frontiers | Frontiers in Natural Products

TYPE Mini Review
PUBLISHED 07 January 2025
DOI 10.3389/fntpr.2024.1524624

OPEN ACCESS

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RECEIVED 13 November 2024
ACCEPTED 16 December 2024
PUBLISHED 07 January 2025

CITATION
Morais V, Suarez N and Silveira F (2025) Methods of saponin purification from *Quillaja* sp. for vaccine adjuvant production.
Front. Nat. Produc. 3:1524624.
doi: 10.3389/fntpr.2024.1524624

Methods of saponin purification from *Quillaja* sp. for vaccine adjuvant production

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Saponins are natural glycosides found in many plant species; they have a hydrophobic region, consisting of a steroid or triterpenoid skeleton called an aglycone, and a hydrophilic region, consisting of sugar chains attached to the aglycone through ether or ester linkages. This combination of polar and nonpolar elements endows saponins with soap-like behaviour in aqueous solutions. Owing to their structural characteristics, the amphiphilic nature of saponins is responsible for their foaming properties, as well as other biological functions, including their haemolytic activity. The adjuvant properties of saponins were known many years ago, but only in recent years have saponins been approved for human vaccine use in this manner. Saponins from *Quillaja saponaria* bark are the only source of approved preparations for human use, but a related species, *Quillaja brasiliensis,* also contains similar saponin compositions that can be obtained from leaves. In this work, we describe the different preparations of saponins used for adjuvants and the purification methods used to obtain each saponin.

KEYWORDS

***Quillaja saponaria*, *Quillaja brasiliensis*, saponin, adjuvant, nanoparticle, QS-21, iscom, matrix**

1 Introduction

Saponins are secondary metabolites found in many plant species. The name derives from the Latin word "sapo," meaning soap, owing to their ability to produce foam in water (Sparg et al., 2004; Vincken et al., 2007). They can be found in the bark, leaves, stems, roots, and even flowers of plants (Moghimipour and Handali, 2015; Rai et al., 2021). Saponins are divided into two main classes, namely, triterpenoids and steroid glycosides, with specific structures characterized by the number and position of the attached sugar units (Sparg et al., 2004; Vincken et al., 2007; Jolly et al., 2024). These natural glycosides have both a hydrophobic region, consisting of a steroid or triterpenoid skeleton called an aglycone, and a hydrophilic region, consisting of a sugar chain containing glucose, glucuronic acid, xylose, rhamnose or methyl pentose attached to the aglycone through ether or ester linkages (Supplementary Figure S1) (Sparg et al., 2004; Vincken et al., 2007; Moghimipour and Handali, 2015). This combination of polar and nonpolar elements endows saponins with soap-like behaviour in aqueous solutions (Sparg et al., 2004; Vincken et al., 2007). Saponins from *Quillaja saponaria* (QSap) contain a triterpenic aglycone, most frequently quillaic acid, which is glycosylated at the C-3 and C-28 positions of the aglycone (Fleck et al., 2019). The specific limitation of the use of saponins is their cytotoxicity, which correlates with their haemolytic activity, which is influenced by the affinity of the aglycone to cholesterol in cell

membranes (Sparg et al., 2004; Lorent et al., 2014). However, saponins are particularly useful as vaccine adjuvants (Kensil, 1996; Sun et al., 2009; Shen et al., 2023).

Adjuvants, substances that aid in the effectiveness of vaccines, have greatly increased the protection provided by vaccine immunizations. Adjuvants play important roles in the type, duration and effectiveness of immune responses to vaccines (Awate et al., 2013; Shi et al., 2019; Verma et al., 2023; Zhao et al., 2023). In 1925, Ramon was the first to describe immunological adjuvants as substances that, when combined with a specific antigen, produce a stronger immune response than produced by the antigen alone. He reported increased yields of tetanus and diphtheria antitoxins produced in horses when the animals developed an abscess at the injection site (Awate et al., 2013; Verma et al., 2023; Chippaux, 2024). By injecting starch, breadcrumbs or tapioca with inactivated toxin, sterile abscesses were induced at the site of injection, increasing antitoxin production. He confirmed that substances able to induce local inflammation at the injection site were also able to enhance the immune response (Di Pasquale et al., 2015; Verma et al., 2023).

Despite numerous advancements in the isolation of antigens and vaccine production, only a limited number of adjuvants have been approved for use in humans. Aluminium salts, which were developed nearly 100 years ago, are still the most widely used adjuvant in licenced human vaccines (Di Pasquale et al., 2015; Shi et al., 2019).

The adjuvant effect of saponins was reported in the initial work of Ramon in 1925; in fact, the adjuvant effects of breadcrumbs, tapioca and starch oil were probably due to the presence of saponins. Years later, in 1951, Espinet used a crude commercially available *Quillaja saponaria* saponin preparation to increase the potency of foot-and-mouth disease vaccines (Espinet, 1951; Barr et al., 1998).

After Espinet's work in foot-and-mouth disease vaccines, in 1974, Dalsgaard successfully isolated the commercially available saponin Quil A® from the cortex of the South American tree *Quillaja saponaria* Molina. He reported that the addition of Quil A® to vaccine preparations stimulated both humoral and cellular immunity and induced differential antibody isotypes (Dalsgaard, 1974; Dalsgaard, 1977). Unfortunately, saponins also have strong haemolytic effects, increasing adverse reactions.

Therefore, efforts have focused on purifying a defined saponin molecule that maintains the adjuvant effect with less haemolytic effects. In 1991, Kensil and coworkers patented QS-21 (Kensil and Marciani, 1991). They further purified saponins by reverse chromatography, and 22 fractions were obtained. The most predominant fractions (QAs 7, 18, 19, and 21) had adjuvant activity (Kensil and Marciani, 1991; Kensil et al., 1991; Wang et al., 2019; Wang, 2021). QA21 (now QS-21) was the fraction that exhibited a better balance between adjuvant activity and low toxicity (Kensil and Marciani, 1991; Kensil, 2001).

Morein and coworkers reported that the formulation of saponins into lipidic nanoparticles, called immune-stimulating complexes (ISCOMs), decreased the haemolytic activity, whereas the adjuvant activity was unaltered (Morein et al., 1984; Barr and Mitchell, 1996). ISCOMs are spherical, open cage-like structures approximately 40 nm in size (Barr and Mitchell, 1996). The formulation of nanoparticles removes the need for complicated purification processes and allows for the safe use of defined mixes of saponins (Barr and Mitchell, 1996). If an antigen is included in a nanoparticle formulation with cholesterol, phospholipids and saponins, the resulting particle is called ISCOM. It is also feasible to create nanoparticles without antigens and combine the nanoparticles and antigens later before delivery. In this case, the nanoparticles are called the ISCOM matrix or simply the matrix (Lövgren Bengtsson et al., 2011). Many studies have shown that adjuvant activity is conserved independently of the use of ISCOM or ISCOM Matrices plus antigen (Fossum et al., 1990; Stertman et al., 2023).

In 1995, Cox, Morein and coworkers patented an ISCOM particle (ISCOM Matrix) that included fractions A and C of saponins from QSap; within the next years, this product was named Matrix-M (Cox et al., 1995). The Matrix-M adjuvant is the third generation of ISCOM technology and consists of two different types of physically stable nanoparticles mixed at a defined ratio (85% Matrix-A + 15% Matrix-C) (Cox et al., 1995; Lövgren Bengtsson et al., 2011; Bai et al., 2024). Matrix-A (nanoparticle of fraction A) and Matrix-C™ (nanoparticle of fraction C) contain different QSap molecules with complementary properties. According to structural analysis, fraction C is composed mainly of QS-21. Compared with those from fraction C, fraction A saponins have weaker adjuvant activity but are less reactive and more easily form nanoparticles. The combination of the two types of particles reduces the reactogenicity while preserving the adjuvant activity (Cox et al., 1995; Lövgren Bengtsson et al., 2011).

Currently, there are two main QSap adjuvant preparations licenced for human use. AS01 and AS02 from GlaxoSmithKline combine QS-21 with other immunostimulatory molecules (Vandepapeliere, 2013). AS01 contains 3-O-desacyl-4′-monophosphoryl lipid A (MPL), and QS-21 is formulated as a liposome, whereas AS02 contains MPL combined with QS-21 in an oil-in-water emulsion; both are designed to induce strong humoral and T-cell-mediated responses (Didierlaurent et al., 2017; Garçon and Di Pasquale, 2017).

AS01 was designed to strengthen the $CD8^+$ response and is included in two licenced human vaccines (Shingrix™ for herpes virus and Mosquirix™ for malaria); it is also currently used in a candidate HIV and tuberculosis vaccine (Garçon and Di Pasquale, 2017; Lacaille-Dubois, 2019; Alving et al., 2020). AS02 has been evaluated in vaccines targeting complex pathogens that require a strong T-cell response and induce strong humoral and cellular immune responses (e.g., against hepatitis B, malaria, and HIV) (Garçon and Di Pasquale, 2017).

Matrix-M (Novavax) is also used in licenced human vaccines such as the Novavax COVID-19 vaccine (Nuvaxovid™) and the antimalarian R21/Matrix-M in collaboration with Oxford University and the Serum Institute of India (Stertman et al., 2023). Nuvaxovid is a recombinant protein vaccine composed of SARS-CoV-2 spike trimer nanoparticles formulated with Matrix-M™ adjuvant (Underwood et al., 2023; Lenart et al., 2024). Matrix-M is also being evaluated in clinical trials for new influenza vaccines (Pedersen et al., 2014; Shinde et al., 2022).

Although QS-21 exhibits limited haemolytic activity *in vitro*, higher doses may lead to side effects. Saponins can disrupt cell membranes, leading to haemolysis, as demonstrated by pore formation in erythrocyte membranes (Petrovsky, 2015). To reduce this toxicity, saponins can be encapsulated in lipid

nanoparticles. This formulation strategy has been shown to reduce saponin-related adverse effects (Ragupathi et al., 2011; Petrovsky, 2015; Bigaeva et al., 2016).

Adverse reactions reported for licensed products, such as QS-21 or ISCOMs, indicate a low incidence of side effects comparable to those of other adjuvants. Saponin-based adjuvants have been associated with local adverse effects, including pain, redness, swelling, and erythema, as well as mild systemic effects such as fever and flu-like symptoms (Ragupathi et al., 2011; Petrovsky, 2015; Bigaeva et al., 2016).

However, owing to overexploitation of the QSap bark of Chilean forests, which has caused important ecological damage and resulted in the scarcity of available supplies, considerable efforts have been undertaken to discover sources of new saponins with improved adjuvant activity and reduced toxicity (Schlotterbeck et al., 2015; Fleck et al., 2019). Another species that belongs to the family Quillajaceae, *Quillaja brasiliensis* (now Quillaja lancifolia D. Don), a native tree distributed in southern Brazil, northern Uruguay, northeastern Argentina and eastern Paraguay, has been shown to be a better source of saponins because the saponins can be purified from leaves (Luebert, 2013; Schlotterbeck et al., 2015; Fleck et al., 2019). At the laboratory scale, saponins from *Quillaja brasiliensis* (QBr) presented characteristics and adjuvant activity similar to those of saponins extracted from QSap, opening a new avenue for the development of saponin-based adjuvants (Cibulski et al., 2016b; Cibulski et al., 2022; Fleck et al., 2019; Magedans et al., 2019; Rivera-Patron et al., 2021).

The precise mechanism of action of QSap or QBr saponins in their role as an adjuvant remains unclear. However, recent investigations have begun to delineate potential modes of action and signalling pathways. Saponins possess the capacity to induce both pro-inflammatory Th1/Th2 and anti-inflammatory Th2 immune responses (Marciani, 2018; Wang, 2021). Structure-activity relationship studies have elucidated that the presence of imine-forming carbonyl groups within the saponin structure is indispensable for T cell activation and the subsequent induction of Th1/Th2 responses. While saponins with diverse triterpenoid aglycons and oligosaccharide chains can activate dendritic cells (DCs) to induce both Th1 and Th2 responses, the presence of fucopyranosyl residues within their oligosaccharide chains can bias DC activation towards a Th2-dominant response through engagement of the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) receptor (Guo et al., 2004; Aguilar and Rodríguez, 2007; Chea et al., 2012; Marciani, 2018; Marciani, 2022; Marciani, 2024). Glycosides like QS-21 can interact separately with T cells and dendritic cells. T cells are co-stimulated by the glycoside's aldehyde, while dendritic cells are activated by interactions with the triterpene group and fucosyl residue, respectively (Soltysik et al., 1995; Liu et al., 2002; Ragupathi et al., 2011; Marciani, 2018; Marciani, 2024).

Lacalle-Dubois propose that QS-21 mechanism of action could be synthetized as follow: stimulation of Th2 humoral and Th1 cell-mediated immune responses through action on antigen presenting cells (APCs) and T cells; release of Th1 cytokines participating in the elimination of intracellular pathogens; activation of the NLRP3 inflammasome and the release of caspase-1 dependent cytokines IL 1β and IL-18 (Marty-Roix et al., 2016; Coccia et al., 2017; Lacaille-Dubois and Wagner, 2017; Marciani, 2018; Lacaille-Dubois, 2019). A growing body of evidence is continually revealing the mechanisms of action of QSap saponins, indicating their complex interactions with multiple signalling pathways.

In this review, we focus on the most common purification methods and formulations of saponins for vaccine adjuvants.

2 Saponin purification

2.1 QSap Quil-A (Quil-A®)

In 1970, commercially available saponins were used as adjuvants in some foot-and-mouth disease (FMD) vaccines. These preparations were introduced by Espinet in 1951 and have since gained considerable popularity among producers of FMD vaccines because some have pronounced adjuvant effects (Espinet, 1951).

In 1973, Dalsgaard published a series of articles concerning the improvement of the FMD vaccine using saponins from *Q. saponaria* as an adjuvant. Previously, poorly defined saponin extracts were used with good results; therefore, Dalsgaard focused on the characterization and possible standardization of saponins for use in FMD vaccines (Dalsgaard, 1974; Dalsgaard, 1977).

Supplementary Figure S2 shows the purification process of Quil-A® proposed by Dalsgaard from an aqueous extract of the cortex of *Quillaja saponaria* Molina (Dalsgaard, 1974). The dialyzed aqueous extract was first separated on an ion exchange DEAE cellulose column equilibrated with 0.1 Tris-HC (pH 7.5). Elution was performed with buffer containing 0.2 M NaCl. The elution peak was subsequently subjected to gel exclusion chromatography on a Sephadex G50 column equilibrated with phosphate buffer at pH 7.5, resulting in 3 peaks. The peak eluted in the second position (middle) was reintroduced into an ion exchange DEAE cellulose column equilibrated with 0.1 Tris-HC (pH 7.5) and was eluted with a linear NaCl gradient increasing from 0 to 1 M, with 2 peaks obtained. Peak F seemed to be a pure substance in gel filtration and thin-layer chromatography analysis. Furthermore, peak F maintained the adjuvant effect at a similar level of activity as the dialyzed aqueous extract and was called Quil-A.

2.2 QSap QS-21

The Quil-A® preparation was a definite improvement over the previously available commercial saponins, although it still showed considerable heterogeneity. Further analysis using high-pressure liquid chromatography revealed that Quil-A® was in fact a heterogeneous mixture of structurally related compounds. However, not all these saponins were active as adjuvants.

The four predominant purified Qsap saponins were QS-7, QS-17, QS-18, and QS-21 (Kensil and Marciani, 1991; Kensil et al., 1991; Kensil, 1996; Kensil, 2001). These saponins were purified by HPLC and low-pressure silica chromatography and were found to be adjuvant-active, although they differed in their biological activities, such as haemolysis and toxicity, in mice. In particular, QS-21 and QS-7 were found to be the least toxic in mice. Owing to its potent adjuvant activity and low toxicity, QS-21 (commercially available as the "Stimulon®" adjuvant) has been identified as a useful

immunological adjuvant (Kensil and Marciani, 1991; Kensil, 2001; Lv et al., 2024).

QS-21 is a complex triterpene glycoside of quillaic acid that is glycosylated at triterpene carbon 3, triterpene carbon 28, and carbon 5 of the second fatty acyl unit in a fatty acid domain (Ragupathi et al., 2011; Marciani, 2024). More recently, QS-21 was further purified using hydrophilic interaction chromatography (HILIC) and resolved into two peaks, QS-21-V1 and QS-21-V2, which have been shown to be chemically different compounds. In C57BL/6 mice immunized with vaccines consisting of ovalbumin and either QS-21 or both the individual components, QS-21-V1, or QS-21-V2, the individual components were comparable in adjuvant effects to that of the original QS-21 peak (containing a mixture of 3:2 QS-21-V1 and QS-21-V2), boosting the IgG subclasses IgG1, IgG2b, and IgG2c, as well as the total IgG titre (Kensil, 2001).

Given its longstanding success as an adjuvant, efforts have been made to improve the extraction and purification processes in order to increase purity and yields.

Baig et al. patented a method for purifying QSap saponins to a purity of at least 93% of QS-21, with impurity peaks outside the QS-21 group below 1% by UV absorbance at 214 nm. The method begins with a crude aqueous extract of QSap bark, typically containing 1–2.8 g/L of QS-21. The method includes three steps: polyvinylpyrrolidone (PVPP) adsorption, diafiltration, and reverse-phase chromatography (Baig et al., 2019).

The first step involves treating the QSap extract with PVPP resin. Typically, the extract is agitated with the resin and subsequently separated from the PVPP resin, along with adsorbed impurities, by filtration. This step of the process generally removes polyphenolic impurities such as tannins. The next step involves purifying the solution by diafiltration, ultrafiltration, or dialysis, preferably diafiltration with a 30 kDa membrane cut-off. This step typically removes salts, sugars, and other low molecular weight materials. The final step involves purifying the solution by reverse-phase chromatography using a polystyrene resin. This step removes non-saponin material and enriches the desired saponins. Alternatively, reverse-phase chromatography can be performed using a phenyl resin (Baig et al., 2019).

In the same way, Qui and Fox presents a novel two-step chromatographic process for purifying the molecular adjuvant QS-21 from QSap bark extract. This method involves a polar reversed-phase (RP) chromatography step followed by hydrophilic interaction chromatography (HILIC). This orthogonal approach significantly improves the purification efficiency, resulting in a product with > 97% purity and high yield (Qi and Fox, 2021).

Moreover, Gao et al. proposed an efficient ultrasound-assisted enzymatic method for extracting the QS-21 from QSap bark. The method utilizes a combination of ultrasound treatment and enzymatic hydrolysis to enhance the extraction efficiency before chromatography purification. They found that the type of enzyme used and the particle size of the QSap bark greatly affect the reaction yield. The best conditions found are using the tannase enzyme and a particle size smaller than 48 μm. The optimized process resulted in a significant increase in QS-21 yield compared to traditional methods, while maintaining high purity (Gao et al., 2022).

2.3 QSap fractions A, B, and C for ISCOM/matrix formulation

Fractions A and C are used by Novavax in ISCOM matrix formulations for human vaccines, and their flow purification is shown in Figure 1. A crude aqueous extract of QSap is pretreated on a C18 column (Sep-Pak). After the column is washed with 10% acetonitrile, lipophilic substances, including saponins, are eluted with 70% acetonitrile. The lipophilic fraction is further fractionated using an HPLC semipreparative C8 column and eluted with an acetonitrile gradient from 25% to 60%. Fractions A, B and C are eluted at approximately 39, 47% and 49% acetonitrile, respectively (Cox et al., 1995; Barr et al., 1998; Lövgren Bengtsson et al., 2011). Fraction A has very high ISCOM-forming activity and low haemolytic activity but medium adjuvant activity. Conversely, fraction C has medium ISCOM-forming activity and high haemolytic activity and adjuvant activity. It has been reported that the ratio of 7 parts of fraction A to 3 parts of fraction C provides very high adjuvant activity, easily forms ISCOMs and has low haemolytic activity (Cox et al., 1995).

2.4 QBr QB-90

The use of saponin fractions of QBr began in 2000. Fleck and colleagues were the first to evaluate the adjuvant power of the QBr saponin-enriched fraction (Fleck et al., 2006). The saponin fraction of this species is remarkably similar to that of the bark of QSap (Magedans et al., 2019). One of the most common protocols for saponin purification was published by Yendo and coworkers in 2017 and is shown in Figure 2 (Yendo et al., 2017). In this case, leaves are used for obtaining aqueous extracts. Further purification includes extraction of the most polar compounds using ethyl acetate and the precipitation of tannins with gelatine. The last step includes fraction purification chromatography with a C18 column and methanol elution. Although fraction QB-90 (eluted in 90% methanol) is the most commonly used fraction, the QB-80 and QB-100 fractions (eluted in 80% and 100% methanol, respectively) also show saponin adjuvant activity (Yendo et al., 2017).

In recent years, the QB-90 fraction alone or in combination with ISCOM or ISCOM matrices has been proven to be effective in many vaccine candidates against influenza, herpes and other viruses at the laboratory scale with notable success (Cibulski et al., 2016a; Cibulski et al., 2016b; Cibulski et al., 2018).

2.5 QBr QB1

Owing to the large similarity between QSap and QBr, efforts have been made to purify QS-21 analogues from QBr. In 2022, Wallace and coworkers purified QB1, which is structurally similar to QS-21 (Wallace et al., 2022). They first purified an immunoadjuvant preparation (named fraction B) from the aqueous extract of QBr leaves by fractionation on a C18 column. Then, fraction B was further fractionated by consecutive separations with silica flash MPLC and reverse-phase C18 HPLC. Two compounds were isolated, and their structures were elucidated using a combination of NMR spectroscopy and mass spectrometry. One of these compounds was triterpene saponin (Qb1), which is an isomer of

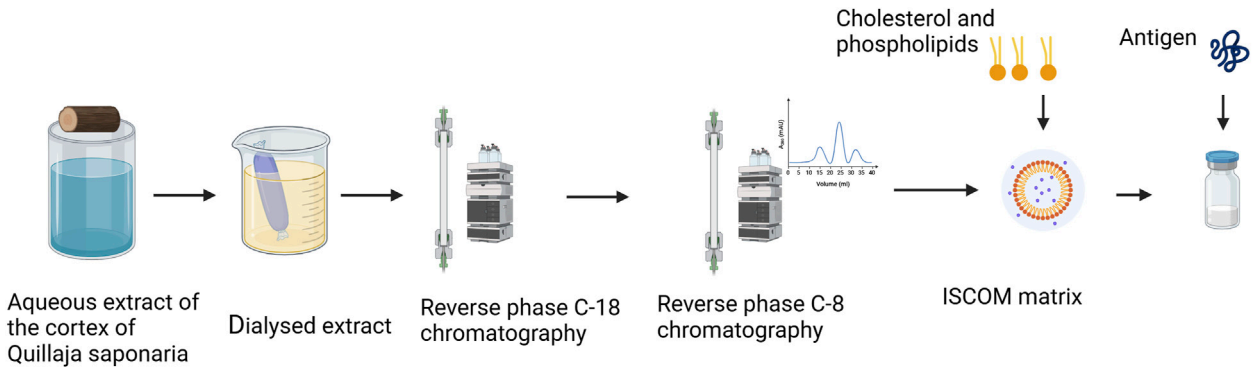

FIGURE 1
Saponin purification for Matrix M production. Created in BioRender. https://BioRender.com/i70i138.

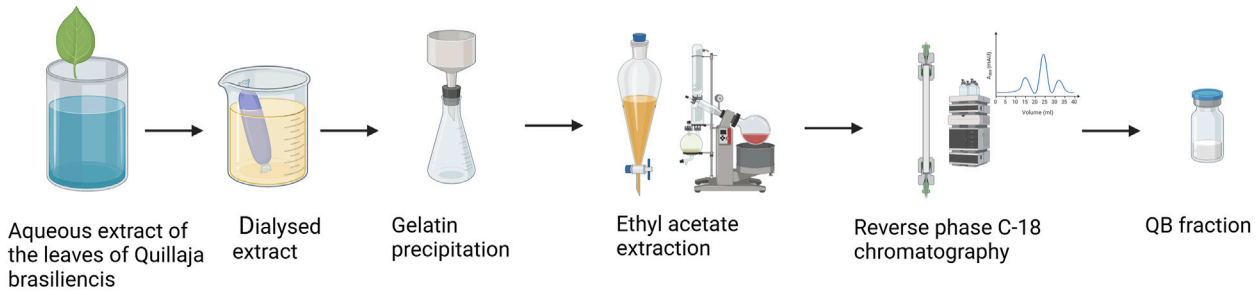

FIGURE 2
Saponins fractions from *Quillaja brasiliensis* obtained by Yendo and coworkers' process. Created in BioRender. https://BioRender.com/e44w417.

TABLE 1 Saponin-based adjuvants and the purification process.

Name	Source	Purification method	Approved for human use	Commercial name (company)	Formulation
Quil-A	*Q. saponaria* bark	Ion exchange and gel filtration chromatography	No		Alone or included in nanoparticles
QS-21	Q. saponaria bark	Reverse phase chromatography	Yes	AS1 and AS2 (GSK)	In combination with MPL. Liposome (AS1); oil in water (AS2)
Fraction 1 and 3 ISCOM	*Q. saponaria* bark	Reverse phase chromatography	Yes	Matrix M (Novavax)	Lipid nanoparticle (ISCOM)
QB-90	*Q. brasiliensis* leaves	Solvent extraction Reverse phase chromatography	No		Alone or included in nanoparticles

AS, Adjuvant system; GSK, GlaxoSmithKline; MPL, monophosphoryl lipid A; ISCOM, Immune-stimulating complexes.

QS-21 (Wallace et al., 2022). The adjuvant activity of this compound has not yet been determined.

3 ISCOM/matrices formulation

There are two classic methods for the preparation of these nanoparticles: dialysis and centrifugation. The protocols are identical for the formulations of the ISCOMs and matrix except that the latter does not introduce the antigen into the nanoparticle; the antigen is added later. Among the two methods, dialysis has gained favour over centrifugation because of its simplicity and ease of scaling up, and currently, it is the widely accepted method for ISCOM/matrix formulation (Barr and Mitchell, 1996). The main important factor in the formation of optimal ISCOMs is the correct ratio of the various components that are combined at the start. According to Barr and Michell, the optimal weight ratios of cholesterol, phospholipid and QSap saponin should be 1:1:5 for the matrix and ratios of 1:1:5:0.1 to 1:1:5:1 when the antigen is included. The dialysis protocol is very simple: solubilized proteins

(in the case of ISCOMs) are added to cholesterol and phospholipid dissolved in a nonionic detergent (MEGA-10 is the most commonly used), and saponins are added and mixed. The detergent is subsequently removed from the mixture by extensive dialysis or diafiltration (Barr and Mitchell, 1996; Barr et al., 1998; Rivera-Patron et al., 2022). Compared to other process, dialysis produces more homogeneous formulations with a narrow particle size distribution (Lendemans et al., 2005; Myschik et al., 2006).

Conversely, the centrifugation method is more complex and includes a sucrose gradient and ultracentrifugation, and it is rarely used (Barr and Mitchell, 1996; Hu et al., 1998). Other methods, such as ether or ethanol injection and lipid-film hydration, are described but are not as popular as the dialysis method (Bangham et al., 1965; Batzri and Korn, 1973; Pons et al., 1993; Copland et al., 2005; Myschik et al., 2006; Pham et al., 2006; Demana et al., 2010; Lendemans et al., 2010).

4 Conclusion

Saponins in combination with other immunostimulants or formulated as lipid nanoparticles have become new protagonists in human vaccine adjuvants (Kensil, 2001; Garçon and Di Pasquale, 2017; Stertman et al., 2023; Marciani, 2024). Table 1 presents the most commonly used saponin preparations as vaccine adjuvants. Adjuvants formulated with saponins have the advantage of generating a strong and balanced immune response, including strong cellular responses that make them suitable for use in viral vaccines (Sjölander et al., 1997; Cibulski et al., 2016a; Verma et al., 2023; Zhao et al., 2023).

With respect to saponin purification for human vaccines, improvements have been made in two different ways. First, the purification or purification/modification of defined saponin molecules can achieve a strong immune response with few side effects (Kensil, 1996; Garçon and Di Pasquale, 2017; Marciani, 2024). Second, the purification of a defined mixture of saponins that can be formulated into nanoparticles maintains immune activity and decreases side effects (Cox et al., 1995; Stertman et al., 2023).

The primary challenges in saponin production for adjuvant use are the sustainability of *Q. saponaria* tree cultivation and the need for better characterized and purified compounds that retain adjuvant potency while minimizing adverse effects (Ragupathi et al., 2011; Fleck et al., 2019).

The extraction of saponins from *Q. brasiliensis* leaves has proven to be the most efficient and environmentally friendly method. Nevertheless, alternative sustainable methods are currently under investigation. Lv and coworkers, reports the successful production of QS-21, through plant cell culture. The study demonstrates that plant cell culture can provide a sustainable and scalable alternative for producing QS-21 with comparable chemical and biological properties to the bark-derived product (Lv et al., 2024).

In this way, Martin et al. propose the complete biosynthesis of QS-21 saponin. The study successfully reconstituted the entire 20-step pathway in tobacco, demonstrating the production of QS-21 in a heterologous expression system (Martin et al., 2024).

In relation with the molecular structure, Marciani emphasizes the need to view adjuvants, particularly saponins like QS-21, as a distinct class of drugs with specific immunopharmacological properties shaped by their functional groups (Marciani, 2024). Specifically, QS-21 has been shown to induce a pro-inflammatory Th1 response through its aldehyde group, challenging the misconception that its adjuvanticity is solely due to its particulate nature (Soltysik et al., 1995; Aguilar and Rodríguez, 2007). The fucose residue is also critical, influencing the type of immune response elicited, although it is often mischaracterized as merely structural. Research into synthetic analogues could help clarify the roles of these components, as changes in sugar moieties may shift immune response (Ragupathi et al., 2011; Marciani, 2024). To fully understand the mechanisms of action of complex adjuvants, it is essential to identify the specific cell receptors they target. Understanding these interactions and their immunological effects can lead to improved adjuvant design (Borriello et al., 2022; Marciani, 2024).

Author contributions

VM: Conceptualization, Visualization, Writing–original draft, Writing–review and editing. NS: Writing–original draft, Writing–review and editing. FS: Conceptualization, Writing–original draft, Writing–review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by PEDECIBA, Química.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fntpr.2024.1524624/full#supplementary-material

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