s
C-25	15.7	[CH_3]	0.75-0.77	s	15.9	[CH_3]	0.80-0.86	s
C-26	17.7	[CH_3]	0.99-1.08	s	17.8	[CH_3]	1.04-1.10	s
C-27	26.3	[CH_3]	1.20-1.26	s	26.5	[CH_3]	1.22-1.30	s
C-28	176.1	[C]	-	-	176.1	[C]	-	-
C-29	107.6	[CH_2]	4.65-4.72	m	107.7	[CH_2]	4.67-4.73	m
			4.72-4.75	m			4.73-4.79	m

Edelmann et al. (Table S2)

Table S2. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignments (126 MHz, pyridine-d_5) of the sugar moieties of betavulgaroside VIII (**5**) and boussingoside A2 (**6**).

	Betavulgaroside VIII				**Boussingoside A2**			
Position	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**
C-1'	107.2	[CH]	4.95-5.00	d [*J* = 8.5]	107.5	[CH]	5.01-5.06	d [*J* = 8.2]
C-2'	74.5	[CH]	4.00-4.07	dd [*J* = 8.3; 8.3]	75.9	[CH]	4.11-4.16	dd [*J* = 8.4; 8.4]
C-3'	84.4	[CH]	4.42-4.49	dd [*J* = 7.3; 7.3]	78.7	[CH]	4.32-4.37	dd [*J* = 8.0; 8.0]
C-4'	72.7	[CH]	4.48-4.55	dd [*J* = 9.1; 9.1]	74.1	[CH]	4.49-4.56	m
C-5'	77.3	[CH]	4.59-4.65	d [*J* = 9.6]	77.6	[CH]	4.58-4.64	m
C-6'	173.1	[C]	-	-	174.4	[C]	-	-
C-1''	176.6	[C]	-	-	-	-	-	-
C-2''	78.3	[CH]	5.56-5.61	d [*J* = 2.8]	-	-	-	-
C-3''	104.7	[CH]	5.92-5.96	d [*J* = 2.8]	-	-	-	-
C-1'''	178.8	[C]	-	-	-	-	-	-
C-2'''	67.5	[CH_2]	4.39-4.47	-	-	-	-	-
			5.19-5.27	-	-	-	-	-
C-1''''	96.1	[CH]	6.22-6.27	d [*J* = 8.3]	96.2	[CH]	6.27-6.32	d [*J* = 8.6]
C-2''''	74.3	[CH]	4.15-4.19	dd [*J* = 8.6; 8.6]	74.5	[CH]	4.18-4.22	dd [*J* = 8.6; 8.6]
C-3''''	79.0	[CH]	4.23-4.29	dd [*J* = 8.8; 8.8]	79.2	[CH]	4.27-4.32	dd [*J* = 8.9; 8.9]
C-4''''	71.4	[CH]	4.26-4.33	dd [*J* = 9.1; 9.1]	71.5	[CH]	4.31-4.38	dd [*J* = 9.0; 9.0]
C-5''''	79.6	[CH]	3.96-4.03	-	79.7	[CH]	4.00-4.05	m
C-6''''	62.5	[CH_2]	4.32-4.38	-	62.7	[CH_2]	4.38-4.43	m
			4.39-4.46				4.43-4.49	m

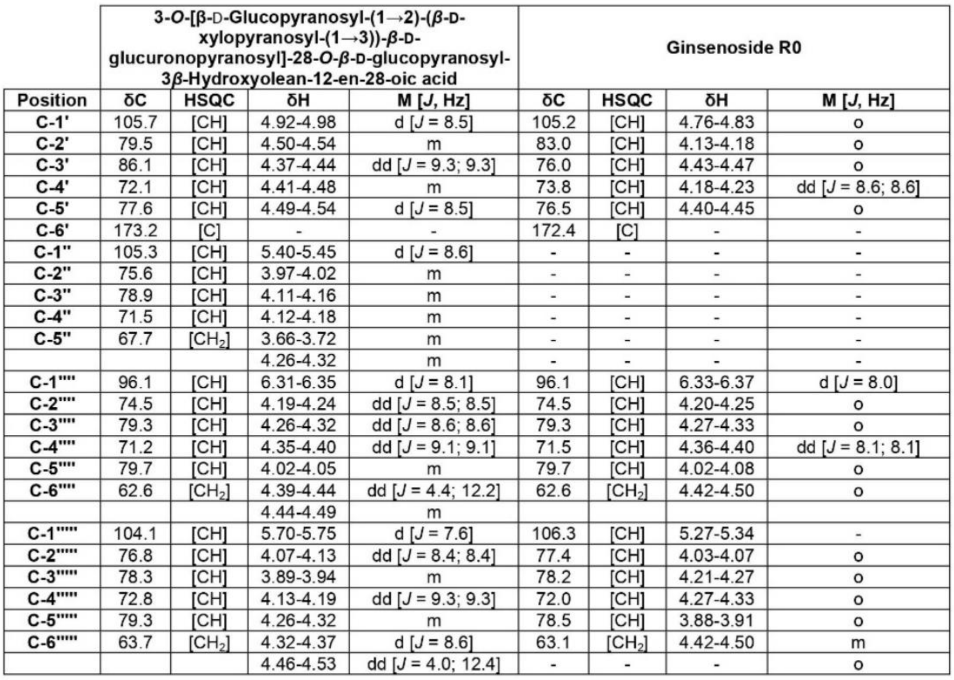
Edelmann et al. (Table S3)

Table S3. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment(126 MHz, pyridine-d_5) of the aglycone moiety of 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-Hydroxyolean-12-en-28-oic acid (**7**) and ginsenoside R0 (**11**).

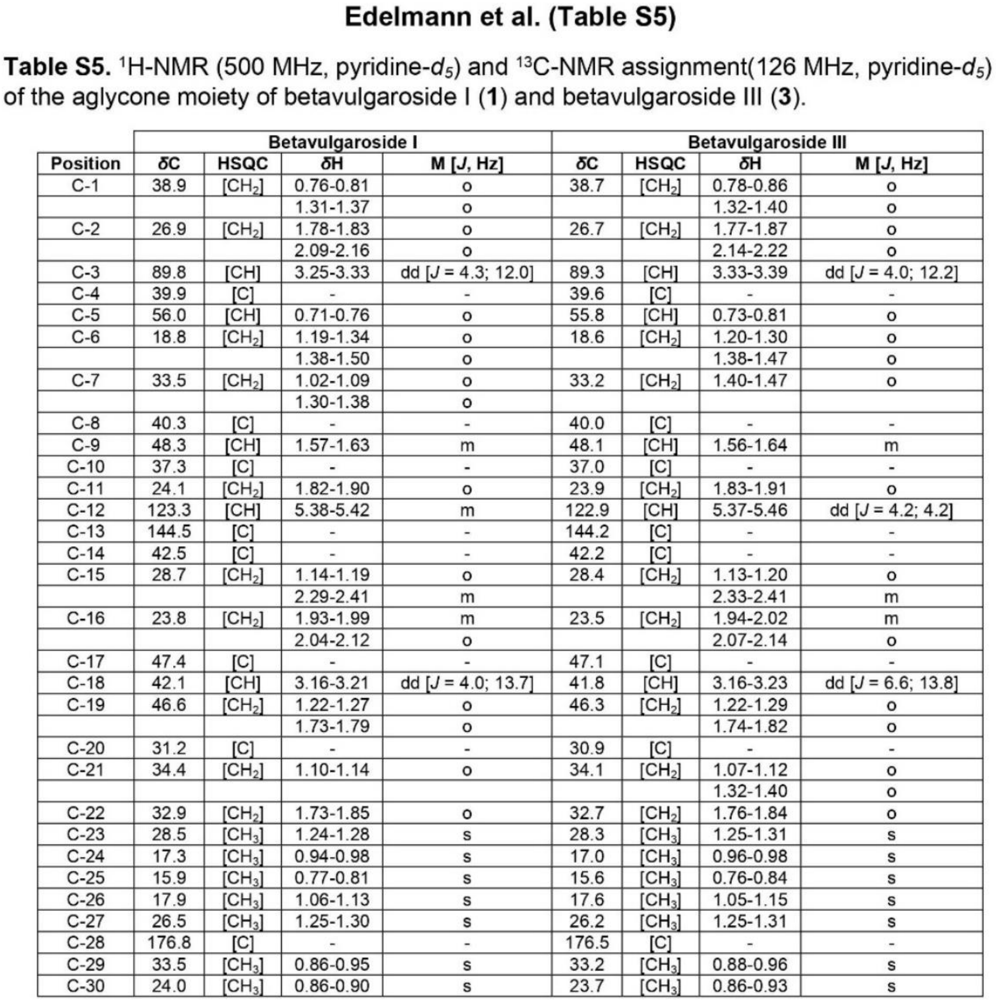
	3-*O*-[*β*-D-Glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-Hydroxyolean-12-en-28-oic acid				**Ginsenoside R0**			
Position	***δ*C**	**HSQC**	***δ*H**	**M [*J*, Hz]**	***δ*C**	**HSQC**	***δ*H**	**M [*J*, Hz]**
C-1	39.0	[CH_2]	0.73-0.81	o	39.0	[CH_2]	0.73-0.77	o
			1.29-1.34	o			1.33-1.38	o
C-2	26.9	[CH_2]	1.76-1.84	o	26.7	[CH_2]	1.78-1.83	o
			2.09-2.19	o			2.20-2.26	o
C-3	90.0	[CH]	3.22-3.28	m	89.7	[CH]	3.17-3.22	o
C-4	39.9	[C]	-	-	39.8	[C]	-	-
C-5	56.1	[CH]	0.68-0.74	m	56.2	[CH]	0.68-0.71	o
C-6	18.8	[CH_2]	1.23-1.31	o	18.9	[CH_2]	1.23-1.29	o
			1.43-1.48	o			1.42-1.47	o
C-7	33.5	[CH_2]	1.29-1.37	o	33.5	[CH_2]	1.28-1.33	o
			1.40-1.46	o			1.41-1.46	o
C-8	40.2	[C]	-	-	40.3	[C]	-	-
C-9	48.3	[CH]	1.54-1.60	m	48.4	[CH]	1.56-1.59	m
C-10	37.2	[C]	-	-	37.3	[C]	-	-
C-11	24.1	[CH_2]	1.80-1.86	o	23.8	[CH_2]	1.95-2.02	o
			1.87-1.92	m			2.09-2.13	m
C-12	122.9	[CH]	5.38-5.42	m	123.5	[CH]	5.43-5.46	m
C-13	144.5	[C]	-	-	144.5	[C]	-	-
C-14	42.5	[C]	-	-	42.5	[C]	-	-
C-15	28.6	[CH_2]	1.12-1.17	o	28.7	[CH_2]	1.13-1.17	o
			2.29-2.36	m			2.31-2.35	m
C-16	23.8	[CH_2]	1.96-2.02	o	24.2	[CH_2]	1.83-1.88	o
			2.06-2.14	m			1.88-1.93	o
C-17	47.4	[C]	-	-	47.4	[C]	-	-
C-18	42.1	[CH]	3.15-3.21	dd [*J* = 5.4; 13.7]	41.2	[CH]	3.17-3.22	m
C-19	46.6	[CH_2]	1.22-1.27	o	46.6	[CH_2]	1.26-1.32	o
			1.73-1.79	o			1.77-1.80	o
C-20	31.1	[C]	-	-	31.2	[C]	-	-
C-21	34.4	[CH_2]	1.05-1.12	o	34.4	[CH_2]	1.06-1.10	o
			1.32-1.37	o			1.33-1.39	o
C-22	32.9	[CH_2]	1.73-1.79	o	32.9	[CH_2]	1.73-1.77	o
			1.79-1.86	o			1.79-1.84	o
C-23	28.3	[CH_3]	1.22-1.30	s	28.6	[CH_3]	1.23-1.27	s
C-24	17.0	[CH_3]	1.04-1.09	s	17.2	[CH_3]	1.05-1.09	s
C-25	15.9	[CH_3]	0.76-0.82	s	16.0	[CH_3]	0.82-0.88	s
C-26	17.8	[CH_3]	1.04-1.09	s	17.9	[CH_3]	1.05-1.09	s
C-27	26.5	[CH_3]	1.25-1.27	s	26.5	[CH_3]	1.22-1.26	s
C-28	176.8	[C]	-	-	176.8	[C]	-	-
C-29	33.5	[CH_3]	0.90-0.92	s	33.6	[CH_3]	0.93-0.96	s
C-30	24.0	[CH_3]	0.86-0.90	s	24.1	[CH_3]	0.88-0.93	s

Edelmann et al. (Table S4)

Table S4. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment(126 MHz, pyridine-d_5) of the sugar moieties of 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-Hydroxyolean-12-en-28-oic acid (**7**) and ginsenoside R0 (**11**).



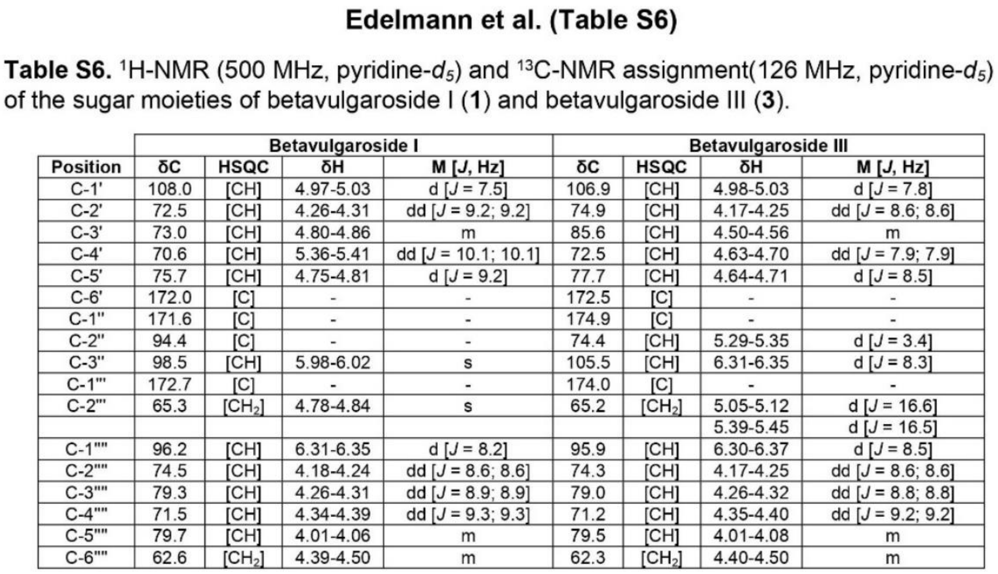
	3-*O*-[β-D-Glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-Hydroxyolean-12-en-28-oic acid				**Ginsenoside R0**			
Position	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**
C-1'	105.7	[CH]	4.92-4.98	d [*J* = 8.5]	105.2	[CH]	4.76-4.83	o
C-2'	79.5	[CH]	4.50-4.54	m	83.0	[CH]	4.13-4.18	o
C-3'	86.1	[CH]	4.37-4.44	dd [*J* = 9.3; 9.3]	76.0	[CH]	4.43-4.47	o
C-4'	72.1	[CH]	4.41-4.48	m	73.8	[CH]	4.18-4.23	dd [*J* = 8.6; 8.6]
C-5'	77.6	[CH]	4.49-4.54	d [*J* = 8.5]	76.5	[CH]	4.40-4.45	o
C-6'	173.2	[C]	-	-	172.4	[C]	-	-
C-1''	105.3	[CH]	5.40-5.45	d [*J* = 8.6]	-	-	-	-
C-2''	75.6	[CH]	3.97-4.02	m	-	-	-	-
C-3''	78.9	[CH]	4.11-4.16	m	-	-	-	-
C-4''	71.5	[CH]	4.12-4.18	m	-	-	-	-
C-5''	67.7	[CH$_2$]	3.66-3.72	m	-	-	-	-
			4.26-4.32	m	-	-	-	-
C-1''''	96.1	[CH]	6.31-6.35	d [*J* = 8.1]	96.1	[CH]	6.33-6.37	d [*J* = 8.0]
C-2''''	74.5	[CH]	4.19-4.24	dd [*J* = 8.5; 8.5]	74.5	[CH]	4.20-4.25	o
C-3''''	79.3	[CH]	4.26-4.32	dd [*J* = 8.6; 8.6]	79.3	[CH]	4.27-4.33	o
C-4''''	71.2	[CH]	4.35-4.40	dd [*J* = 9.1; 9.1]	71.5	[CH]	4.36-4.40	dd [*J* = 8.1; 8.1]
C-5''''	79.7	[CH]	4.02-4.05	m	79.7	[CH]	4.02-4.08	o
C-6''''	62.6	[CH$_2$]	4.39-4.44	dd [*J* = 4.4; 12.2]	62.6	[CH$_2$]	4.42-4.50	o
			4.44-4.49	m				
C-1'''''	104.1	[CH]	5.70-5.75	d [*J* = 7.6]	106.3	[CH]	5.27-5.34	-
C-2'''''	76.8	[CH]	4.07-4.13	dd [*J* = 8.4; 8.4]	77.4	[CH]	4.03-4.07	o
C-3'''''	78.3	[CH]	3.89-3.94	m	78.2	[CH]	4.21-4.27	o
C-4'''''	72.8	[CH]	4.13-4.19	dd [*J* = 9.3; 9.3]	72.0	[CH]	4.27-4.33	o
C-5'''''	79.3	[CH]	4.26-4.32	m	78.5	[CH]	3.88-3.91	o
C-6'''''	63.7	[CH$_2$]	4.32-4.37	d [*J* = 8.6]	63.1	[CH$_2$]	4.42-4.50	m
			4.46-4.53	dd [*J* = 4.0; 12.4]	-	-	-	o



Edelmann et al. (Table S5)

Table S5. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment(126 MHz, pyridine-d_5) of the aglycone moiety of betavulgaroside I (**1**) and betavulgaroside III (**3**).

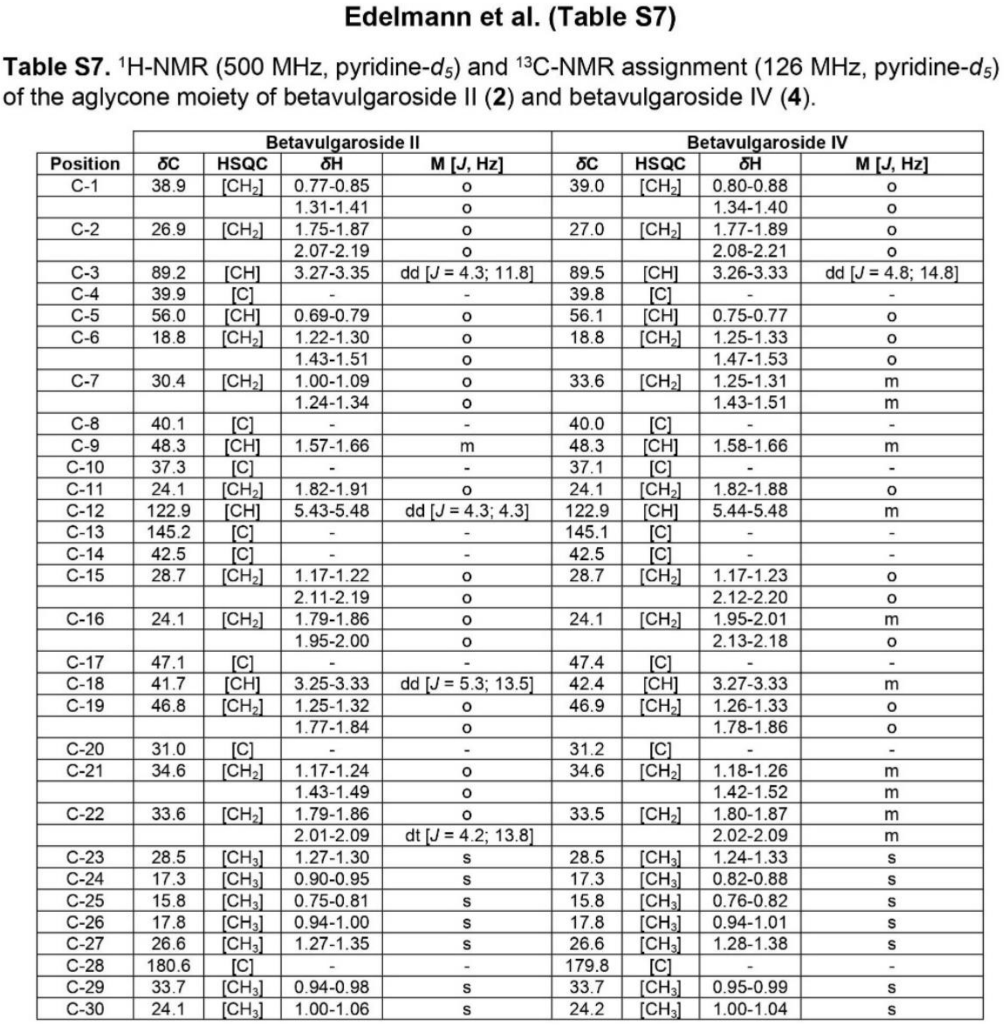
	Betavulgaroside I				**Betavulgaroside III**			
Position	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**
C-1	38.9	[CH_2]	0.76-0.81	o	38.7	[CH_2]	0.78-0.86	o
			1.31-1.37	o			1.32-1.40	o
C-2	26.9	[CH_2]	1.78-1.83	o	26.7	[CH_2]	1.77-1.87	o
			2.09-2.16	o			2.14-2.22	o
C-3	89.8	[CH]	3.25-3.33	dd [*J* = 4.3; 12.0]	89.3	[CH]	3.33-3.39	dd [*J* = 4.0; 12.2]
C-4	39.9	[C]	-	-	39.6	[C]	-	-
C-5	56.0	[CH]	0.71-0.76	o	55.8	[CH]	0.73-0.81	o
C-6	18.8	[CH_2]	1.19-1.34	o	18.6	[CH_2]	1.20-1.30	o
			1.38-1.50	o			1.38-1.47	o
C-7	33.5	[CH_2]	1.02-1.09	o	33.2	[CH_2]	1.40-1.47	o
			1.30-1.38	o				
C-8	40.3	[C]	-	-	40.0	[C]	-	-
C-9	48.3	[CH]	1.57-1.63	m	48.1	[CH]	1.56-1.64	m
C-10	37.3	[C]	-	-	37.0	[C]	-	-
C-11	24.1	[CH_2]	1.82-1.90	o	23.9	[CH_2]	1.83-1.91	o
C-12	123.3	[CH]	5.38-5.42	m	122.9	[CH]	5.37-5.46	dd [*J* = 4.2; 4.2]
C-13	144.5	[C]	-	-	144.2	[C]	-	-
C-14	42.5	[C]	-	-	42.2	[C]	-	-
C-15	28.7	[CH_2]	1.14-1.19	o	28.4	[CH_2]	1.13-1.20	o
			2.29-2.41	m			2.33-2.41	m
C-16	23.8	[CH_2]	1.93-1.99	m	23.5	[CH_2]	1.94-2.02	m
			2.04-2.12	o			2.07-2.14	o
C-17	47.4	[C]	-	-	47.1	[C]	-	-
C-18	42.1	[CH]	3.16-3.21	dd [*J* = 4.0; 13.7]	41.8	[CH]	3.16-3.23	dd [*J* = 6.6; 13.8]
C-19	46.6	[CH_2]	1.22-1.27	o	46.3	[CH_2]	1.22-1.29	o
			1.73-1.79	o			1.74-1.82	o
C-20	31.2	[C]	-	-	30.9	[C]	-	-
C-21	34.4	[CH_2]	1.10-1.14	o	34.1	[CH_2]	1.07-1.12	o
							1.32-1.40	o
C-22	32.9	[CH_2]	1.73-1.85	o	32.7	[CH_2]	1.76-1.84	o
C-23	28.5	[CH_3]	1.24-1.28	s	28.3	[CH_3]	1.25-1.31	s
C-24	17.3	[CH_3]	0.94-0.98	s	17.0	[CH_3]	0.96-0.98	s
C-25	15.9	[CH_3]	0.77-0.81	s	15.6	[CH_3]	0.76-0.84	s
C-26	17.9	[CH_3]	1.06-1.13	s	17.6	[CH_3]	1.05-1.15	s
C-27	26.5	[CH_3]	1.25-1.30	s	26.2	[CH_3]	1.25-1.31	s
C-28	176.8	[C]	-	-	176.5	[C]	-	-
C-29	33.5	[CH_3]	0.86-0.95	s	33.2	[CH_3]	0.88-0.96	s
C-30	24.0	[CH_3]	0.86-0.90	s	23.7	[CH_3]	0.86-0.93	s



Edelmann et al. (Table S6)

Table S6. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment(126 MHz, pyridine-d_5) of the sugar moieties of betavulgaroside I (**1**) and betavulgaroside III (**3**).

	Betavulgaroside I				**Betavulgaroside III**			
Position	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**
C-1'	108.0	[CH]	4.97-5.03	d [*J* = 7.5]	106.9	[CH]	4.98-5.03	d [*J* = 7.8]
C-2'	72.5	[CH]	4.26-4.31	dd [*J* = 9.2; 9.2]	74.9	[CH]	4.17-4.25	dd [*J* = 8.6; 8.6]
C-3'	73.0	[CH]	4.80-4.86	m	85.6	[CH]	4.50-4.56	m
C-4'	70.6	[CH]	5.36-5.41	dd [*J* = 10.1; 10.1]	72.5	[CH]	4.63-4.70	dd [*J* = 7.9; 7.9]
C-5'	75.7	[CH]	4.75-4.81	d [*J* = 9.2]	77.7	[CH]	4.64-4.71	d [*J* = 8.5]
C-6'	172.0	[C]	-	-	172.5	[C]	-	-
C-1''	171.6	[C]	-	-	174.9	[C]	-	-
C-2''	94.4	[C]	-	-	74.4	[CH]	5.29-5.35	d [*J* = 3.4]
C-3''	98.5	[CH]	5.98-6.02	s	105.5	[CH]	6.31-6.35	d [*J* = 8.3]
C-1'''	172.7	[C]	-	-	174.0	[C]	-	-
C-2'''	65.3	[CH_2]	4.78-4.84	s	65.2	[CH_2]	5.05-5.12	d [*J* = 16.6]
							5.39-5.45	d [*J* = 16.5]
C-1''''	96.2	[CH]	6.31-6.35	d [*J* = 8.2]	95.9	[CH]	6.30-6.37	d [*J* = 8.5]
C-2''''	74.5	[CH]	4.18-4.24	dd [*J* = 8.6; 8.6]	74.3	[CH]	4.17-4.25	dd [*J* = 8.6; 8.6]
C-3''''	79.3	[CH]	4.26-4.31	dd [*J* = 8.9; 8.9]	79.0	[CH]	4.26-4.32	dd [*J* = 8.8; 8.8]
C-4''''	71.5	[CH]	4.34-4.39	dd [*J* = 9.3; 9.3]	71.2	[CH]	4.35-4.40	dd [*J* = 9.2; 9.2]
C-5''''	79.7	[CH]	4.01-4.06	m	79.5	[CH]	4.01-4.08	m
C-6''''	62.6	[CH_2]	4.39-4.50	m	62.3	[CH_2]	4.40-4.50	m



Edelmann et al. (Table S7)

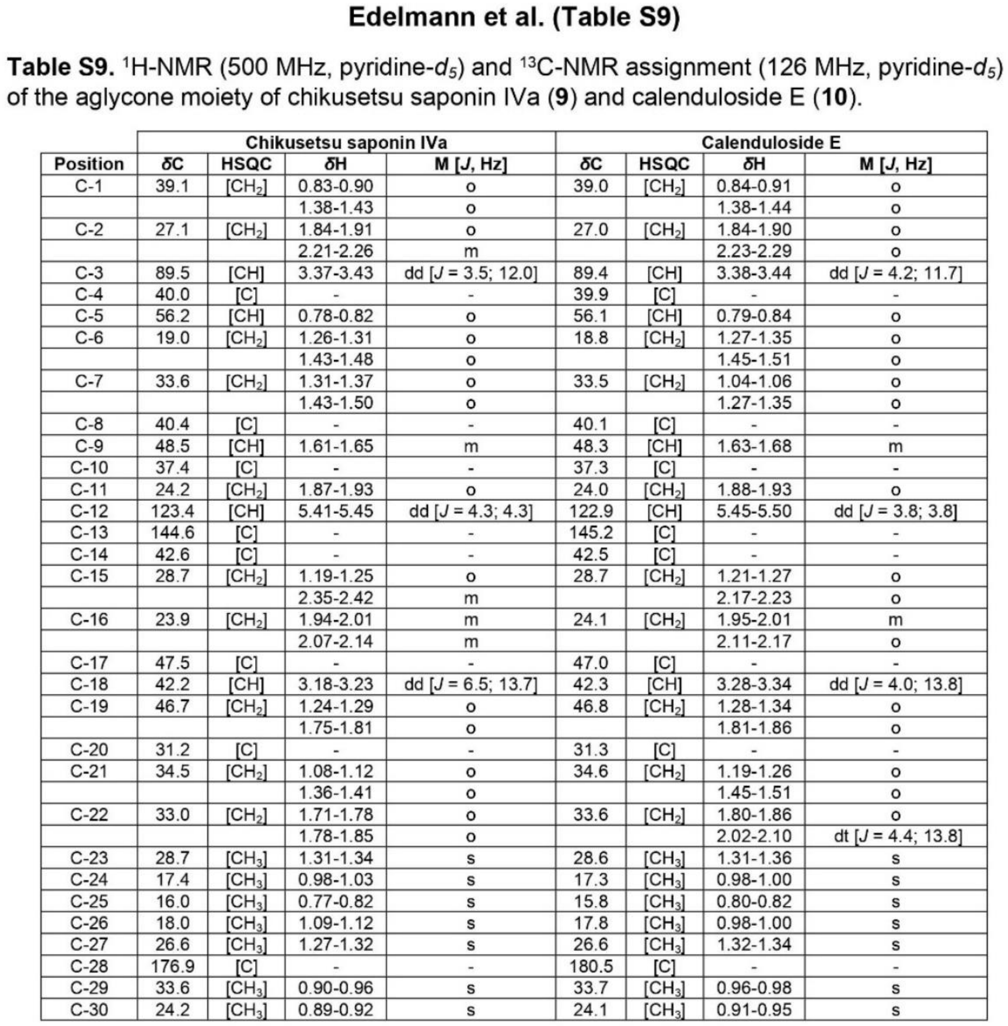
Table S7. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment (126 MHz, pyridine-d_5) of the aglycone moiety of betavulgaroside II (**2**) and betavulgaroside IV (**4**).

	Betavulgaroside II				**Betavulgaroside IV**			
Position	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**
C-1	38.9	[CH_2]	0.77-0.85	o	39.0	[CH_2]	0.80-0.88	o
			1.31-1.41	o			1.34-1.40	o
C-2	26.9	[CH_2]	1.75-1.87	o	27.0	[CH_2]	1.77-1.89	o
			2.07-2.19	o			2.08-2.21	o
C-3	89.2	[CH]	3.27-3.35	dd [*J* = 4.3; 11.8]	89.5	[CH]	3.26-3.33	dd [*J* = 4.8; 14.8]
C-4	39.9	[C]	-	-	39.8	[C]	-	-
C-5	56.0	[CH]	0.69-0.79	o	56.1	[CH]	0.75-0.77	o
C-6	18.8	[CH_2]	1.22-1.30	o	18.8	[CH_2]	1.25-1.33	o
			1.43-1.51	o			1.47-1.53	o
C-7	30.4	[CH_2]	1.00-1.09	o	33.6	[CH_2]	1.25-1.31	m
			1.24-1.34	o			1.43-1.51	m
C-8	40.1	[C]	-	-	40.0	[C]	-	-
C-9	48.3	[CH]	1.57-1.66	m	48.3	[CH]	1.58-1.66	m
C-10	37.3	[C]	-	-	37.1	[C]	-	-
C-11	24.1	[CH_2]	1.82-1.91	o	24.1	[CH_2]	1.82-1.88	o
C-12	122.9	[CH]	5.43-5.48	dd [*J* = 4.3; 4.3]	122.9	[CH]	5.44-5.48	m
C-13	145.2	[C]	-	-	145.1	[C]	-	-
C-14	42.5	[C]	-	-	42.5	[C]	-	-
C-15	28.7	[CH_2]	1.17-1.22	o	28.7	[CH_2]	1.17-1.23	o
			2.11-2.19	o			2.12-2.20	o
C-16	24.1	[CH_2]	1.79-1.86	o	24.1	[CH_2]	1.95-2.01	m
			1.95-2.00	o			2.13-2.18	o
C-17	47.1	[C]	-	-	47.4	[C]	-	-
C-18	41.7	[CH]	3.25-3.33	dd [*J* = 5.3; 13.5]	42.4	[CH]	3.27-3.33	m
C-19	46.8	[CH_2]	1.25-1.32	o	46.9	[CH_2]	1.26-1.33	o
			1.77-1.84	o			1.78-1.86	o
C-20	31.0	[C]	-	-	31.2	[C]	-	-
C-21	34.6	[CH_2]	1.17-1.24	o	34.6	[CH_2]	1.18-1.26	m
			1.43-1.49	o			1.42-1.52	m
C-22	33.6	[CH_2]	1.79-1.86	o	33.5	[CH_2]	1.80-1.87	m
			2.01-2.09	dt [*J* = 4.2; 13.8]			2.02-2.09	m
C-23	28.5	[CH_3]	1.27-1.30	s	28.5	[CH_3]	1.24-1.33	s
C-24	17.3	[CH_3]	0.90-0.95	s	17.3	[CH_3]	0.82-0.88	s
C-25	15.8	[CH_3]	0.75-0.81	s	15.8	[CH_3]	0.76-0.82	s
C-26	17.8	[CH_3]	0.94-1.00	s	17.8	[CH_3]	0.94-1.01	s
C-27	26.6	[CH_3]	1.27-1.35	s	26.6	[CH_3]	1.28-1.38	s
C-28	180.6	[C]	-	-	179.8	[C]	-	-
C-29	33.7	[CH_3]	0.94-0.98	s	33.7	[CH_3]	0.95-0.99	s
C-30	24.1	[CH_3]	1.00-1.06	s	24.2	[CH_3]	1.00-1.04	s

Edelmann et al. (Table S8)

Table S8. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment(126 MHz, pyridine-d_5) of the sugar moieties of betavulgaroside II (**2**) and betavulgaroside IV (**4**).

	Betavulgaroside II				**Betavulgaroside IV**			
Position	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**
C-1'	107.5	[CH]	4.98-5.03	d [*J* = 8.5]	107.3	[CH]	5.00-5.06	d [*J* = 7.9]
C-2'	72.0	[CH]	4.25-4.31	m	74.7	[CH]	4.16-4.20	dd [*J* = 8.6; 8.6]
C-3'	72.4	[CH]	4.81-4.84	d [*J* = 8.6; 8.6]	85.6	[CH]	4.51-5.56	m
C-4'	70.0	[CH]	5.36-5.40	m	72.7	[CH]	4.64-4.70	dd [*J* = 9.4; 9.4]
C-5'	75.0	[CH]	4.76-4.80	d [*J* = 7.5]	77.5	[CH]	4.67-4.70	d [*J* = 9.3]
C-6'	172.5	[C]	-	-	172.9	[C]	-	-
C-1''	172.1	[C]	-	-	175.1	[C]	-	-
C-2''	94.4	[C]	-	-	74.3	[CH]	5.30-5.36	m
C-3''	98.1	[CH]	5.97-6.01	s	105.5	[CH]	6.31-6.35	m
C-1'''	173.1	[C]	-	-	174.7	[C]	-	-
C-2'''	64.9	[CH_2]	4.78-4.84	s	65.3	[CH_2]	5.08-5.13	d [*J* = 8.4]
							5.38-5.44	d [*J* = 3.8]



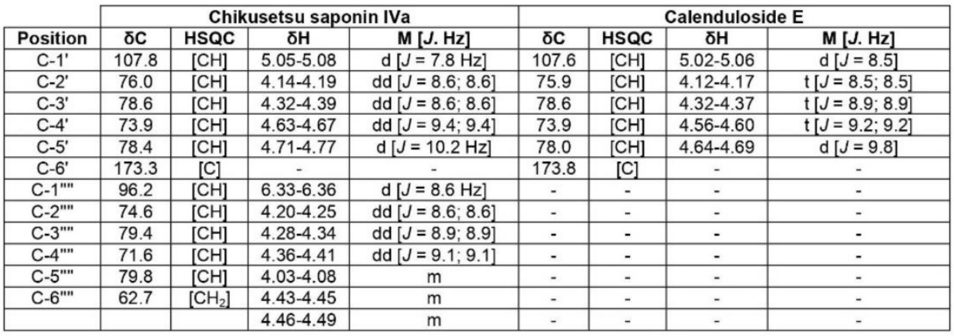
Edelmann et al. (Table S9)

Table S9. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment (126 MHz, pyridine-d_5) of the aglycone moiety of chikusetsu saponin IVa (**9**) and calenduloside E (**10**).

	Chikusetsu saponin IVa				**Calenduloside E**			
Position	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**	**δC**	**HSQC**	**δH**	**M [*J*, Hz]**
C-1	39.1	[CH_2]	0.83-0.90	o	39.0	[CH_2]	0.84-0.91	o
			1.38-1.43	o			1.38-1.44	o
C-2	27.1	[CH_2]	1.84-1.91	o	27.0	[CH_2]	1.84-1.90	o
			2.21-2.26	m			2.23-2.29	o
C-3	89.5	[CH]	3.37-3.43	dd [*J* = 3.5; 12.0]	89.4	[CH]	3.38-3.44	dd [*J* = 4.2; 11.7]
C-4	40.0	[C]	-	-	39.9	[C]	-	-
C-5	56.2	[CH]	0.78-0.82	o	56.1	[CH]	0.79-0.84	o
C-6	19.0	[CH_2]	1.26-1.31	o	18.8	[CH_2]	1.27-1.35	o
			1.43-1.48	o			1.45-1.51	o
C-7	33.6	[CH_2]	1.31-1.37	o	33.5	[CH_2]	1.04-1.06	o
			1.43-1.50	o			1.27-1.35	o
C-8	40.4	[C]	-	-	40.1	[C]	-	-
C-9	48.5	[CH]	1.61-1.65	m	48.3	[CH]	1.63-1.68	m
C-10	37.4	[C]	-	-	37.3	[C]	-	-
C-11	24.2	[CH_2]	1.87-1.93	o	24.0	[CH_2]	1.88-1.93	o
C-12	123.4	[CH]	5.41-5.45	dd [*J* = 4.3; 4.3]	122.9	[CH]	5.45-5.50	dd [*J* = 3.8; 3.8]
C-13	144.6	[C]	-	-	145.2	[C]	-	-
C-14	42.6	[C]	-	-	42.5	[C]	-	-
C-15	28.7	[CH_2]	1.19-1.25	o	28.7	[CH_2]	1.21-1.27	o
			2.35-2.42	m			2.17-2.23	o
C-16	23.9	[CH_2]	1.94-2.01	m	24.1	[CH_2]	1.95-2.01	m
			2.07-2.14	m			2.11-2.17	o
C-17	47.5	[C]	-	-	47.0	[C]	-	-
C-18	42.2	[CH]	3.18-3.23	dd [*J* = 6.5; 13.7]	42.3	[CH]	3.28-3.34	dd [*J* = 4.0; 13.8]
C-19	46.7	[CH_2]	1.24-1.29	o	46.8	[CH_2]	1.28-1.34	o
			1.75-1.81	o			1.81-1.86	o
C-20	31.2	[C]	-	-	31.3	[C]	-	-
C-21	34.5	[CH_2]	1.08-1.12	o	34.6	[CH_2]	1.19-1.26	o
			1.36-1.41	o			1.45-1.51	o
C-22	33.0	[CH_2]	1.71-1.78	o	33.6	[CH_2]	1.80-1.86	o
			1.78-1.85	o			2.02-2.10	dt [*J* = 4.4; 13.8]
C-23	28.7	[CH_3]	1.31-1.34	s	28.6	[CH_3]	1.31-1.36	s
C-24	17.4	[CH_3]	0.98-1.03	s	17.3	[CH_3]	0.98-1.00	s
C-25	16.0	[CH_3]	0.77-0.82	s	15.8	[CH_3]	0.80-0.82	s
C-26	18.0	[CH_3]	1.09-1.12	s	17.8	[CH_3]	0.98-1.00	s
C-27	26.6	[CH_3]	1.27-1.32	s	26.6	[CH_3]	1.32-1.34	s
C-28	176.9	[C]	-	-	180.5	[C]	-	-
C-29	33.6	[CH_3]	0.90-0.96	s	33.7	[CH_3]	0.96-0.98	s
C-30	24.2	[CH_3]	0.89-0.92	s	24.1	[CH_3]	0.91-0.95	s

Edelmann et al. (Table S10)

Table S10. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR assignment (126 MHz, pyridine-d_5) of the sugar moiety of chikusetsu saponin IVa (**9**) and calenduloside E (**10**).



	Chikusetsu saponin IVa				**Calenduloside E**			
Position	**δC**	**HSQC**	**δH**	**M [*J*. Hz]**	**δC**	**HSQC**	**δH**	**M [*J*. Hz]**
C-1'	107.8	[CH]	5.05-5.08	d [*J* = 7.8 Hz]	107.6	[CH]	5.02-5.06	d [*J* = 8.5]
C-2'	76.0	[CH]	4.14-4.19	dd [*J* = 8.6; 8.6]	75.9	[CH]	4.12-4.17	t [*J* = 8.5; 8.5]
C-3'	78.6	[CH]	4.32-4.39	dd [*J* = 8.6; 8.6]	78.6	[CH]	4.32-4.37	t [*J* = 8.9; 8.9]
C-4'	73.9	[CH]	4.63-4.67	dd [*J* = 9.4; 9.4]	73.9	[CH]	4.56-4.60	t [*J* = 9.2; 9.2]
C-5'	78.4	[CH]	4.71-4.77	d [*J* = 10.2 Hz]	78.0	[CH]	4.64-4.69	d [*J* = 9.8]
C-6'	173.3	[C]	-	-	173.8	[C]	-	-
C-1''''	96.2	[CH]	6.33-6.36	d [*J* = 8.6 Hz]	-	-	-	-
C-2''''	74.6	[CH]	4.20-4.25	dd [*J* = 8.6; 8.6]	-	-	-	-
C-3''''	79.4	[CH]	4.28-4.34	dd [*J* = 8.9; 8.9]	-	-	-	-
C-4''''	71.6	[CH]	4.36-4.41	dd [*J* = 9.1; 9.1]	-	-	-	-
C-5''''	79.8	[CH]	4.03-4.08	m	-	-	-	-
C-6''''	62.7	[CH_2]	4.43-4.45	m	-	-	-	-
			4.46-4.49	m	-	-	-	-

Structure Elucidation of Sugar Beet Saponins (9-11). *Chikusetsu Saponin IVa (**9**)*. LC-MS (ESI$^-$) analysis revealed *m/z* 793.5 as the pseudo molecular ion ([M−H]$^-$), thus suggesting a molecular mass of 794.5 Da for **9**. An empirical formula of $C_{42}H_{66}O_{14}$ was indicated by UPLC-ToF-MS, based on the measured *m/z* 793.4374. Accurate mass and sum formula showed that the substance is not betavulgaroside IV but rather chikusetsu saponin IVa, which is another literature known sugar beet saponin.[27] MS/MS analysis revealed the neutral losses of 176 amu and 162 amu, which demonstrated the presence of one hexose, and one uronic acid. They were identified as D-glucuronic acid and D-glucose via the hydrolysis described above. Indications for oleanolic acid as the aglycone were based on the daughter ion *m/z* 455.3.[39] The structure of compound **9** was confirmed with supplementary NMR experiments. These showed that glucose is linked to the aglycon with an ester bond that was observed through the HMBC coupling of H-C(1””) to C-28. D-glucuronic acid is linked to the aglycone with an ether bond between C-3 and C-1’ that was also observed in HMBC spectrum. With the exception of carbon atom C-21 and C-22, which were interchanged and carbon atoms C-3'''' and C-5'''' as well as hydrogen atoms H-C(3''') and H-C(5''') which were interchanged, all data are well in line to the literature.[53]

*Calenduloside E (**10**)*. The UPLC-ToF-MS analysis of fractions A-I to A-X gave hints for the presence of a further literature known sugar beet saponin (**10**).[26] Comparison of accurate mass, fragments, retention time and CCS-values obtained with a commercial reference by UPLC-ToF-MS confirmed the identity and revealed an empirical formula of $C_{36}H_{56}O_9$, based on the measured *m/z* 631.3851. MS/MS analysis led to the identification of the daughter ion *m/z* 455.3 [M−176−H]$^-$, thus demonstrating the presence of one uronic acid, which is typical for calenduloside E.[26] D-Glucuronic acid was identified as the only saccharide. A daughter ion at *m/z* 455.3 Da indicated that oleanolic acid is the aglycone of compound no. **10**.[39] NMR experiments confirmed the chemical structure of compound **10**. For example, the connection between oleanolic acid and the D-glucuronic acid moiety was verified by HMBC experiments. After complete assignment of all atoms **10** was confirmed to be calenduloside E.[58-60]

*Ginsenoside R0 (**11**).* Indications about the occurrence of compound **11** that has never been observed within sugar beet were found by means of UPLC-ToF-MS analysis of fractions A-I to A-X.[57] Accurate mass, retention time and CCS-value are the same as the ones of a commercial reference and, therefore, confirmed the identity. Furthermore, a molecular mass of 956.4 Da was obtained through LC-MS analysis that measured a pseudo molecular ion ($[M-H]^-$) of *m/z* 955.4. It was confirmed by UPLC-ToF-MS indicating an empirical formula of $C_{48}H_{76}O_{19}$, based on the measured *m/z* 955.4911. These additional data also matched to compound **11**, which is a substructure of compound **7** and compound **8**.[61] Further LC-MS/MS experiments led to the identification of the daughter ions *m/z* 793.3 $[M-162-H]^-$, 731.3 $[M-162-44-18-H]^-$, 631.3 $[M-162-162-H]^-$, 569.3 $[M-162-162-44-18-H]^-$, and 455.2 $[M-176-162-162-H]^-$, thus demonstrating the presence of an acid group, two hexoses, and one uronic acid, which is typical for compound **11**.[61] The corresponding saccharides were identified as D-glucuronic acid and D-glucose. Daughter ion *m/z* 455.2 indicates that oleanolic acid is the aglycone of compound no. **11**.[39] The structure of compound **11** was confirmed with supplementary NMR experiments and a complete assignment was carried out. It showed that oleanolic acid is part of the aglycone and saccharide side chains comply with the literature, although a difference of about 0.3 ppm between the literature values and the data shown in Table S3 and S4 was found for almost all protons.[61] The chemical shifts shown for compound **11** are comparable to the other compounds, analyzed by us.

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9.2 Fast and Sensitive LC-MS/MS Method for the Quantitation of Saponins in Various Sugar Beet Materials

Summary:

A fast and sensitive LC-MS/MS method for the simultaneously quantification of saponins in various sugar beet materials and plant compartments was developed in this work. In total, eleven different saponins were quantified, these being: betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1→2)-(*β*-D-xylopyranosyl-(1→3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsusaponin IVa (**9**), calenduloside E (**10**) and ginsenoside R0 (**11**). Overall, the beets and leaves of twelve cultivars were examined for the presence of these saponins, with one variety being studied in particular detail. Distinct compartments of this variety were examined with respect to their saponin distribution. It was found that the quantity of saponins not only varies between different compartments but also between plant varieties. The total amount of saponin found in various sugar beet root varieties was in the range of 862 mg/kg to 2,452 mg/kg. The amount in leaves was generally higher, with concentrations ranging from 907 mg/kg to 5,398 mg/kg. Among other sugar beet-related materials, a total saponin quantity of 10.3 g/kg was determined in the by-product stream dried sugar beet pulp. This quantity is only exceeded by sugar beet fiber, with 12.7 g/kg. Finally, the occurrence of individual saponins in sugar beet and sugar beet materials was published for the first time.

The individual contribution of Matthias Edelmann is specified below:

Matthias Edelmann developed the investigation concept and carried out the laboratory work. The development of the LC–MS/MS method was supported by Dr. Christian Schmid and Katharina Booz. Matthias Edelmann analyzed all the data, discussed the results, designed the figures, developed the story of the paper, wrote the first draft of the manuscript, and partially revised the paper.

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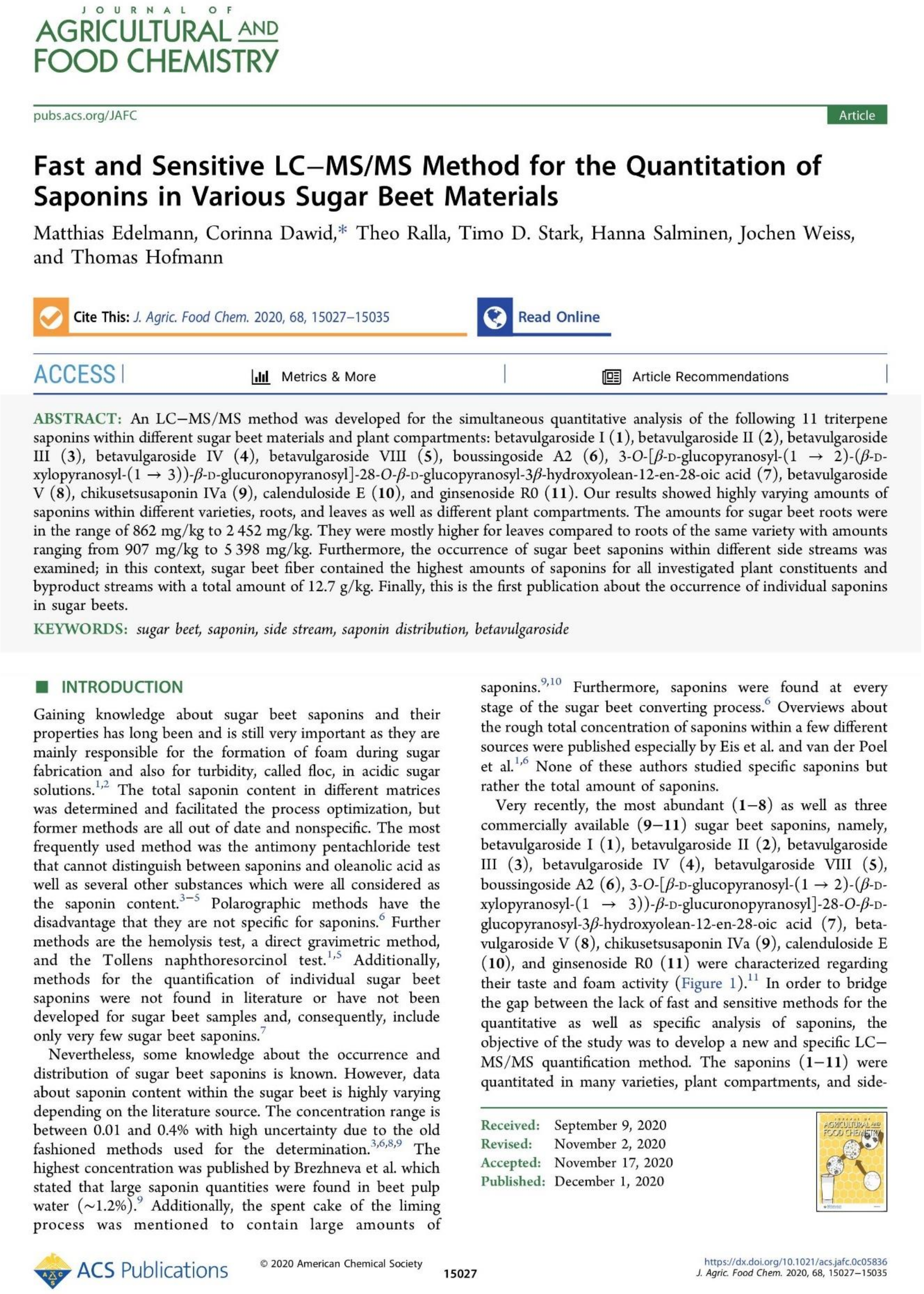

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Fast and Sensitive LC–MS/MS Method for the Quantitation of Saponins in Various Sugar Beet Materials

Matthias Edelmann, Corinna Dawid,* Theo Ralla, Timo D. Stark, Hanna Salminen, Jochen Weiss, and Thomas Hofmann

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ABSTRACT: An LC–MS/MS method was developed for the simultaneous quantitative analysis of the following 11 triterpene saponins within different sugar beet materials and plant compartments: betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsusaponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**). Our results showed highly varying amounts of saponins within different varieties, roots, and leaves as well as different plant compartments. The amounts for sugar beet roots were in the range of 862 mg/kg to 2 452 mg/kg. They were mostly higher for leaves compared to roots of the same variety with amounts ranging from 907 mg/kg to 5 398 mg/kg. Furthermore, the occurrence of sugar beet saponins within different side streams was examined; in this context, sugar beet fiber contained the highest amounts of saponins for all investigated plant constituents and byproduct streams with a total amount of 12.7 g/kg. Finally, this is the first publication about the occurrence of individual saponins in sugar beets.

KEYWORDS: *sugar beet, saponin, side stream, saponin distribution, betavulgaroside*

■ INTRODUCTION

Gaining knowledge about sugar beet saponins and their properties has long been and is still very important as they are mainly responsible for the formation of foam during sugar fabrication and also for turbidity, called floc, in acidic sugar solutions.[1,2] The total saponin content in different matrices was determined and facilitated the process optimization, but former methods are all out of date and nonspecific. The most frequently used method was the antimony pentachloride test that cannot distinguish between saponins and oleanolic acid as well as several other substances which were all considered as the saponin content.[3−5] Polarographic methods have the disadvantage that they are not specific for saponins.[6] Further methods are the hemolysis test, a direct gravimetric method, and the Tollens naphthoresorcinol test.[1,5] Additionally, methods for the quantification of individual sugar beet saponins were not found in literature or have not been developed for sugar beet samples and, consequently, include only very few sugar beet saponins.[7]

Nevertheless, some knowledge about the occurrence and distribution of sugar beet saponins is known. However, data about saponin content within the sugar beet is highly varying depending on the literature source. The concentration range is between 0.01 and 0.4% with high uncertainty due to the old fashioned methods used for the determination.[3,6,8,9] The highest concentration was published by Brezhneva et al. which stated that large saponin quantities were found in beet pulp water (~1.2%).[9] Additionally, the spent cake of the liming process was mentioned to contain large amounts of saponins.[9,10] Furthermore, saponins were found at every stage of the sugar beet converting process.[6] Overviews about the rough total concentration of saponins within a few different sources were published especially by Eis et al. and van der Poel et al.[1,6] None of these authors studied specific saponins but rather the total amount of saponins.

Very recently, the most abundant (**1**−**8**) as well as three commercially available (**9**−**11**) sugar beet saponins, namely, betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsusaponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**) were characterized regarding their taste and foam activity (Figure 1).[11] In order to bridge the gap between the lack of fast and sensitive methods for the quantitative as well as specific analysis of saponins, the objective of the study was to develop a new and specific LC–MS/MS quantification method. The saponins (**1**−**11**) were quantitated in many varieties, plant compartments, and side-

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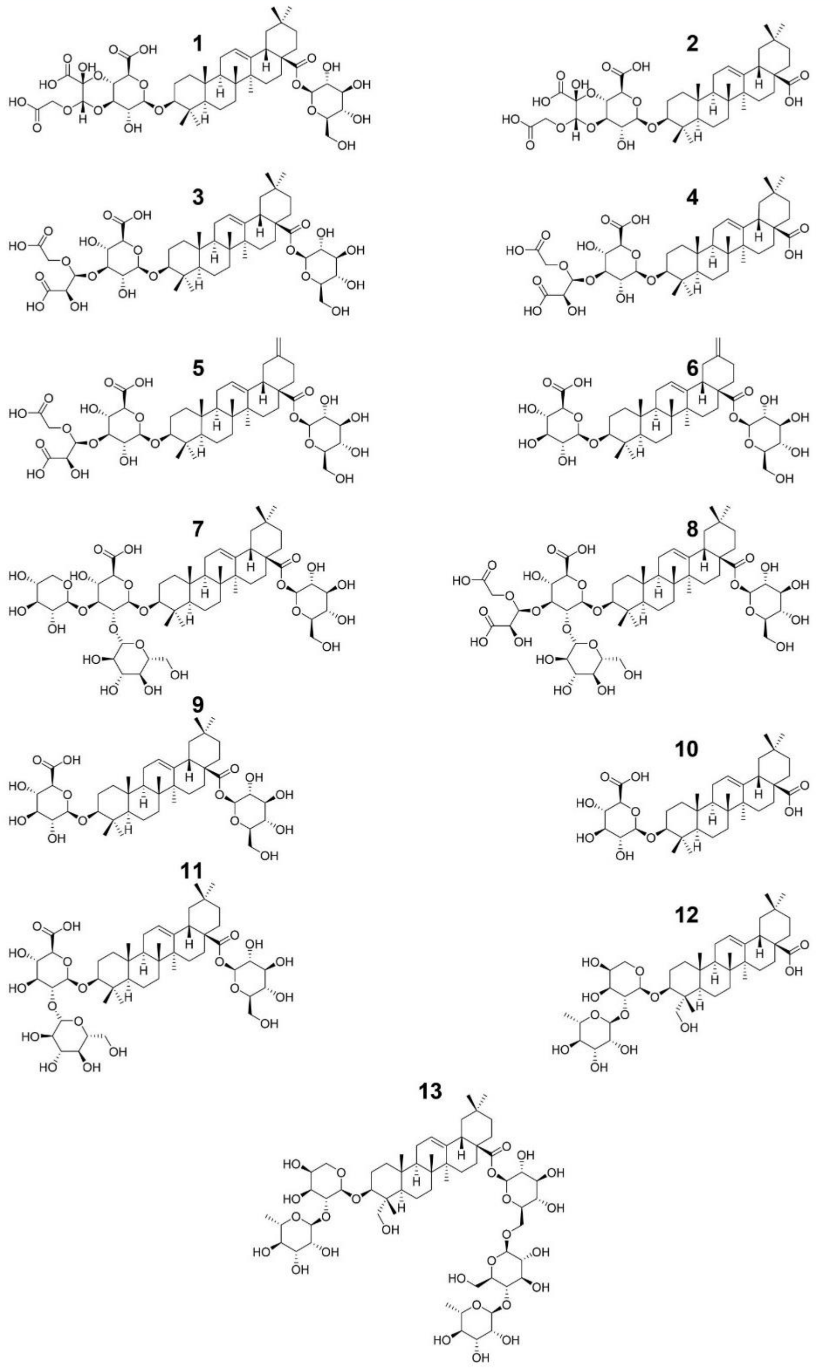

Figure 1. Sugar beet saponins (**1**−**11**) and internal standards (**12** and **13**): betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), betavulgaroside V (**8**), chikusetsu saponin IVa (**9**), calenduloside (**10**), ginsenoside R0 (**11**), *α*-hederin (**12**), and hederacoside C (**13**).

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streams occurring during the sugar beet converting process as well as within the beetroot, sugar beet fiber, and Goldsaft.

■ MATERIALS AND METHODS

Chemicals. Ultrapure water was obtained from a Milli-Q Integral 3 system (Millipore, Molsheim, France). HPLC grade methanol was obtained from J. T. Baker (Deventer, Netherlands), acetonitrile was of LC−MS grade (Honeywell, Seelze, Germany), and formic acid (98−100%) was obtained from Merck (Darmstadt, Germany). Betavulgaroside I (**1**), betavulgaroside II (**2**), betavulgaroside III (**3**), betavulgaroside IV (**4**), betavulgaroside VIII (**5**), boussingoside A2 (**6**), 3-*O*-[*β*-D-glucopyranosyl-(1 → 2)-(*β*-D-xylopyranosyl-(1 → 3))-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid (**7**), and betavulgaroside V (**8**) were isolated from sugar beet material as recently described.[11] Chikusetsusaponin IVa (**9**) and ginsenoside R0 (**11**) were obtained from PhytoLab (Vestenbergsgreuth, Germany). Calenduloside E (**10**) was obtained from Carbosynth (Compton, U.K.). *α*-Hederin (**12**) and hederacoside C (**13**) were obtained from Extrasynthese (Lyon, France), and all of them are shown in Figure 1.

Plant Material. The two sugar beet varieties Annemaria and Artus were obtained as freeze-dried material and the varieties Beretta, Daphna, Eva Maria, Kleist, Rhinema, as well as Hannibal as fresh samples (Pfeifer & Langen GmbH Co. KG, Köln, Germany). The fresh varieties BTS, Lisanna, and Marley were thankfully provided by a local sugar beet farmer (county Munich). Fresh materials were delivered from September to November 2018. They were washed, sliced, and crushed with the help of liquid nitrogen (2.5 L per kg) shortly after delivery. All sample materials were kept at −25 °C until further use. The variety Hannibal was separated with a knife into different compartments yielding leaves, stalk, pod (top, middle, and bottom part), as well as the inner part (top, middle, and bottom part; see Figure 2) before they were crushed. Stalk (compressed stem) and top (hypocotyl) of the root were defined according to Biancardi.[12] The bottom part of the root was defined as the lower third of the root, and the middle part was the remaining part. The inner part of the root was obtained by cutting off the pod, which was defined to be about 5 mm thick.

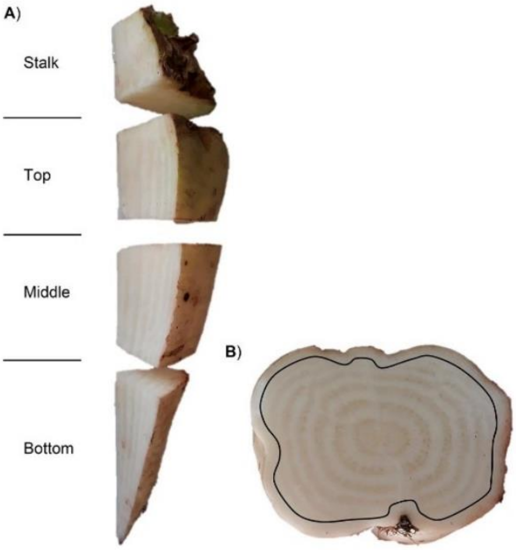

Figure 2. (A) Sugar beet variety Hannibal that was split into different parts (leaves; stalk; top, middle, and bottom of the inner part; as well as top, middle, and bottom of the pod) for the investigation of the saponin distribution. (B) Cross-section of the sugar beet plant. The black circle illustrated the part where the pod was separated from the inner part.

Other Sample Materials. Raw juice, dried sugar beet pulp, flume water, and process water were obtained from Pfeifer & Langen GmbH Co. KG. Thick juice and carbonation sludge were obtained from Südzucker AG (Mannheim, Germany). Beetroot and Goldsaft were obtained from local supermarkets (Freising, Germany), and molasses (Königshofer Melasse) as well as sugar beet fiber (Fibrex) were obtained via the Internet.

Purity Control/Concentration Determination of the Standard Substances and Internal Standards with ^{1}H NMR. Concentration determination was performed by means of ^{1}H NMR spectra, acquired on a 500 MHz Bruker Avance III spectrometer, equipped with a triple-resolution cryoprobe (Bruker, Rheinstetten, Germany). Analysis of the spectra was performed with MestReNova 12.0.0-20080 (Mestrelab Research, Santiago de Compostela, Spain).

UHPLC−MS/MS. The analysis was performed on a Triple Quad 6500$^+$ System (AB Sciex, Darmstadt, Germany) combined with an ExionLC system (consisting of an ExionLC AD Pump, ExionLC Degaser, ExionLC AD Autosampler, ExionLC AC Column Oven, ExionLC Controller, and ExionLC Solvent Valve; AB Sciex Instruments, Darmstadt, Germany). Analyst 1.6.3 was used to control the devices. The temperature of the column oven was set at 60 °C, and the autosampler temperature was 15 °C. A Nucleodur C_{18} Pyramid column (150 mm × 2.0 mm, 1.8 *μ*m, Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used for the separation. Formic acid (1%, solvent A) and acetonitrile with 1% formic acid (solvent B) were used as the mobile phases. The following solvent gradient (0.4 mL/min) was used after sample injection (1 *μ*L): 0 min, 100% A; 1 min, 100% A; 2 min, 42.5% B; 3 min, 42.5% B; 7 min, 55% B; 9 min, 80% B; 9.5 min, 100% B; 11 min, 100% B; 12 min, 100% A; and 15 min, 100% A. The MS-system was operated in the multiple reaction monitoring (MRM) mode using the negative electrospray ionization (ESI$^-$) mode. Ion spray voltage was set at −4500 V, entrance potential at −10 V, and source temperature at 450 °C. Nitrogen served as the curtain- and collision gases. Curtain gas, 35 psi; gas 1, 55 psi; and gas 2, 65 psi. Mass transitions of the pseudomolecular ions ($[M - H]^-$) into specific product ions are shown in Figure 3 and summarized in Table 1. Optimized fragmentation parameters were obtained by injection of tuning solutions containing the analytes in a mixture of acetonitrile/water (50/50; v/v, each) and flow injection (10 *μ*L/min).

Quantitative Analysis and Method Validation. *Internal Standard Preparation.* A stock solution of the internal standards *α*-hederin (8 mg/100 mL) and hederacoside C (8 mg/100 mL) was prepared in 75% methanol and stored at 5 °C until further use. The exact concentration was obtained by preparing the stock solution with saponin solutions consisting of *α*-hederin (methanol-d_4) as well as hederacoside C (80/20%, acetonitrile-d_3/D_2O) and the measurement of their concentrations with the help of qNMR according to Frank et al.[13]

Calibration Curve and Linear Range. A calibration stock solution was prepared containing compounds **1**−**11** which were dissolved in different mixtures of deuterated water and deuterated methanol. Their concentrations were determined by means of qNMR according to Frank et al.[13] Calibration solutions were prepared by 1:1 stepwise dilution of the stock solution in order to get 17 calibration points over a concentration range from 50 *μ*mol/L to 0.8 nmol/L and keeping constant levels of the internal standards *α*-hederin (10.7 *μ*g/mL; **12**) and hederacoside C (10.7 *μ*g/mL; **13**). Calibrations curves were prepared after UHPLC−MS/MS analysis by plotting the normalized peak area ratios (peak area of the corresponding analyte divided by the peak area of the internal standard) of the analytes against their concentrations (each sample in triplicate) showing a linear response with correlation coefficients of ≥0.98 for all components with the exception of **10** ($r^2 \geq 0.95$) which quickly reached detector saturation.

Analysis of Saponins. Each sample was analyzed in triplicate. Root and leaf samples of freeze-dried material (~750 or ~170 mg) were

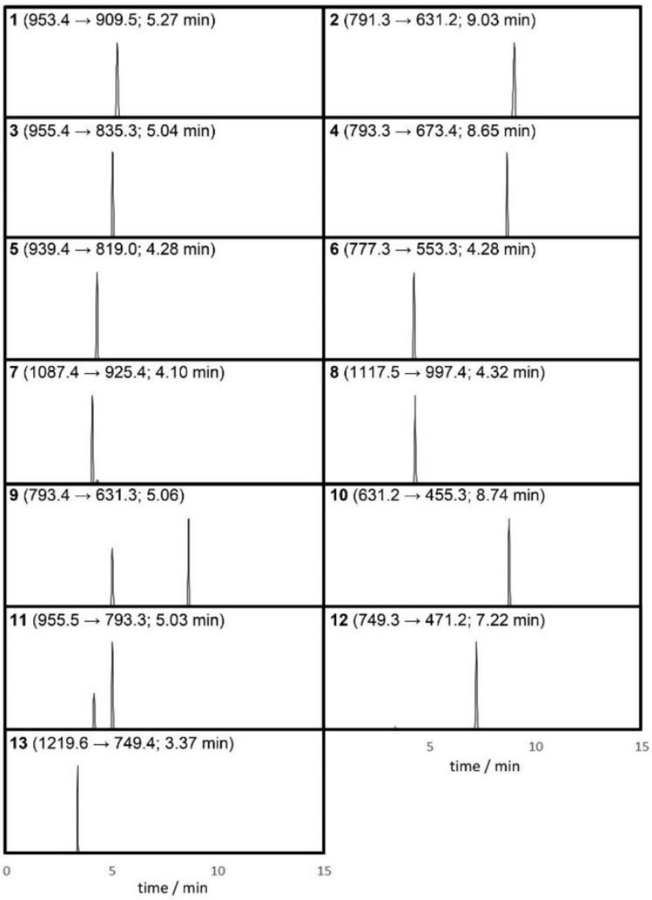

Figure 3. LC−MS/MS analysis of a sugar beet sample, showing the mass transition traces of the sugar beet saponins (**1**−**11**) and the internal standards (**12** and **13**). The signal intensity of each mass transition is normalized.

weighed into a precellys lysing kit (CK28_15 mL; Bertin Technologies SAS, Montigny-le-Bretonneux, France). The same lysing kit was used for freeze-dried flume water (~50 mg) and freeze-dried process water (~40 mg). Dried sugar beet pulp and sugar beet fiber (~100 mg, each) were weighed into a different precellys lysing kit (CK68-R_15 mL; Bertin Technologies SAS). All samples were immediately spiked with the combined internal standards α-hederin and hederacoside C (each 80 μg/mL, 1 mL) after homogenization. Subsequently, methanol (70%) was given to the dry and methanol (75%) was given to water containing samples (6.5 mL, each), then repeatedly homogenized (4 × 30 s, 6500 rpm, 60 s delay time) using a precellys evolution homogenizer (Bertin Technologies SAS). After an equilibration time of 60 min, the mixture was centrifuged (4.4 krpm for 10 min) and membrane filtered (Minisart RC 15, 0.45 μm, Sartorius, Göttingen, Germany). Samples from raw juice (~25 mg), thick juice (~40 mg), molasses (~50 mg), and Goldsaft (~80 mg) were prepared without using the homogenizer. They were spiked with the internal standards solution (0.4 mL) and methanol (2.6 mL, 70%). Finally, after shaking, they were membrane filtered (Minisart RC 15, 0.45 μm, Sartorius). Additionally, different sugar products originating from sugar beet were dissolved in water with a concentration of 10 mg mL^{-1}.

Recovery. Recovery experiments were performed with sugar beet root material of the variety Hannibal. A specific compartment was selected which originated from the upper part of the inner plant material. The corresponding samples (~750 mg) were spiked with three defined but different concentration levels of each analyte (**1**−**5**, **7**, and **8**) as well as fixed concentrations of the internal standards (**12** and **13**). The total volume (7.5 mL) was adjusted with methanol (75%), and the extraction was carried out as detailed above. All samples were prepared in triplicate together with samples not including spiked saponins.

Interday Precision. One sugar beet root sample of the Daphna variety was repeatedly analyzed on different days (10×, each).

■ RESULTS AND DISCUSSION

So far, sugar beet saponins have never been quantified at the molecular level. This has several reasons, on the one hand only chikusetsusaponin IVa (**9**), calenduloside E (**10**), and ginsenoside R0 (**11**) are commercially available. On the other hand, it is quite challenging to isolate these compounds. Furthermore, it is known that some saponins are derivatized into methyl esters during extraction with methanol under

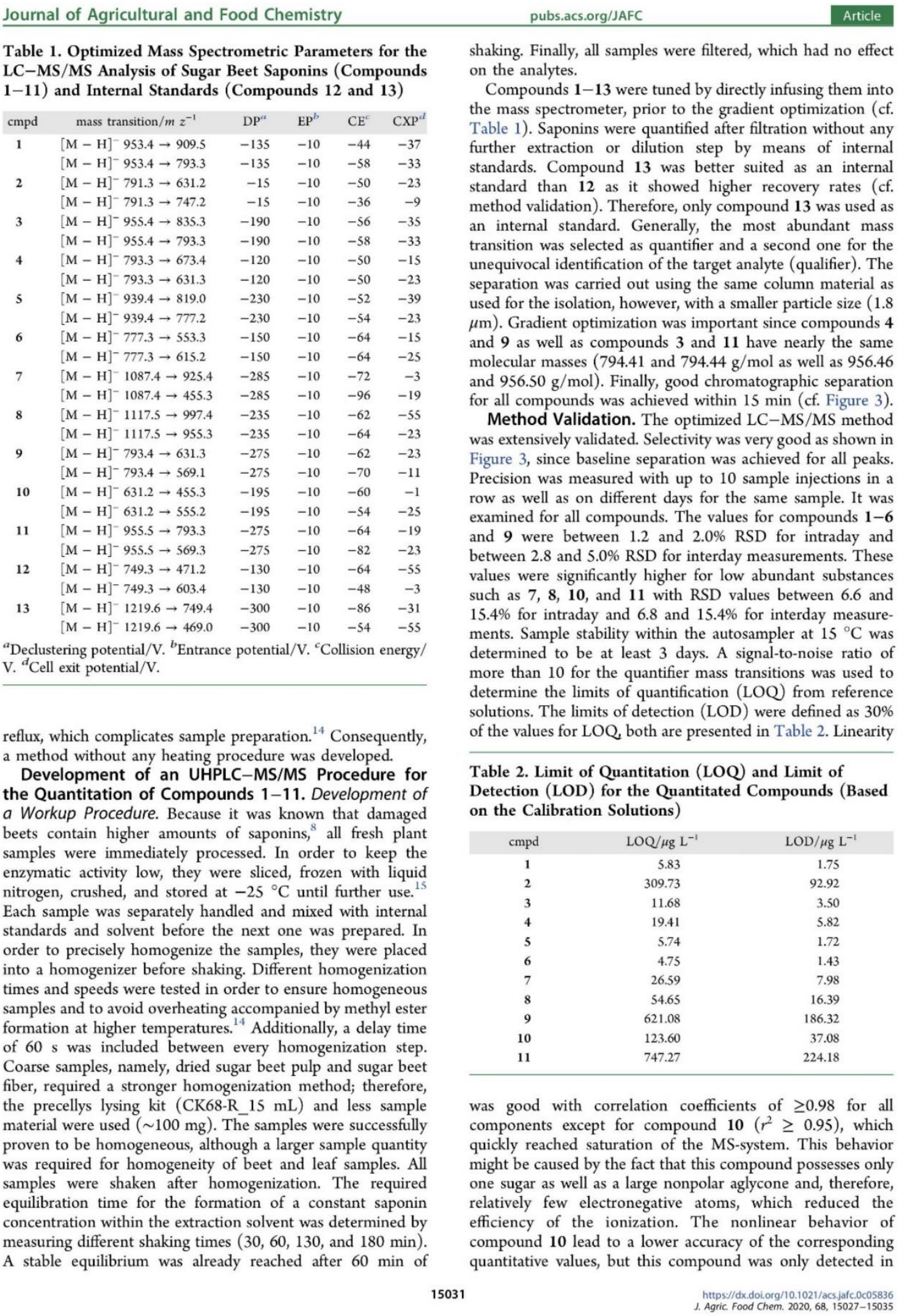

Table 1. Optimized Mass Spectrometric Parameters for the LC−MS/MS Analysis of Sugar Beet Saponins (Compounds 1−11) and Internal Standards (Compounds 12 and 13)

cmpd	mass transition/$m\ z^{-1}$	DP[a]	EP[b]	CE[c]	CXP[d]
1	$[M - H]^-$ 953.4 → 909.5	−135	−10	−44	−37
	$[M - H]^-$ 953.4 → 793.3	−135	−10	−58	−33
2	$[M - H]^-$ 791.3 → 631.2	−15	−10	−50	−23
	$[M - H]^-$ 791.3 → 747.2	−15	−10	−36	−9
3	$[M - H]^-$ 955.4 → 835.3	−190	−10	−56	−35
	$[M - H]^-$ 955.4 → 793.3	−190	−10	−58	−33
4	$[M - H]^-$ 793.3 → 673.4	−120	−10	−50	−15
	$[M - H]^-$ 793.3 → 631.3	−120	−10	−50	−23
5	$[M - H]^-$ 939.4 → 819.0	−230	−10	−52	−39
	$[M - H]^-$ 939.4 → 777.2	−230	−10	−54	−23
6	$[M - H]^-$ 777.3 → 553.3	−150	−10	−64	−15
	$[M - H]^-$ 777.3 → 615.2	−150	−10	−64	−25
7	$[M - H]^-$ 1087.4 → 925.4	−285	−10	−72	−3
	$[M - H]^-$ 1087.4 → 455.3	−285	−10	−96	−19
8	$[M - H]^-$ 1117.5 → 997.4	−235	−10	−62	−55
	$[M - H]^-$ 1117.5 → 955.3	−235	−10	−64	−23
9	$[M - H]^-$ 793.4 → 631.3	−275	−10	−62	−23
	$[M - H]^-$ 793.4 → 569.1	−275	−10	−70	−11
10	$[M - H]^-$ 631.2 → 455.3	−195	−10	−60	−1
	$[M - H]^-$ 631.2 → 555.2	−195	−10	−54	−25
11	$[M - H]^-$ 955.5 → 793.3	−275	−10	−64	−19
	$[M - H]^-$ 955.5 → 569.3	−275	−10	−82	−23
12	$[M - H]^-$ 749.3 → 471.2	−130	−10	−64	−55
	$[M - H]^-$ 749.3 → 603.4	−130	−10	−48	−3
13	$[M - H]^-$ 1219.6 → 749.4	−300	−10	−86	−31
	$[M - H]^-$ 1219.6 → 469.0	−300	−10	−54	−55

[a]Declustering potential/V. [b]Entrance potential/V. [c]Collision energy/V. [d]Cell exit potential/V.

reflux, which complicates sample preparation.[14] Consequently, a method without any heating procedure was developed.

Development of an UHPLC−MS/MS Procedure for the Quantitation of Compounds 1−11. *Development of a Workup Procedure.* Because it was known that damaged beets contain higher amounts of saponins,[8] all fresh plant samples were immediately processed. In order to keep the enzymatic activity low, they were sliced, frozen with liquid nitrogen, crushed, and stored at −25 °C until further use.[15] Each sample was separately handled and mixed with internal standards and solvent before the next one was prepared. In order to precisely homogenize the samples, they were placed into a homogenizer before shaking. Different homogenization times and speeds were tested in order to ensure homogeneous samples and to avoid overheating accompanied by methyl ester formation at higher temperatures.[14] Additionally, a delay time of 60 s was included between every homogenization step. Coarse samples, namely, dried sugar beet pulp and sugar beet fiber, required a stronger homogenization method; therefore, the precellys lysing kit (CK68-R_15 mL) and less sample material were used (∼100 mg). The samples were successfully proven to be homogeneous, although a larger sample quantity was required for homogeneity of beet and leaf samples. All samples were shaken after homogenization. The required equilibration time for the formation of a constant saponin concentration within the extraction solvent was determined by measuring different shaking times (30, 60, 130, and 180 min). A stable equilibrium was already reached after 60 min of shaking. Finally, all samples were filtered, which had no effect on the analytes.

Compounds **1**−**13** were tuned by directly infusing them into the mass spectrometer, prior to the gradient optimization (cf. Table 1). Saponins were quantified after filtration without any further extraction or dilution step by means of internal standards. Compound **13** was better suited as an internal standard than **12** as it showed higher recovery rates (cf. method validation). Therefore, only compound **13** was used as an internal standard. Generally, the most abundant mass transition was selected as quantifier and a second one for the unequivocal identification of the target analyte (qualifier). The separation was carried out using the same column material as used for the isolation, however, with a smaller particle size (1.8 μm). Gradient optimization was important since compounds **4** and **9** as well as compounds **3** and **11** have nearly the same molecular masses (794.41 and 794.44 g/mol as well as 956.46 and 956.50 g/mol). Finally, good chromatographic separation for all compounds was achieved within 15 min (cf. Figure 3).

Method Validation. The optimized LC−MS/MS method was extensively validated. Selectivity was very good as shown in Figure 3, since baseline separation was achieved for all peaks. Precision was measured with up to 10 sample injections in a row as well as on different days for the same sample. It was examined for all compounds. The values for compounds **1**−**6** and **9** were between 1.2 and 2.0% RSD for intraday and between 2.8 and 5.0% RSD for interday measurements. These values were significantly higher for low abundant substances such as **7**, **8**, **10**, and **11** with RSD values between 6.6 and 15.4% for intraday and 6.8 and 15.4% for interday measurements. Sample stability within the autosampler at 15 °C was determined to be at least 3 days. A signal-to-noise ratio of more than 10 for the quantifier mass transitions was used to determine the limits of quantification (LOQ) from reference solutions. The limits of detection (LOD) were defined as 30% of the values for LOQ, both are presented in Table 2. Linearity was good with correlation coefficients of ≥0.98 for all components except for compound **10** ($r^2 \geq 0.95$), which quickly reached saturation of the MS-system. This behavior might be caused by the fact that this compound possesses only one sugar as well as a large nonpolar aglycone and, therefore, relatively few electronegative atoms, which reduced the efficiency of the ionization. The nonlinear behavior of compound **10** lead to a lower accuracy of the corresponding quantitative values, but this compound was only detected in

Table 2. Limit of Quantitation (LOQ) and Limit of Detection (LOD) for the Quantitated Compounds (Based on the Calibration Solutions)

cmpd	LOQ/μg L^{-1}	LOD/μg L^{-1}
1	5.83	1.75
2	309.73	92.92
3	11.68	3.50
4	19.41	5.82
5	5.74	1.72
6	4.75	1.43
7	26.59	7.98
8	54.65	16.39
9	621.08	186.32
10	123.60	37.08
11	747.27	224.18

four samples (cf. Table 3). Finally, recovery rates were checked by spiking three different amounts of reference standards to the same sample material. The results, obtained with compound **13** as an internal standard, were in the range of 48.6% to 59.0% for compounds **1**, **3**, **5**, **7**, and **8** (bidesmosidic saponins) as well as 244.3% and 162.7% for compounds **2** and **4** (monodesmosidic saponins). Recovery rates were in the same order of magnitude and revealed difficulties with the sample matrix, as it was too low for bidesmosidic saponins and too high for monodesmosidic ones. The used method is a compromise to simultaneously determine mono- and bidesmosidic saponins. Better suited internal standards, e.g., stable isotopically labeled, might be the best way to improve recovery since the two different types of saponins (mono- and bisdesmosidic ones) highlighted divergent recovery rates.

Appearance of Saponins in Different Sugar Beet (Related) Materials, Varieties, Compartments, and Beetroot. Not only differences between sugar beet leaves and root for 11 different varieties were investigated but also the localization of saponins within different plant compartments (Table 3). Furthermore, different side-streams or constituents formed during the fabrication of sugar and the commercial products sugar beet fiber and Goldsaft were analyzed. Beetroot was selected as it is known to contain to some extent the same saponins as sugar beet and both belong to the same subspecies.[16,17] In particular, the interested reader will find useful information about the sugar beet converting process in the *Beet-Sugar Handbook*.[8]

Saponin Distribution between Sugar Beet Roots and Leaves. Total saponin amounts varied highly, for roots as well as for leaves. The lowest amount was found for the root of variety Kleist with 862 mg/kg, followed by the leaves of variety BTS with 907 mg/kg. The highest amount for roots was found within the variety Annemaria with an amount of 2 452 mg/kg. The concentrations within leaves were mostly higher with quantities of up to 5 398 mg/kg for the variety Daphna. These results partly explain the range between 0.01% and 0.4% for sugar beet saponins published within the literature.[3,6,8,9]

The saponin distribution of individual saponins between leaves and roots has already been investigated in other plants. Shimoyamada et al. found that the amount of soyasaponin I within soybean plants is much higher within leaves compared to roots for six different varieties.[18] Other authors with small data sets showed the opposite for *allium nigrum* L. and *glycyrrhiza glabra*, which means that the amount of saponins within roots is higher compared to leaves.[19,20] It might be possible that different plants behave variable. Huhman et al. showed that a seemingly random distribution between leaves and roots in the plant *medicago truncatula* also exist.[21] Our results demonstrated for sugar beet that always only compound **2** indicated a lower content within sugar beet leaves compared to roots. The opposite was observed for compounds **7** and **8** which always revealed higher values within leaves. Compounds **2** and **9**−**11** only occurred in small quantities within leaves. Exclusively, the variety Annemaria exhibited compound **11**. Compounds **2** and **6** were present in relatively small quantities within leaves. The major compounds for leaves are compound **1**, **3**−**5**, **8**, and especially compound **7**. Although, compound **7** was the most abundant compound within leaves, only very small amounts were quantified in roots. The two major compounds for roots are **1** and **3**, followed by compounds **4**, **2**, and **5**. Compounds **6** and **8** only occurred in traces within roots.

Saponin Distribution within Different Plant Compartments. Apart from the different distribution between leaves and root, it is known that saponin abundance in the plant kingdom varies in individual organs and tissues,[22] as well as on geography, season, plant age, soil conditions, and light levels.[23,24] Nevertheless, several conclusions can be drawn from our data, since one variety, the variety Hannibal, was separated into different plant compartments. The amount of the four different saponins **5**−**8** highly varied between different plant compartments. The lowest amounts were measured for the inner parts of the root, significantly higher for the pod and the highest for stalk and leaves. In contrast, the saponin content for compounds **1**−**4** tended to be the highest for the pod and was clearly lower for the inner part as well as stalk and leaves. The same observation was indicated for asparagus.[25] However, our data revealed two opposing trends, and it was not possible to detect **9**−**11** within the variety Hannibal above their limit of detection.

Saponins within Sugar Beet Pulp and Sugar Beet Fiber. As suggested in our previous studies, large amounts of saponins are found in sugar beet pulp (sugar-exhausted sugar beet).[11] These cannot be completely removed by extraction with warm water and this is not intended (during the sugar beet converting process) due to their disturbing foam activity.[6] Sugar beet pulp could be a particularly good saponin source as it occurs in large amounts and contains the second-highest concentration of saponins within the tested samples. The total amount of saponins was 10.3 g/kg. The highest saponin amount (12.7 g/kg) was found within commercially available sugar beet fiber. It was the only sample material that contained all 11 saponins and some of them with the highest amount of individual saponins (Table 3). Compound **10** occurred in particularly large quantities and might be generated due to the intensive treatment of the material during its production process.[26]

Saponin Occurrence in Raw and Thick Juices. Raw juice, which is the desired product after beet extraction, contained only tiny amounts of saponins, although large quantities of some saponins were found in many types of beet. It was not possible to quantify compounds **5**−**8**, which was not expected due to their occurrence in sugar beet root. Moreover, the amounts of compounds **1**−**4** are completely different than supposed. The sparingly soluble compounds **2** and **4** occurred in particularly high quantities, whereas the two major compounds of roots, compounds **1** and **3**, in low quantities. This is remarkable because raw juice was only thermally treated.[6] The much lower amounts within thick juice can be explained by the complex sugar purification process and especially the liming and carbonation steps.[6] In particular, the content of poorly soluble substances **2** and **4** was significantly reduced. Compounds **1** and **3** remained within the thick juice at lower concentrations.

Saponin Occurrence in Beet Sugar. It is known that saponins cause floc in acidic sugar solutions.[2] However, this does not seem to be a problem anymore. Presumably, the optimization of the sugar beet converting process has resulted in less saponins to be present in the final product. Low amounts were already observed for thick juice, the intermediate product of sugar, and crystallization further reduces their concentration.[6] Consequently, we have not found any of the 11 saponins within several table sugar products originating from sugar beet.

Table 3. Quantitative Data about Sugar Beet Roots, Leaves, Sugar Beet Compartments, Side Streams, Commercial Products, and Beetroot[a]

	mass fraction in mg kg^{-1}										
	betavulgaroside I (1)	betavulgaroside II (2)	betavulgaroside III (3)	betavulgaroside IV (4)	betavulgaroside VIII (5)	boussingoside A2 (6)	3-*O*-[β-D-gluco... (7)	betavulgaroside V (8)	chikusetsusaponin IVa (9)	calenduloside E (10)	ginsenoside R0 (11)
Annarosa leaves	545.9 ± 5.3	13.0 ± 0.3	1724.1 ± 23.1	114.2 ± 1.9	464.4 ± 5.9	3.5 ± 0.1	1602.8 ± 22.5	538.5 ± 6.7	19.8 ± 0.2		15.3 ± 0.2
Annarosa root	317.3 ± 3.5	135.2 ± 1.9	360.3 ± 6.0	189.6 ± 1.7	102.6 ± 3.1	1.0 ± 0.0	25.8 ± 0.7	18.3 ± 0.4			
Annemaria leaves[b]	274.1 ± 1.9	271.2 ± 1.0	1454.9 ± 9.7	1959.7 ± 15.6	75.2 ± 0.9	1.8 ± 0.0	391.7 ± 3.3	309.6 ± 1.7	(8.4 ± 0.5)	18.5 ± 0.1	
Annemaria root[b]	538.0 ± 6.3	515.0 ± 1.6	534.0 ± 7.2	689.5 ± 3.1	105.7 ± 1.7	3.3 ± 0.0	39.4 ± 0.3	27.5 ± 0.3			
Artus leaves	88.0 ± 0.9	132.3 ± 0.7	190.3 ± 1.0	457.6 ± 1.4	55.2 ± 1.9	2.0 ± 0.1	275.0 ± 0.8	64.6 ± 0.3			
Artus root	287.1 ± 3.6	747.9 ± 2.5	112.1 ± 1.4	455.0 ± 3.1	36.3 ± 0.3	1.3 ± 0.0	19.7 ± 0.6	3.8 ± 0.1			
Beretta leaves	139.9 ± 0.5	5.4 ± 0.3	764.0 ± 1.8	95.8 ± 2.1	644.4 ± 6.4	4.1 ± 0.1	2343.7 ± 14.8	363.2 ± 1.3	(6.1 ± 0.1)		37.4 ± 0.5
Beretta root	402.2 ± 9.9	187.0 ± 3.6	285.6 ± 3.2	176.6 ± 4.9	89.8 ± 0.6	0.7 ± 0.0	20.8 ± 0.2	6.5 ± 0.1			
BTS leaves	73.4 ± 0.9	14.0 ± 0.1	208.6 ± 2.6	78.0 ± 0.2	104.5 ± 2.1	0.9 ± 0.0	286.4 ± 4.7	141.5 ± 2.1			
BTS root	476.7 ± 4.5	58.3 ± 1.5	374.1 ± 3.3	90.5 ± 1.4	69.0 ± 0.7	0.5 ± 0.0	35.3 ± 0.3	18.0 ± 0.2			
Daphna leaves[b]	369.5 ± 1.2	19.0 ± 0.5	1209.6 ± 6.4	157.2 ± 3.0	873.7 ± 12.2	5.1 ± 0.1	2183.9 ± 6.6	550.3 ± 1.3	(7.1 ± 0.2)		22.2 ± 0.2
Daphna root[b]	445.7 ± 9.3	159.5 ± 0.6	812.0 ± 14.5	384.4 ± 2.5	141.6 ± 1.3	0.9 ± 0.0	13.8 ± 0.3	9.9 ± 0.1			
Eva Maria leaves	562.1 ± 6.8	21.1 ± 1.5	1426.1 ± 16.7	120.2 ± 6.4	434.1 ± 2.4	2.9 ± 0.0	1850.5 ± 29.7	365.2 ± 4.8	17.8 ± 0.5		(11.2 ± 0.3)
Eva Maria root	600.3 ± 9.8	104.3 ± 1.8	553.2 ± 13.2	130.3 ± 1.6	155.7 ± 3.9	1.2 ± 0.0	14.5 ± 0.3	6.8 ± 0.2			
Kleist leaves	326.0 ± 3.0	14.4 ± 0.3	967.2 ± 11.1	104.8 ± 1.4	447.1 ± 4.9	2.9 ± 0.0	2903.2 ± 35.2	459.2 ± 3.5	12.9 ± 0.4		53.7 ± 0.8
Kleist root	344.8 ± 4.7	58.6 ± 0.6	277.4 ± 5.6	69.7 ± 0.8	87.3 ± 1.6	0.6 ± 0.0	17.5 ± 0.0	6.3 ± 0.2			
Lisanna leaves	97.8 ± 0.5	26.3 ± 0.5	119.0 ± 3.0	54.6 ± 0.5	85.9 ± 2.8	0.5 ± 0.0	628.8 ± 2.1	103.3 ± 0.3			
Lisanna root	460.2 ± 5.6	75.8 ± 0.8	536.1 ± 3.4	147.0 ± 0.9	97.2 ± 0.5	0.7 ± 0.0	38.8 ± 0.4	23.1 ± 0.2			
Marley leaves	44.4 ± 1.0	6.3 ± 0.2	154.5 ± 4.8	54.4 ± 0.3	73.9 ± 3.0	0.5 ± 0.0	531.0 ± 14.8	137.7 ± 3.9			
Marley root	367.6 ± 5.5	46.7 ± 0.5	373.0 ± 6.8	85.2 ± 0.7	64.3 ± 0.6	0.4 ± 0.0	40.5 ± 0.6	26.7 ± 0.4			
Rhinema root	401.0 ± 3.6	105.5 ± 4.3	301.0 ± 4.6	113.6 ± 3.6	79.8 ± 2.2	0.7 ± 0.0	16.4 ± 0.2	13.8 ± 0.3			
Rhinema leaves	372.3 ± 2.1	23.1 ± 0.5	1348.9 ± 4.6	166.7 ± 2.3	322.7 ± 3.9	2.2 ± 0.0	965.6 ± 1.4	394.5 ± 2.2	12.9 ± 0.1		(5.5 ± 0.1)
Hannibal leaves	8.8 ± 0.4	8.2 ± 0.1	91.7 ± 3.3	177.7 ± 2.0	195.5 ± 11.5	1.2 ± 0.1	309.6 ± 8.3	52.6 ± 1.7			
Hannibal stalk	85.8 ± 2.4	37.2 ± 0.4	161.0 ± 4.6	120.9 ± 0.9	213.6 ± 6.0	1.3 ± 0.0	95.6 ± 2.8	58.7 ± 1.7			
Hannibal inner top	186.1 ± 3.5	49.2 ± 0.6	75.2 ± 1.7	38.1 ± 0.3	26.8 ± 0.7	0.1 ± 0.0	14.4 ± 0.3	9.6 ± 0.2			
Hannibal inner middle	314.9 ± 8.6	137.8 ± 1.0	107.6 ± 0.7	71.8 ± 0.7	17.2 ± 0.1	(0.1 ± 0.0)	11.0 ± 0.0	4.4 ± 0.0			
Hannibal inner bottom	195.4 ± 1.4	418.4 ± 2.9	59.7 ± 0.7	188.6 ± 1.1	7.6 ± 0.1	(0.1 ± 0.0)	4.7 ± 0.1	0.8 ± 0.0			
Hannibal pod top	152.3 ± 5.6	147.5 ± 2.0	182.6 ± 5.1	280.0 ± 5.1	67.9 ± 2.4	0.5 ± 0.0	28.6 ± 1.0	22.4 ± 0.7			
Hannibal pod middle	436.9 ± 8.6	323.4 ± 4.8	352.2 ± 4.0	364.8 ± 4.3	69.8 ± 1.4	0.5 ± 0.0	45.0 ± 0.2	24.9 ± 0.4			
Hannibal pod bottom	721.0 ± 4.8	347.8 ± 5.4	513.5 ± 5.8	353.2 ± 6.9	94.0 ± 1.4	0.6 ± 0.0	45.1 ± 0.5	22.0 ± 0.3			
Raw juice	3.3 ± 0.9	482.9 ± 5.9	2.9 ± 1.0	525.8 ± 6.5							
Thick juice	0.3 ± 0.1	-	0.5 ± 0.1	23.2 ± 0.7							
Molasse			2.2 ± 0.1	135.0 ± 1.7			(0.9 ± 0.1)			165.2 ± 1.7	
Dried sugar beet pulp	3871.2 ± 56.8	767.9 ± 34.7	3028.3 ± 55.1	1187.1 ± 71.5	646.3 ± 13.5	68.7 ± 1.7	243.3 ± 3.4	118.6 ± 3.1	391.6 ± 10.7		
Flume water	434.4 ± 7.4	133.2 ± 1.2	916.6 ± 12.5	371.9 ± 6.5	135.7 ± 1.8	2.0 ± 0.0	56.5 ± 0.6	5.6 ± 0.4			
Process water	51.6 ± 0.9		98.6 ± 0.5	231.4 ± 1.4	32.3 ± 0.4	(1.0 ± 0.1)	(2.7 ± 0.1)				

Table 3. continued

	mass fraction in mg kg^{-1}										
	betavulgaroside I (1)	betavulgaroside II (2)	betavulgaroside III (3)	betavulgaroside IV (4)	betavulgaroside VIII (5)	boussingoside A2 (6)	3-*O*-[*β*-D-gluco... (7)	betavulgaroside V (8)	chikusetsusaponin IVa (9)	calenduloside E (10)	ginsenoside R0 (11)
Sugar beet fiber	5246.4 ± 27.9	1113.6 ± 33.8	1006.5 ± 4.2	622.5 ± 11.9	188.4 ± 3.9	351.4 ± 8.4	329.3 ± 4.0	45.7 ± 0.9	2823.7 ± 19.9	943.9 ± 25.8	12.5 ± 0.9
Goldsaft	629.2 ± 10.8	29.3 ± 1.0		3.1 ± 0.9		78.8 ± 1.8	53.1 ± 1.5		592.9 ± 13.1	18.6 ± 0.9	
Beetroot	205.4 ± 0.1	4.6 ± 0.1	442.3 ± 2.0	33.6 ± 0.2	2.7 ± 0.1	0.0 ± 0.0	65.3 ± 0.7	40.7 ± 0.3			

[a]Values lower than the limit of quantification are in parentheses. Concentrations are the mean of three biological replicates with the standard deviation. [b]Obtained from freeze-dried-material.

Saponins within Molasses and Goldsaft. Molasses which is a side-stream during sugar crystallization showed significant amounts of saponins and is well-known to contain saponins.[2] This can be explained by the fact that it consists of many less polar substances compared to sugar.[6] Particularly high amounts of compound **10** were found in molasses, which potentially have been formed by thermal heating that might lead to the degradation of higher molecular weight saponins into smaller ones. Another commercially available product is Goldsaft that is similar to molasses and indicated some saponins. It was surprising that it did not reveal compound **3** which is one of the most abundant saponins within sugar beet root.

Saponin Occurrence in Carbonation Sludge. Another side-stream which might be a good saponin source is carbonation sludge.[10] However, sugar beet saponins were only observed in traces within this material. This difference might be caused by changes in the sugar beet converting process since this observation was published in 1955.[10]

Saponins within Flume and Process Water. Only very few saponins were detected in flume water that was mentioned to be a good saponin source by Brezhneva et al.[9] The values summarized in Table 3 were related to the dry masses of flume and process water. Dry mass amounts were in the range of 2.3 to 15.8 g per liter. The results shown in Table 3 were much lower if these were converted to a liter of liquid. Even for dry matter, the amounts do not even come close to the value mentioned by Brezhneva et al.[9]

Saponin Occurrence in Beetroot. Beetroot as another plant was chosen for analysis as it originates from the same subspecies.[16,17] The amounts of saponins were similar to those of sugar beet, with the difference that the concentration of **5** was significantly lower and concentrations of **7** and **8** were slightly higher than in most sugar beet varieties.

■ CONCLUSION

In summary, a sensitive and fast LC−MS/MS-based method for the quantification of 11 different sugar beet saponins was developed. Using this method, the occurrence of many different saponins within root and leaves of different sugar beet varieties was investigated. In addition, the distribution inside one variety was analyzed. A large data set was obtained about the amounts of saponins within different varieties as well as plant compartments and side-streams. This knowledge could be very useful for breeding new sugar beet varieties that are more resistant against different types of biotic stress factors.[27] For instance, some saponins are known to possess antifungal activities.[28] Additionally, the occurrence of saponins within different side streams was explored that could facilitate the utilization of sugar beet saponins as food ingredients. Finally, for the first-time, quantitative data about individual sugar beet saponins within commercial products were provided.

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Notes

The authors declare no competing financial interest.

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