Identification of lipophilic bioproduct portfolio from bioreactor samples of extreme halophilic Archaea with HPLC-MS/MS

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Abstract

Extreme halophilic Archaea are a yet unexploited source of natural carotenoids. At elevated salinities, however, material corrosivity issues occur and the performance of analytical methods is strongly affected.

The goal of this study was to develop a method for identification and downstream processing of potentially valuable bioproducts produced by Archaea. To circumvent extreme salinities during analysis, a direct sample preparation method was established to selectively extract both the polar and the non-polar lipid contents of extreme halophiles with hexane, acetone and the mixture of MeOH/MTBE/water, respectively. Halogenated solvents, as used in conventional extraction methods, were omitted due to environmental considerations and potential process scale-up.

The HPLC-MS/MS method using atmospheric pressure chemical ionization was developed and tuned with three commercially available C_{40} carotenoid standards, covering the wide polarity range of natural carotenoids, containing different number of OH-groups. The chromatographic separation was achieved on a C_{30} RP-HPLC column with a MeOH/MTBE/water gradient. Polar lipids, the geometric isomers of the C_{50} carotenoid bacterioruberin and vitamin MK-8 were the most valuable products found in bioreactor samples. In contrast to literature on shake flask cultivations, no anhydrous analogues of bacterioruberin, as by-products of the carotenoid biosynthesis, were detected in bioreactor samples.

This study demonstrates the importance of sample preparation and the applicability of HPLC-MS/MS methods on real samples from extreme halophilic strains. Furthermore, from a biotechnological point-of-view, this study would like to reveal the relevance of using controlled and defined bioreactor cultivations instead of shake flask cultures in the early stage of potential bioproduct profiling.

Keywords

Halophiles, Bacterioruberin, Archaeal lipids, Menaquinone-8, controlled bioprocessing, HPLC-MS/MS, preparation of biological samples

Introduction

Products of biotechnological processes for new, commercially valuable materials from alternative biological resources are gaining more and more importance in science and industry. Suitable analytical techniques are required for the identification of products of natural origin. For small molecules, compound identification with LC-MS requires orthogonal spectroscopic methods or multidimensional MS techniques [1]. However, only compound identification from biological matrices using tandem MS techniques have been reported [2-4]. Referring to the spectroscopic techniques in the case of unknown molecules, where no e.g. UV/Vis spectroscopic data is available, multidimensional LC-MS techniques may help the compound identification. It is a fact that “structure elucidation” is impossible using MS techniques alone, and must be accomplished by NMR techniques. The terms “structure identification” and “structure elucidation”, however, are used to describe different grades of structure investigation. The term “structure elucidation” usually refers to full de novo structure identification of a compound, including stereochemical assignments [5]. Thus, a combination of chromatographic techniques with multidimensional, namely tandem MS offers an adequate solution for bioproduct screening and compound identification. In case further e.g. stereochemical information is required, HPLC-NMR coupled techniques can help with structural investigation of compounds of natural origin [6].

Many halophilic Archaea have a high content of yellow to red colored carotenoid pigments, making their natural habitats, solar salterns or soda lakes pink or even bright red [7,8]. The extreme halophilic Archaea can thrive with 20 % w/w to saturated sodium chloride concentrations in hypersaline environments [9]. Some halophilic Archaea are even able to cope with high salinity and high alkalinity at the same time, and were therefore named Haloalkalophiles. Although there is great potential in the carotenoid production by extreme halophiles, there is very little information in the literature about

the analysis of the carotenoid profile of these microorganisms. Gas chromatography cannot be used due to the temperature sensitivity of carotenoids. Due to the strong color of the analytes, Vis detection with a suitable HPLC method could be a selective and sensitive alternative for carotenoid analysis, in case all carotenoid standards, which are present in the sample, are commercially available [10]. However, this is not the case in the first identification step of a bioproduct portfolio. The three-fingered absorption maxima are characteristic for carotenoids in general and the respective absorption maxima for several carotenoid compounds are known [11]; these are, however, not considered as a sufficient technique for compound identification per se. Recently, the analysis of carotenoids with HPLC-MS/MS has been reported for non-halophilic microorganisms [12,13]. Archaeal and halophilic polar lipids have been analyzed with electrospray ionization (ESI) [14] and MALDI-TOF MS [15] techniques. Regarding bioproduct screening of extreme halophiles, some outdated examples exist for NMR bioproduct analysis [16,17] but the extracted bioproducts are typically analyzed by TLC [16,18,19]. A recent example shows HPLC-MS/MS analysis of carotenoids produced by various extremophilic microorganisms [20].

The coupling of HPLC with atmospheric pressure tandem mass spectrometry offers a robust, highly selective and sensitive method for the analysis of components present in low concentration level in biological matrices. Due to the low polarity of the group of the targeted analytes, the sample extracts were analyzed by HPLC-APCI-MS/MS technique. APCI is especially suitable for the ionization of compounds with moderate polarity. For carotenoids it has become widely used due to its high sensitivity [21]. The industrial and commercial relevance of natural archaeal lipid compounds, especially carotenoid compounds, is very high, for example as an alternative to synthetic food colorants [22]. According to the most general lipid classification of halophilic Archaea,

both the polar lipids (glycolipids and phospholipid derivatives) and the representatives of non-polar lipids (C_{30} isoprenoids, C_{40} and C_{50} carotenoids, retinal and vitamin Menaquinone-8 abbreviated as MK-8) can be classified as lipid components [23]. Carotenoids can be used not only as food colorants, but also as precursors for vitamin A synthesis and even as antioxidant or anticancer compounds due to their scavenging activity for active oxygen [24]. The main compound of the carotenoid content of extreme halophile Archaea is bacterioruberin (BR), an acyclic C_{50} carotenoid with four hydroxyl groups [17] (Figure 1). Its anhydrous derivatives have also been reported. As BR has more conjugated double bonds than C_{40} carotenoids and also has 4 hydroxyl groups, its increased scavenging capacity was reported [20]. The biosynthetic pathway of BR production is still not completely elucidated. In human diet, the uptake of different forms of menaquinone is of high importance in the prevention of osteoporosis [25]. In general, lipids are widely used in cosmetic and food industries [26]; moreover, the archaeal polar lipids may be useful in the fight against cancer: mixtures of these polar lipids can form special membrane vesicles, named archaeosomes which may function as safe cancer vaccine adjuvants in mammals [27]. It is also noteworthy that the archaeal polar lipids are ethers and not esters. Their chiral centers are mirror images of the analogous diester glycerolphospholipids found in all other organisms, since archaeal dietherphospholipids contain glycerol-1-phosphate (S configuration) instead of glycerol-3-phosphate (R configuration).

This study aimed at establishing an integrated method for the identification of the bioproduct portfolio for halophilic Archaea from bioreactor cultivations – in an innovative interdisciplinary approach. We have implemented direct and easy sample preparation methods to selectively extract both the polar and the non-polar lipid content from samples of extreme halophile strains. In contrast to the conventional extraction

methods, the compound extractions were all carried out with organic solvents avoiding the use of halogenated solvents. Substituting the use of chlorinated solvents for the scalability of sustainable pharmaceutical and food technological processes is of highest interest. Using the developed sample preparation methods, the lipophilic bioproduct scan of the bioreactor samples was accomplished. An HPLC-MS/MS method was developed and tuned by using three commercially available carotenoid standards, which cover the polarity range of the targeted lipophilic products of extreme halophiles. The method then was adjusted for extreme halophilic sample extracts. In the bioreactor samples, we could identify seven geometric isomers of the carotenoid bacterioruberin, saturated and monounsaturated form of menaquinone, nonderived core polar lipids and their glycosylated or phosphorylated derivatives.

Materials and methods

Chemicals

The solvents hexane, methanol (MeOH), methyl-tert-buthyl-ether (MTBE) were of HPLC grade (Merck, Darmstadt, Germany). Acetone was of HPLC grade (Carlo Erba Reagenti, Rodano, Italy). Ammonium acetate was puriss. grade (Fluka, Sigma Aldrich, St. Louis, MO, USA). The synthetic standards were purchased in photometric purity (>95%): Beta-carotene (Sigma Aldrich, St. Louis, MO, USA), Astaxanthin and Canthaxanthin (Dr. Ehrenstorfer GmbH, Augsburg, Germany). Carotenoid standards were handled under reduced light in glass covered with aluminium foil in order to avoid light degradation or possible *cis-trans* photoisomerization.

Strains and cultivation procedures

Haloferax mediterranei (HFX, DSMZ 1411) and *Natronobacterium gregoryi* (NGR, DSMZ 3393) wild type strains were purchased from DSMZ – German collection of Microorganisms and cell cultures. The bioreactor samples were harvested from batch

cultivations exactly after substrate depletion on suitable defined media. The conditions of inoculation shake flask and bioreactor cultivations are described elsewhere [28].

Sample preparation method

The bioreactor broth samples were centrifuged at 20400 g for 20 min and then the pellets were washed with 20% NaCl solution and then re-centrifuged (Sorvall RC6+ Centrifuge, Thermo Scientific, Waltham, MA, USA). The washed pellets were frozen for subsequent analysis. 0.1 g of the thawed biomass samples was weighed into glass tubes in triplicates and 2 mL of the respective extraction solvent was pipetted onto them. After vortex homogenization of the samples, they were placed into an ultrasonic waterbath (Realsonic 40-S, Budapest, Hungary) for 30 min to enhance the extraction of the compounds. Subsequently, the samples were centrifuged (Sigma 204 centrifuge, Osterode am Harz, Germany) at 3000 g for 10 min to separate biomass from the extraction solvent. Aiming at only qualitative analysis, the supernatant was brought to dryness at 40°C in a TurboVap LV evaporator under gentle N_2 stream (Zymark, Hopkinton, MA, USA) to recover as much sample as possible for further analysis. The dry residue was then reconstituted with 200 µL extraction solvent prior to injection. Thus, an approximately tenfold sample concentration was achieved considering the partial entrapment of solvent in the pellet.

In order to extract compounds with different polarities, the following extraction solvents with increasing polarity were utilized: a) hexane, b) acetone, c) HPLC eluent A (91% MeOH/5% MTBE/4% water containing 5 mM ammonium acetate).

HPLC- method

The chromatographic separation was achieved on a Dionex Acclaim C_{30}e (250x4.6 mm, 5 µm) reversed phase (RP) column (Thermo Scientific, Waltham, MA, USA) with a Perkin Elmer Series 200 HPLC System (Perkin Elmer, Shelton, CT, USA). The initial eluent condition was 95 % v/v of eluent A (91% MeOH/5% MTBE/4% water

containing 5 mM ammonium acetate). A linear gradient elution was started from the 18th min to 40th min with eluent B (50% MTBE/46% MeOH/4% water) to 95 % v/v, and was further kept at this composition for 10 min. The column was reequilibrated to initial solvent conditions before each injection; reequilibration time was 15 min. Injection volume: 50 µL for Q1 scan, 100 µL for product ion scan. The flow rate was 1 mL min^{-1}. The autosampler temperature was kept at 10°C to prevent degradation of the sample components and the chromatographic separation was carried out at 25°C.

HPLC-DAD detection

According to the reported absorption wavelength of carotenoid compounds [29], HPLC-Vis measurements were performed with a Perkin Elmer Series 200 HPLC System (Perkin Elmer, Shelton, CT, USA) with Vis detection at 495 nm using the previously described HPLC method. Moreover, DAD data was obtained with Merck Hitachi Elite LaChrome 2000 HPLC System (Tokyo, Japan) to help the identification of the chromatographic peaks with the recorded UV/Vis spectra.

HPLC-MS detection

The eluent from the chromatographic column was introduced directly into a Perkin Elmer Sciex API 365 triple quadrupole mass spectrometer with unit resolution (Perkin Elmer, Toronto, ON, Canada). The data evaluation was accomplished with Analyst software version 1.4.2 (AB Sciex, Palo Alto, CA, USA). Before the MS detection, no splitting was applied. The 1 mL min^{-1} flow of highly volatile eluent (MeOH-MTBE-water gradient) could be completely evaporated by the ion source. The APCI ion source was used in positive mode with the following parameters: curtain gas 10 L min^{-1}, nebulizer gas 10 L min^{-1}, nebulizer current: 2 µA, ion source temperature: 400°C, declustering potential: 10 V, focusing potential: 150 V, entrance potential: 3 V. Regarding the larger molecular mass of the targeted lipophilic bioproducts, the mass spectrometer was operated in Q1 scan mode in the range of 500-2000 m/z. Product ion

scans were recorded from the selected parent ions with collision energy: 20 V and CAD gas: 1 unit. Parameters in negative mode were the same as in positive mode. In some cases, in order to restrict the production of ion adducts with NH_4^+ (from ammonium acetate), Na^+ or K^+ or the solvent adducts with water, the declustering potential was set from +10 V to +60 V. According to the equipment resolution, the obtained m/z values were rounded to the corresponding integer.

Results and discussion

Liquid phase extraction: Salinity and polarity

The goal of this work was to find a widely applicable workflow for bioreactor cultivations of extreme halophilic Archaea by simple but powerful analytical methods to analyze the lipophilic bioproducts. After the bioreactor batch cultivations, subsamples of the biomass were extracted. The use of chlorinated solvents is widely documented specifically for lipid extractions based on the method of Bligh and Dyer [30] which were modified for extreme halophiles [31]. Polar and non-polar lipids can, however, only be extracted with different methods [32], for instance, hexane and methanol for non-polar lipids and chloroform-methanol-water (1:2:0.8 v/v) for polar lipids [33]. It has previously been reported that acetone can be used as an alternative extraction solvent for polar lipids in the case of extreme halophiles [34], and ethyl acetate and methanol are suitable for the extraction of carotenoids [20]. In order to cope with high salinities without chlorinated solvents, we have modified the available carotenoid extraction methods of non-halophiles or moderate halophiles [35,36] for the extraction of a wide range of lipophilic compounds from extreme halophilic samples. In order to selectively extract compounds with different polarities, the following extraction solvents with increasing polarity were utilized: a) hexane, to extract compounds with the lowest polarity, presumably only non-polar lipids; b) acetone, to extract polar lipids;

c) HPLC eluent A (91% MeOH/5% MTBE/4% water containing 5 mM ammonium acetate), to extract both a wide range of polar lipids and the carotenoids. On one hand, the extraction step with suitable organic solvents is inevitable to avoid the high salinity of the samples, since it may render further bioprocessing impossible. Therefore, methanol and acetone, which are only slightly protic solvents, i.e. they can only dissolve low amounts of NaCl, were chosen to overcome the high salinity. On the other hand, since the potential bioproducts from extreme halophilic Archaea have very different polarities, it was expected that each extraction solvent would open a different window on the bioproducts from the bioreactor samples. The extracts obtained using the three extraction solvents were then analyzed using HPLC-MS/MS to verify that different bioproducts could be extracted.

Choice of standards - RP-HPLC: polarity selection and the gradient method

A solvent gradient RP-HPLC method was developed which was suitable to elute all the expected components. In RP-HPLC, separation occurs mainly on the basis of polarity differences. More polar compounds are eluted first while the least polar compounds have the longest retention times. Furthermore, the applied C_{30} column has enhanced shape selectivity which enables to separate even different geometrical isomers [21]. As commercial availability of analytical grade standards of carotenoids of natural origin is very limited, for method development three synthetic carotenoids were chosen. These compounds cover the wide polarity range typical for natural carotenoids and have been reported to be produced by halophilic microoorganisms [29]. It should be noted, that whereas the extreme halophilic archaeal lipohilic products analyzed in this work have widely different polarities, they are all relatively nonpolar compounds. This apparent contradiction is understood if we look at Table 1: Here the logP values of the three compounds which were used for method development are given; they range between 8 and 15. Compared to this, typical non-polar organic solvents have logP values below 5.

Part A of the Supplementary data shows the chemical formulae of the three compounds of Table 1. Data on the logP values of the archaeal polar lipids is not available; the published logP values of common lipids are in the range of 6-11 [37]. Hence, the gradient method was designed to have the capacity to measure more lipophilic as well as more hydrophilic compounds of the sample extracts, by positioning the retention times of the three chosen standards in the middle of the chromatogram.

HPLC-MS for bioproduct scan

In MS, polarity plays a role in ionization, in the formation of ion-adducts with ions from the eluent and to some extent also in fragmentation patterns. If the analyte exhibits adequate ionization in APCI, it should be preferred to ESI. The major advantages of APCI over ESI are that it is less dependent on the pre-ionization of the analytes and that solvent clusters, which could cover the valuable ions of interest, are less likely to be formed. In our case, we can add that APCI creates an inherent selectivity against extremely hydrophilic components which can be ionized only by ESI. Therefore they are excluded from an APCI-conducted mass spectrometric method for scanning a wide range of lipophilic products. An APCI method was developed using the standards. Subsequently, since during analysis different sub-groups of compounds were targeted, the methods were further adjusted for the different compound groups in the sample extracts. For example, as negative ion source mode is favorable for the ionization of sugar derivatives, the negative ion source mode was used to study components with possible glycoside or phosphatide moieties.

Figure 2 shows the positive mode total ion chromatograms (TIC) from different strains and extraction solvents. One can immediately note that eluent A did not extract the very lipophilic products which are eluted between about 36 and 50 minutes in the acetone extract of NGR and HFX, respectively. At the beginning of the chromatograms (about

5-6 min) we observed larger peaks which were not studied in detail for several reasons. They were thought to be too polar to be relevant for this study, they represented overlapping peaks of many molecules with similar molar masses and the spectra of these molecules were unlikely to belong to important lipophilic bioproducts from the biomass sample extracts. Peaks with retention times longer than about 9 show that the number of products giving relatively high MS signal was moderate. This unusually simple TIC of a very complex sample may be attributed to two factors: one is that the molecular masses were scanned only from 500 m/z upwards because the expected lipophilic products - extreme halophilic lipid compounds - are in this range. A definite advantage of using MS detection in this work is that smaller molecules did not interfere with screening. The other reason for obtaining simple TICs is that the samples contain only a limited variety of high molecular mass bioproducts. Any peak in the TICs is actually due to the integrated intensities of all molecules in the scanned mass range being eluted at a particular retention time. Therefore, for each peak the primarily responsible protonated molecule or deprotonated molecule was identified. As it could be expected from the simple TICs, the TIC peaks were usually belonging to a single protonated molecule or deprotonated molecule. The compounds producing each peak were attempted to be identified based on the literature about what kinds of compounds may mainly be expected and the characteristic traits of these molecules were studied by different mass spectrometric techniques. The details and the results of this work are presented below.

Development of HPLC-MS/MS method

Admixture of 5 mM ammonium acetate in eluent A has proven beneficial for increasing the signal intensity [12]. Methanol was utilized as protic solvent, because it was found [38] that the solvent composition influenced carotenoid ionization during APCI and the use of protic solvents enhanced the formation of protonated carotenoids. The MS-MS

instrument was used in a variety of operational modes, to allow for finding and identification of the products and perform crosschecks on the results. The operation parameters for the coupled mass spectrometry method were tuned during sequential chromatographic runs with the mixture of the three carotenoid standards. The goal was to gain maximal intensities of $[M+H]^+$ ions of the 3 standards in Q1 scan mode. However, it was realized that both formation of adducts and some in-source fragmentation also occurred in Q1 scan mode (data not shown). The obtained product ion spectra were in good agreement with the previously reported product spectra of the standards [13]. Therefore, it can be seen that the developed chromatographic method with MS detection was suitable for covering the range of polarity of diverse natural carotenoids and also thereby other lipids. Furthermore, this proposed method might also be used for the identification of other, unknown natural products produced by halophilic organisms having similar polarities. For method development, only C_{40} standards were available, which have slightly but significantly different polarities than C_{50} compounds due to the chain length. Estimation of quantity of C_{50} compounds based on a method with C_{40} standards cannot be performed, since ionization efficiency of APCI is very sensitive to compound polarities [39].

In quantitative analysis the method repeatability can be improved in various well-known ways, e.g., spiking, internal standards, etc. For qualitative analysis, the reproducibility of the TIC chromatograms can however not supply enough information about the repeatability of the method. The consistency of chromatographic separation is however very important for obtaining stable TICs with stable retention times and slight changes in the retention times of the compounds between Figure 2 and 3 can be observed.

Identification of the carotenoid BR

The main carotenoid compounds reported in extreme halophilic samples, BR and its derivatives, contain several hydroxyl groups which render them moderately polar [20]. However, other naturally produced carotenoids are glycosides and this may influence their extractability [40]. The results of the mass spectrometric identification of BR isomers are presented on the NGR samples extracted with eluent A, as more than 10-fold intensive signals were detected for NGR than for HFX (data not shown). Figures 2-5, as well as, Table 2 show the assignment of the seven chromatographic peaks with the use of TICs in positive and negative mode, product ion spectrum of m/z 742 (positive mode) and the HPLC-Vis data. Seven peaks were assigned as BR peaks (Figure 2), with m/z 742 of the protonated molecule $[M+H]^+$ (Figure 3). We have observed ion source fragmentations in Q1 scan mode and have also studied the product ion scans (Table 2, Figure 3). The obtained losses and fragmentations were in agreement with the general characteristic losses and fragments of BR found in literature [20] (Table 2 and Supplementary data B). With water losses occurring during MS fragmentation, the anhydrous analogues of BR were formed (1, 2, 3 and 4 water losses as BR contains four OH-groups). The characteristic hydrocarbon losses from the polygenic chain of the carotenoids were also detected (58 - C_4H_{10} loss, 70 - C_5H_{10} loss, 92 - toluene loss, 106 - xylene loss).

Whereas the application of positive mode enabled to detect the nonderived carotenoids without glycosylation, negative ionization mode is suitable for the ionization of components with ester groups, sugar moieties and glycosylated derivatives. Different glycosides of C_{50}-carotenoids were reported to be produced by halophilic organisms [41]. Depending on the oxygen content of the carotenoid, mono- and diglycosides are most commonly formed with glucose, mannose or rhamnose. In the negative ionization mode we have not found any significant peaks which might be attributed to glycosides

of BR. Carotenoid glycosides were only detected with very low signal intensity in samples of both strains with the mass 901 as $[M-H]^-$ which may correspond to BR with a C_6-sugar moiety, as the loss of an anhydrous C_6-sugar (mass reduction by 162) resulted in the BR residue (data not shown). On the other hand, in the negative mode only mono-acetate adducts were detected in the cases of the listed seven geometric isomers. In Figure 4 seven peaks were found as the acetate adduct of BR isomers with m/z 800 $[M+CH_3COO]^-$. If any of the peaks had been the monoanhydrous derivative of BR, the m/z 800 $[M+CH_3COO]^-$ could not have been intensive, but peaks as the acetate adduct of the monoanhydrous derivative with m/z 782 $[M+CH_3COO]^-$ could have been detected. Table 2 summarizes the data for the acetate adducts. TICs in negative ion mode could only be obtained with low intensity. Hence, it is more difficult to obtain fragmentation from these less intensive negatively charged peaks (in this case acetate adducts of BR) than from the more abundant peaks in the positive mode. However, the presence of the seven $[M+CH_3COO]^-$ peaks and the occurrence of ion source fragmentation could help with the correlation of the seven BR geometric isomers (Figure 4). As shown, no anhydrous derivative of BR could be detected in any samples with either polarity.

The HPLC-Vis detection has also shown the existence of seven peaks at the same retention time range as the BR peaks identified by MS (Figure 5), though slight retention time differences were observed between the different chromatographic systems. The HPLC-Vis detection was carried out at 495 nm, where bacterioruberin exhibits the largest absorption [19]. In order to provide more information on the identification of the BR geometric isomers, Table 3 shows additional results from HPLC-DAD measurements. The seven identified BR peaks resulted in UV/Vis absorption spectra with three fingered maxima in the 460-530 nm range and two

maxima under 400 nm. For BR, characteristic spectral peaks of three fingered peaks at 467, 493 and 527 nm; and two *cis* peaks at 370 and 385 nm have been reported [10]. The spectra of all of the seven detected peaks were in good agreement with the published three fingered maxima results for BR. The existence of seven geometric isomers of BR in the sample extracts can be supported by the fact that BR is synthesized from the lycopene [42]. Regarding their different molecular energies due to the position of the *cis*- bond in the conjugated chain, their stability is also different, and reported as: 5-*cis* ≥ all-*trans* > 9-*cis* > 13-*cis* > 15-*cis*> 7-*cis* > 11-*cis*. A study on the bioavailability of the different natural geometric isomers of lycopene also showed that all seven isomers can be present [43]. Up to now, only 4 geometric isomers of BR were detected from halophilic organisms (all-*trans*-bacterioruberin, 5-*cis*-bacterioruberin, 9-*cis*-bacterioruberin, 13-*cis* bacterioruberin [16]). Hence, some of them may have been decomposed or not produced at all in earlier reported experiments. The current approaches to bioproduct profiling are shake-flask cultivations; where, however, undesired limitations may occur [44]. Anhydrous derivatives of BR are likely to arise due to incomplete hydroxylation. This may be a result of insufficient environmental conditions or lack of nutritional supplementations, which did not occur in our bioreactor experiments possibly leading to the absence of by-products; namely anhydrous derivatives of BR.

Identification of polar lipid components

Polar lipid structures of extreme halophiles described in the literature were searched with the help of product ion scans of the relevant peaks of the Q1 scans. As illustrated by Figures 2 and Figure 6 and by the Supplementary data C and D, a large number of polar lipids were found in the bioreactor samples of both strains by using two extraction methods (acetone and eluent A). The identification procedure for different groups of bioproducts was performed as follows. In the fragmentation of different lipid

compounds, the core fragment for the justification of the presence of lipid structure was m/z 373 which is the protonated form of the C_{20}-monoether residue (Figure 7). Some characteristic losses could also support the identification (372: C_{20}-monoether loss, 280: C_{20} alkene loss, etc.). It should be mentioned that the UV/Vis absorption maxima of the other investigated compounds have not been reported yet. Hence, HPLC-Vis data cannot support further identification. However, in Figure 5 certain peaks are visible in the retention time range of 20-35 min at 495 nm which might be attributed to unsaturated lipids. Unsaturations in the lipid side chains might induce conjugation and thereby also absorption at 495 nm.

The lipid profiles of halophilic Archaea are so varied that they served as chemotaxonomic information before the advent of genetic taxonomy. Nowadays, however, the major criterion for taxonomy is the sequence of the 16S rRNA genes [45]. Therefore, differences between the lipid profiles of the sample extracts of the halophilic strain HFX and the haloalkalophilic strain NGR can be also seen in Figure 6 and in Supplementary data C and D. The core form and different derivatives of $C_{20}C_{20}$-diether were found in both sample extracts. However, the $C_{20}C_{25}$-diether lipid could only be found in the NGR extracts in detectable amount. Since this study only aims to provide qualitative profiling of bioproducts of extreme halophiles, further chemotaxonomic information was not targeted by the analysis.

Glycolipids were only detected in the HFX extracts in reasonable amounts. Although the glycosylation of lipids from haloalkalophilic strain isolates has been observed before [34], in the bioreactor sample extracts of the strain NGR the presence of glycolipids was not detected with our analytical method.

Both HFX and NGR sample extracts also revealed unsaturated forms of lipids. Desaturation of phytoene, carotenoids and alkane chains were reported to be

thermodynamically favored due to the creation of conjugated double bond systems [46]. As analogue phenomena, the desaturations of the long alkane glycerol ether chains of the lipids can be hypothesized. With the desaturation of as much as 4 bonds in the alkane chains, a conjugated double bond system is created in the C_{20} carbon chain. The detection of mono- to four times unsaturated lipids in the analyzed sample extracts is therefore in good agreement with the hypothesis of a thermodynamically preferred phenomenon to formulate conjugated systems in the side chains of the lipids.

Vitamin MK-8, a valuable non-polar lipid compound

The intensive peaks between 43 and 50 min (Figure 6) have been identified by their fragment ion spectrum (Figure 8) as saturated and monounsaturated forms of Vitamin MK-8 (Supplementary material part E). Eluent A could not extract these apolar compounds (Figure 2 and 4), but acetone and to a lesser extent hexane could extract them (Figure 4). The key point for the peak assignments was the characteristic fragment of the core menaquinone unit of the molecules, which can be observed as neutral loss from the molecule ion (m/z 186) and also as protonated residue (m/z 187) in the fragment ion spectrum (Figure 8). The 8 in MK-8 stands for the length of the isoprenoid chain on the quinone molecule and the losses of each isoprene unit (68 as saturated and 66 as unsaturated) can be followed in the fragment ion spectra.

The monounsaturated form of MK-8 was also identified in the bioreactor sample extracts in agreement with earlier results on halophilic extracts [47]. This observation is similar to the one made above in relation to the polar lipids. As the analyzed samples were of bioreactor origin, the well-defined and controlled cultivation conditions could also trigger the production of the unsaturated form of MK-8. A recent report describes a microbial platform for the bioproduction of MK-8 with a metabolically engineered *E.*

coli strain [48]. Bioproduction of MK-8 with extreme halophiles offers a nonsterile alternative for vitamin MK-8 production of natural origin.

Conclusions

Extreme halophilic Archaea are known to produce a variety of lipophilic compounds which may also be valuable natural products with many possible applications from food coloring agents to anticancer materials. The main aim of this study is to emphasize the developed analytical method for extreme halophilic bioreactor samples. We here present the capacity of the developed methods for extractions and qualitative bioproduct profiling of a halophilic and a haloalkalophilic strain, respectively. The HPLC-MS/MS method was developed using commercially available carotenoid standards, and then for the first time, HPLC-MS/MS technique was applied for the analysis of extreme halophilic bioreactor samples.

The different bioproduct portfolio identified in this study compared to earlier reports is likely caused by limitations occuring during shake-flask culture; here defined bioreactor fermentations were performed. In the present work a more uniform carotenoid bioproduct was identified. It could be predicted that the hydration steps of BR were completely carried out in our bioreactor studies, due to the defined and controlled cultivation conditions. Reducing separation and purification costs of the bioproducts is of high importance for the production of biobased products with high added value.

As a summary, the key points of this work are: i) chlorinated solvents were replaced for sample preparation of extreme halophilic lipid compounds from biological samples; ii) a qualitative method was implemented to identify some compounds the lipophilic bioproduct portfolio from bioreactor samples of extreme halophilic Archaea; and iii) in the bioreactor samples, seven BR geometric isomers were identified, including new ones compared to the literature on shake flask sample extracts.

Based on this study, detailed analyses of the carotenoid production over the bioreactor processes are planned under different cultivation conditions. Furthermore, the feasibilities of quantitative analysis will also be addressed with developing supporting analytics, e.g. antioxidant assays.

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Appendices
Supplementary data

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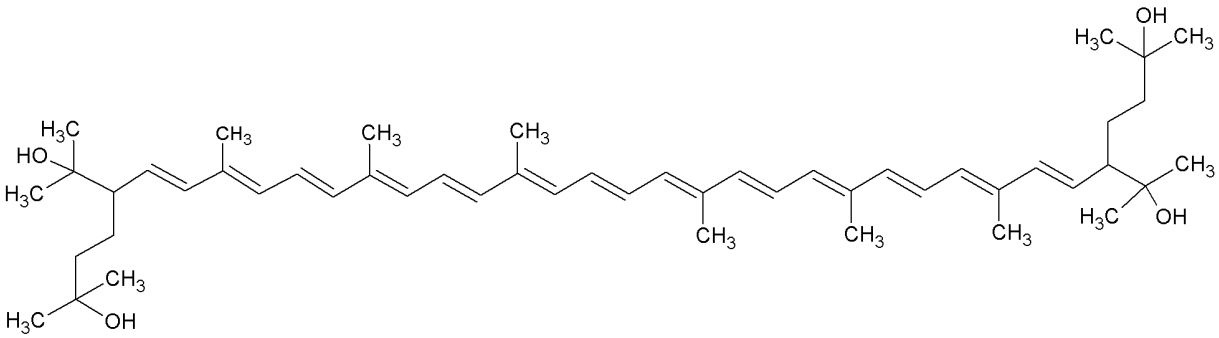

Figure 1. Chemical structure of Bacterioruberin (BR).

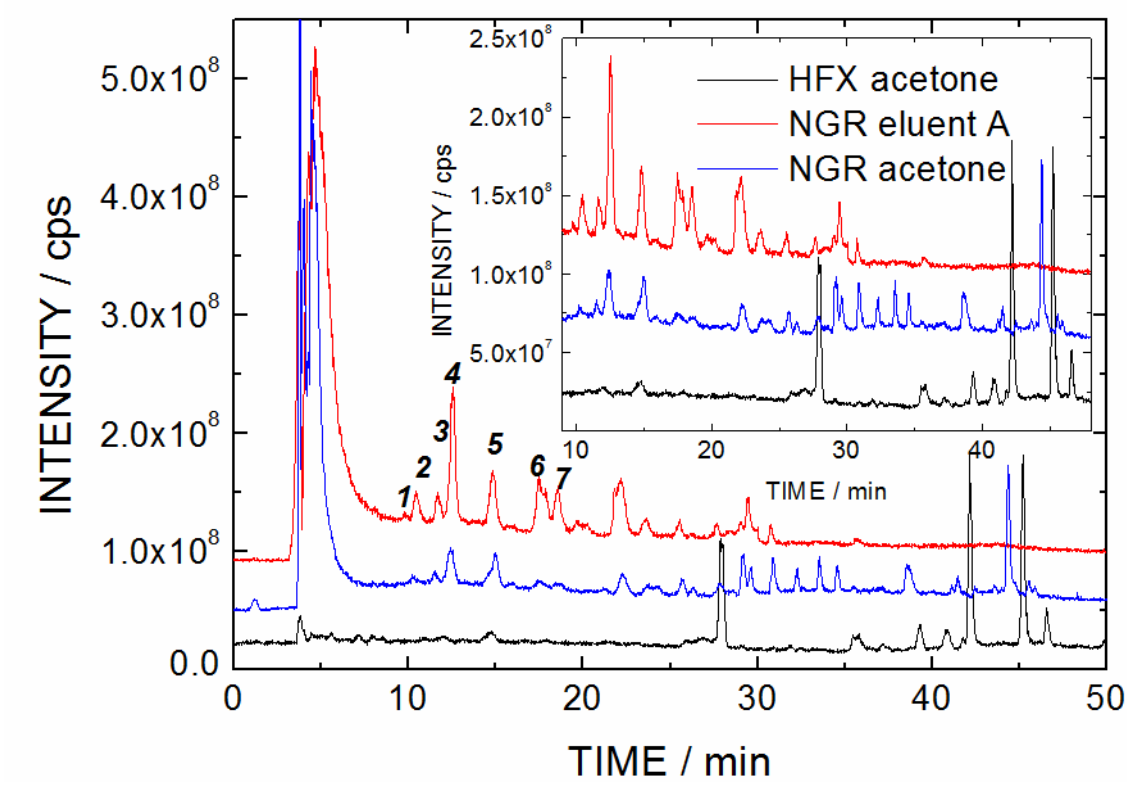

Figure 2. TICs of HFX and NGR samples extracted with different solvents, in APCI positive ionization mode. For better visibility NGR acetone and NGR eluent A samples are shifted upwards with 4×10^7 and 8×10^7 values, respectively. Inset figure shows the magnified TICs from the 9th minute. The carotenoid compounds can be found in the 9-20 min retention time range. The seven peaks lined up (t_{ret} 9.77, 10.45, 11.66, 12.56, 14.86, 17.46, 18.57 mins, respectively) indicate the seven geometric isomers of bacterioruberin.

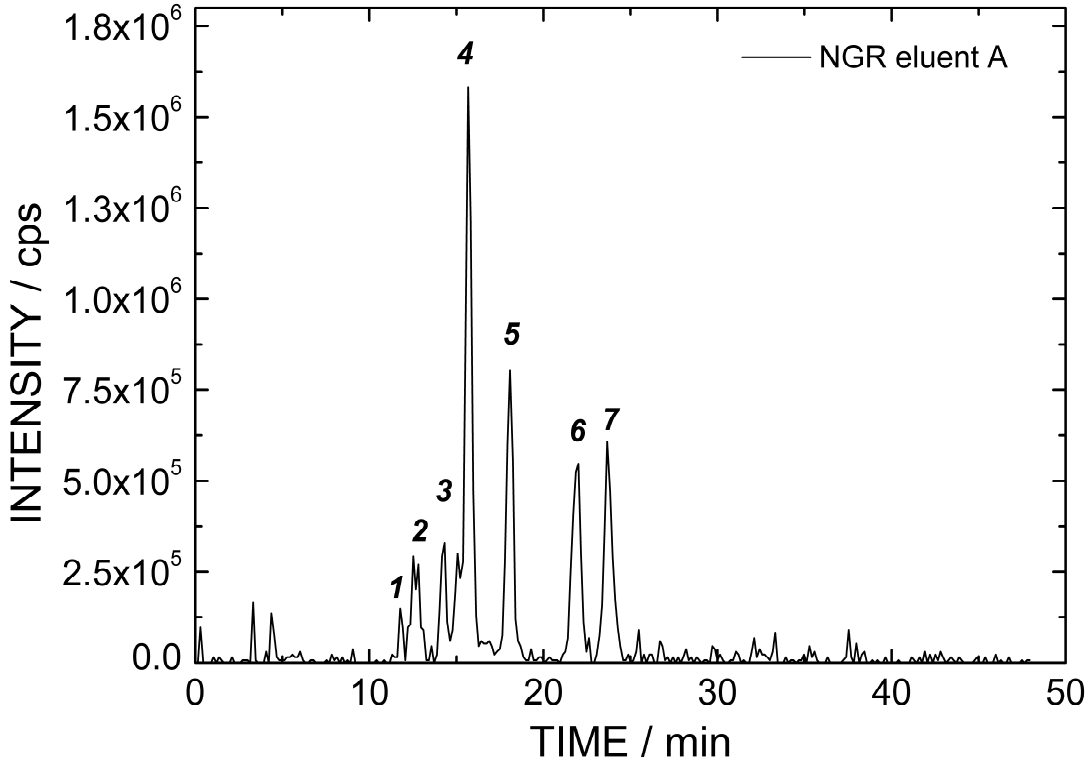

Figure 3. Product ion scan of m/z 742 of NGR sample extracted with eluent A as an extraction solvent. The seven peaks lined up (t_{ret} 11.79, 12.54, 14.27, 15.70, 18.08, 21.95, 23.69 mins, respectively) indicate the seven geometric isomers of bacterioruberin.

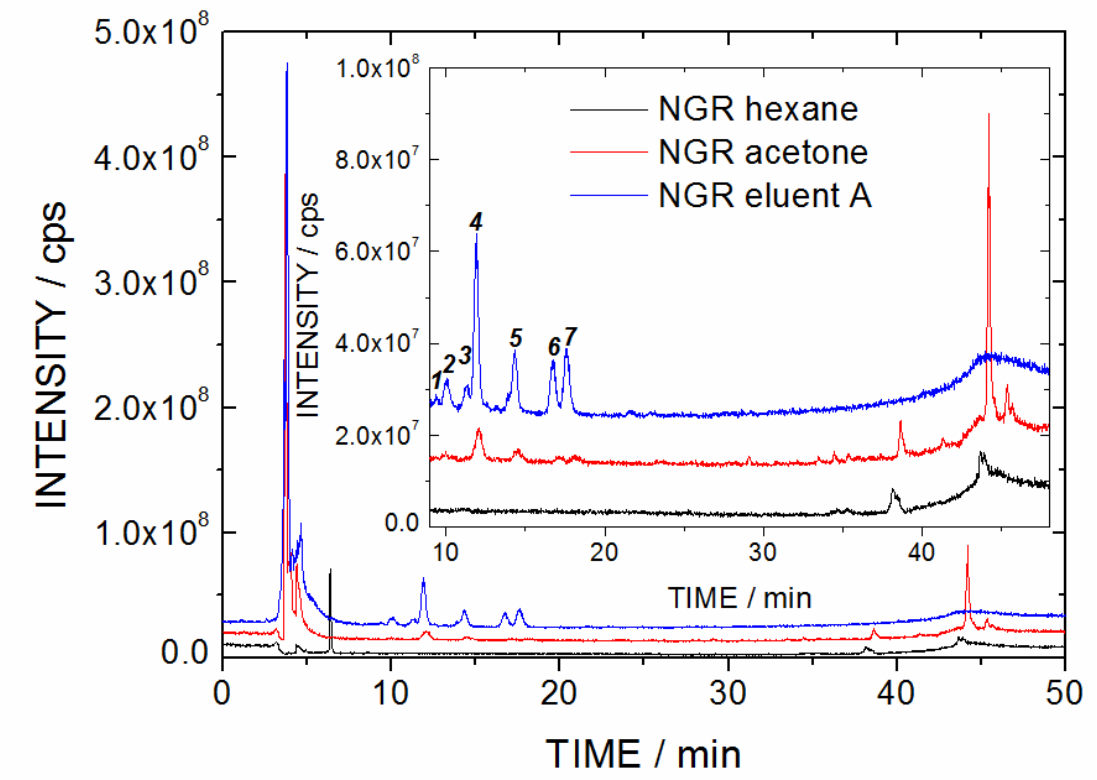

Figure 4. TICs of NGR samples extracted in different extraction solvents in APCI negative ionization mode. For better visibility NGR acetone and NGR eluent A samples are shifted upwards with 1×107 and 2×107 values, respectively. Inset figure shows the magnified TICs from the 9th minute. Compared to the sample extract with eluent A, in the sample extracts with hexane as well as acetone, only the least polar compounds with the greater retention times are present. The seven peaks lined up indicate the seven geometric isomers of bacterioruberin.

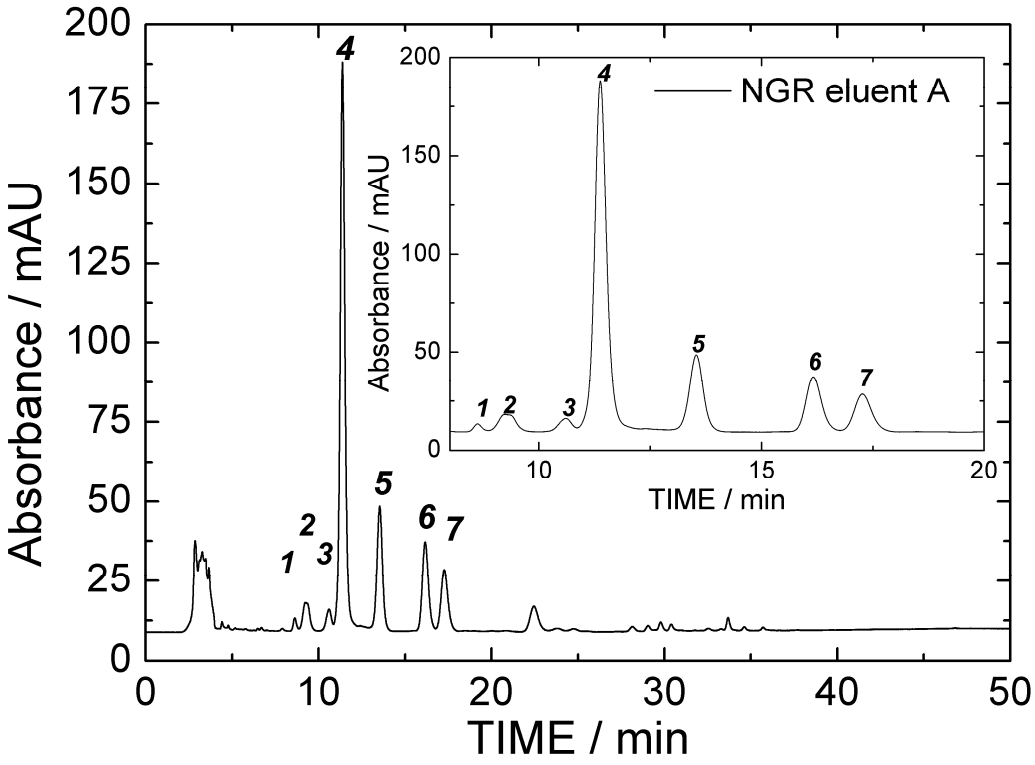

Figure 5. HPLC-Vis data of NGR sample extract with eluent A at 495 nm detection wavelength (495 nm is the greatest among the three-fingered absorption maxima of bacterioruberin). The seven bacterioruberin peaks are lined up.

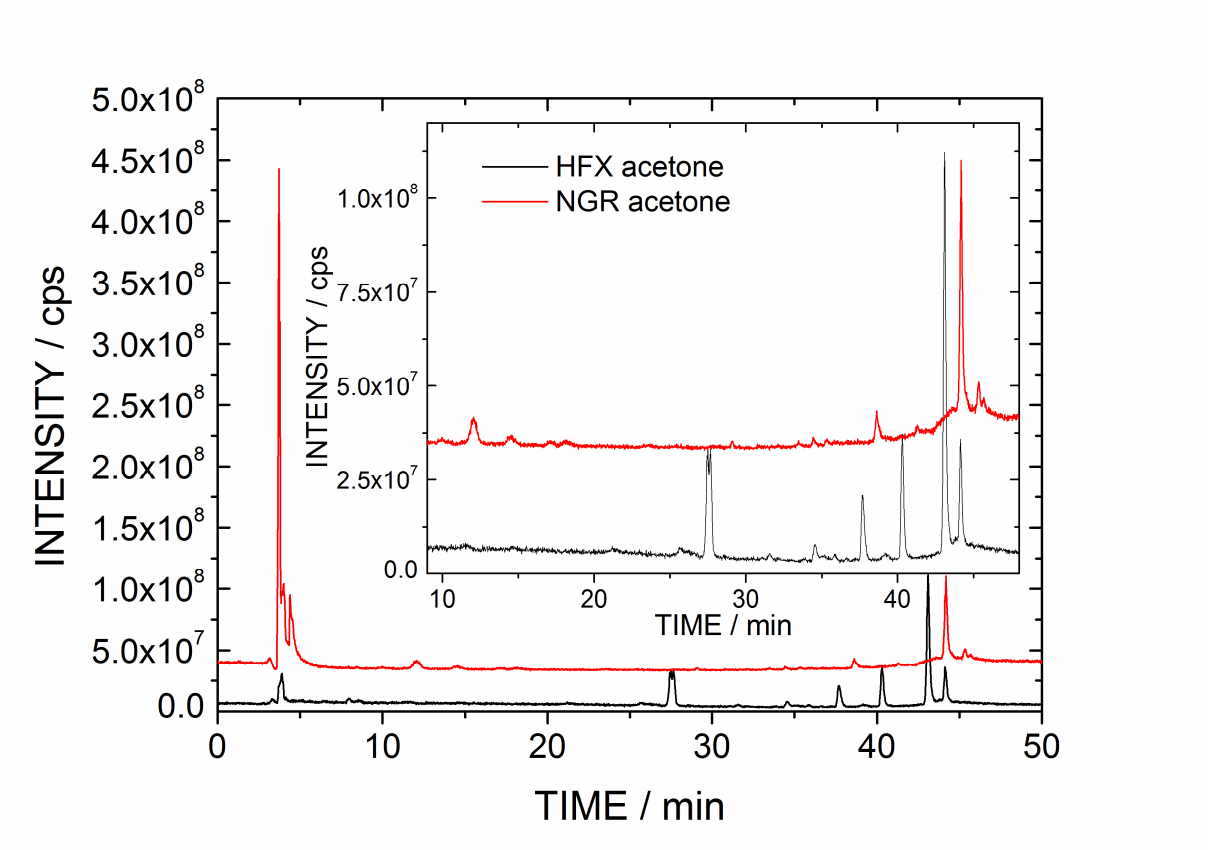

Figure 6. TICs of HFX and NGR samples extracted in *acetone* in APCI negative ionization mode. For better visibility NGR sample is shifted upwards with 3×10^7 value. Inset figure shows the magnified TICs from the 9th minute. The peaks observed in HFX but not in NGR can be attributed to the the glycolipid components present.

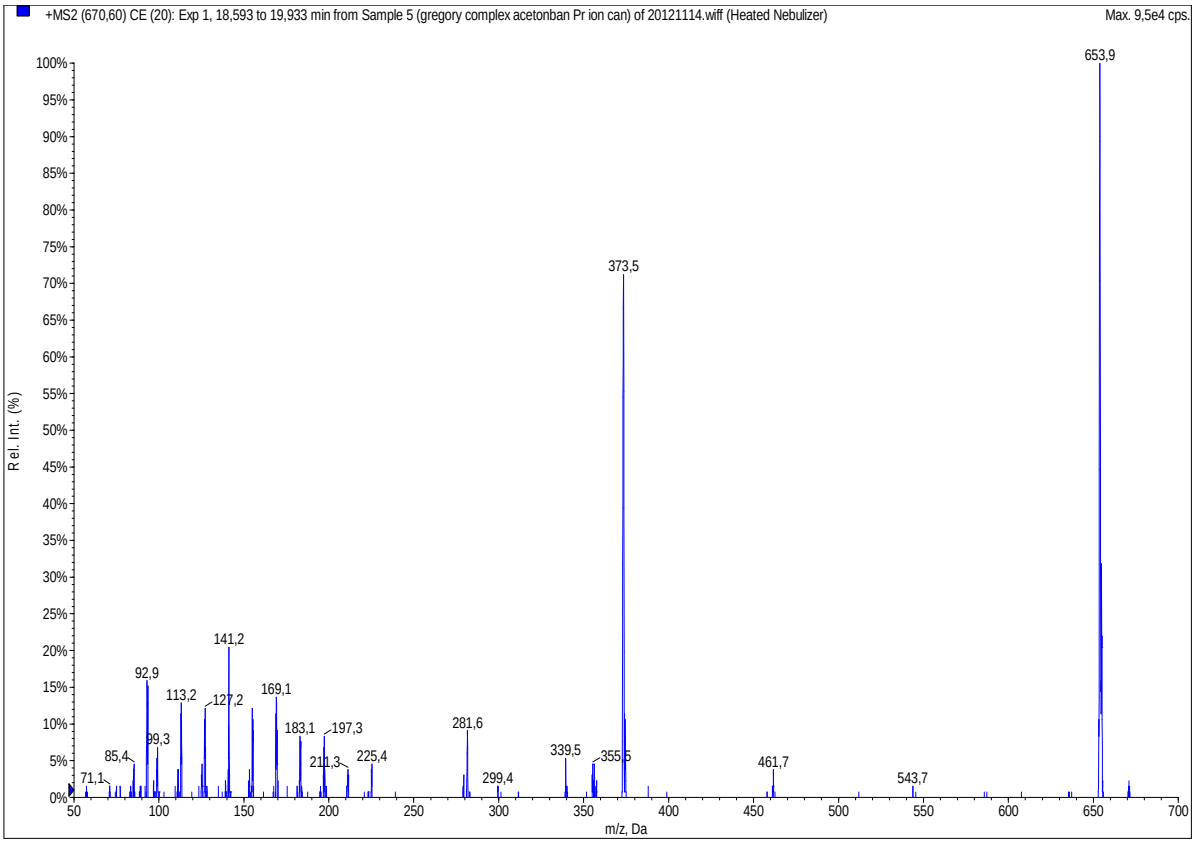

Figure 7. Fragment ion spectrum of the *m/z* 671 parent ion: the ammonium adduct of C_{20}-diether as an example for lipid identification in the sample of NGR extracted with acetone. The presence of the characteristic fragment (374: C_{20}-monoether) and loss (654-373=281: C_{20}-chain) can verify the identified lipids.

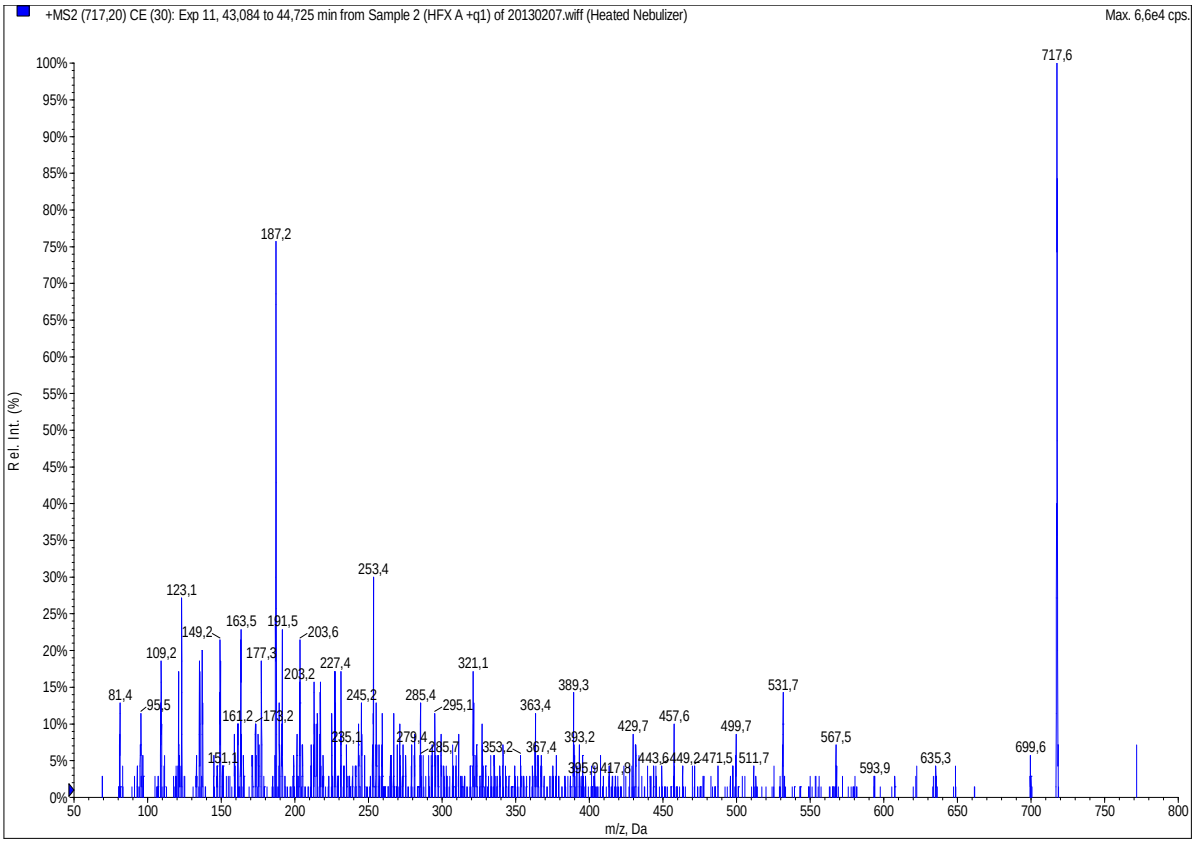

Figure 8 Fragment ion spectrum of the unsaturated form of vitamin MK-8 in HFX sample extracted with acetone, measured in positive mode.

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Identification of lipophilic bioproduct portfolio from bioreactor samples of extreme halophilic Archaea with HPLC-MS/MS

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Tables

Table 1 Carotenoid standards: In the method development, with the increasing oxygen content of the standards, a wide polarity range of natural carotenoid compounds was covered.

Component	Composition	Molecular mass (g/mol)	logP value	Measured concentrations
Beta-carotene	$C_{40}H_{56}$	536.4	14.7	20 mg/L, 10 mg/L
Cantaxanthin	$C_{40}H_{52}O_2$	564.8	9.52	20 mg/L, 10 mg/L
Astaxanthin	$C_{40}H_{52}O_4$	596.8	8.16	20 mg/L, 10 mg/L

Table 2 Mass spectrometric data of the observed geometric isomers of Bacterioruberin (BR), as the main carotenoid component of the sample extracts with **eluent A** as determined by HPLC-MS/MS with APCI in positive and in negative ionization mode. The order of retention of the geometric isomers of BR is reasoned by the different shapes of the geometric isomers. Referring to the unit resolution of the equipment, the m/z values were rounded to the corresponding integer.

Compound	Ret. time Fig. 2 (Fig. 3)	m/z in negative ionization	m/z in positive ionization	Fragments in positive mode	Comments

Peak 1	9.77 (11.79)	800 Monoacetate adduct of BR	**742** Molecular ion of BR	724 688 582 564 496	Water loss from the $[M+H]^+$ Triple water loss Triple water loss and -106 (xylene loss) Four times water loss and -106 Four times water loss and -3x58 (C_4H_{10})
Peak 2	10.45 (12.54)	800 Monoacetate adduct of BR	**742** Molecular ion of BR	724 706 684 666 630 612 596 494	Water loss from the $[M+H]^+$ Double water loss -58 Water loss and -58 Triple water loss and -58 Four times water loss and -58 Triple water loss and -92 (toluene) Double water loss and -2x106
Peak 3	11.66 (14.27)	800 Monoacetate adduct of BR	**742** Molecular ion of BR	724 706 684 666 582 564 432	Water loss from the $[M+H]^+$ Double water loss -58 Water loss and -58 Triple water loss and -106 Four times water loss and -106 Triple water loss and -106; -92; -58

Peak 4	12.56 (15.70)	800 Monoacetate adduct of BR	**742** Molecular ion of BR	724 688 670 648	Water loss from the $[M+H]^+$ Triple water loss Four times water loss Double water loss and -58
Peak 5	14.86 (18.08)	800 Monoacetate adduct of BR	**742** Molecular ion of BR	724 706 684 666 648 564 512	Water loss from the $[M+H]^+$ Double water loss -58 Water loss and -58 Double water loss and -58 Four times water loss and -106 Water loss and -2x106
Peak 6	17.46 (21.95)	800 Monoacetate adduct of BR	**742** Molecular ion of BR	724 706 684 666 648 582 544 494	Water loss from the $[M+H]^+$ Double water loss -58 Water loss and -58 Double water loss -58 Triple water loss and -106 (xylene loss) -106 and -92 Double water loss and -2x106

Peak 7	18.57 (23.69)	800 Monoacetate adduct of BR	**742** Molecular ion of BR	724 706 684 666 650 600 582	Water loss from the $[M+H]^+$ Double water loss -58 Water loss and -58 -92 Double water loss and -106 Triple water loss and -106

Table 3 Absorption peak maxima obtained by HPLC-DAD results with NGR sample extract in eluent A. The seven carotenoid peaks resulted in UV/Vis absorption spectra with three fingered maxima in the 460-530 nm range and two maxima under 400 nm. For BR, characteristic spectral peaks of three fingered peaks at 467, 493 and 527 nm; and two *cis* peaks at 370 and 385 nm have been reported.

Compound	**t_R at HPLC-Vis (min)**	**Three-fingered absorption maxima (nm)**			**Two maxima under 400 nm (nm)**	
Peak 1	8.6	467	494	525	305	316
Peak 2	9.2	459	492	520	372	398
Peak 3	10.5	471	496	529	303	317
Peak 4	11.4	467	495	528	304	316

Peak 5	13.5	463	489	518	369	386
Peak 6	16.1	483	493	525	308	314
Peak 7	17.2	471	496	529	303	317

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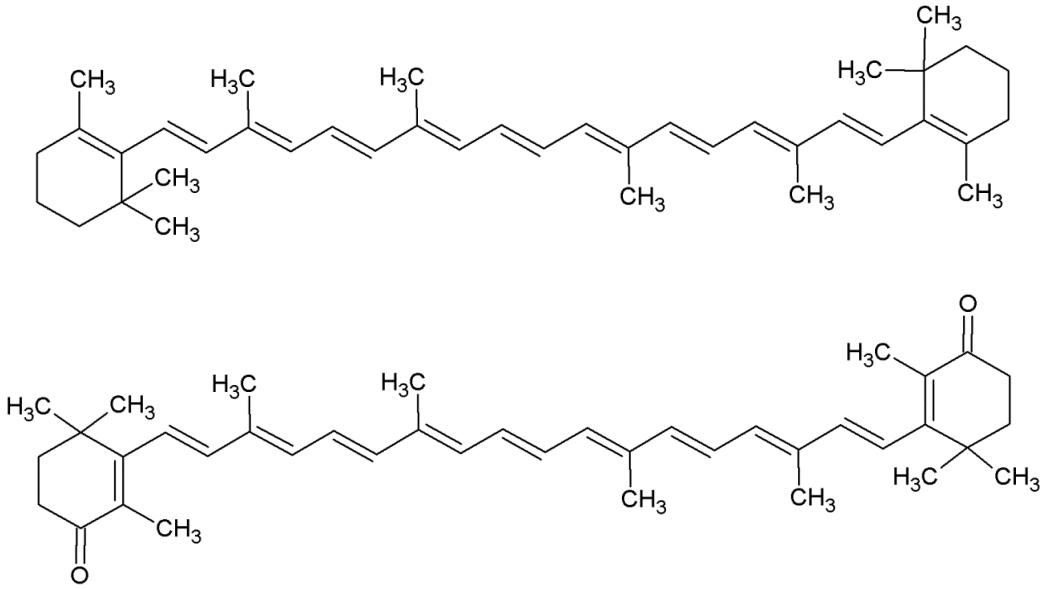
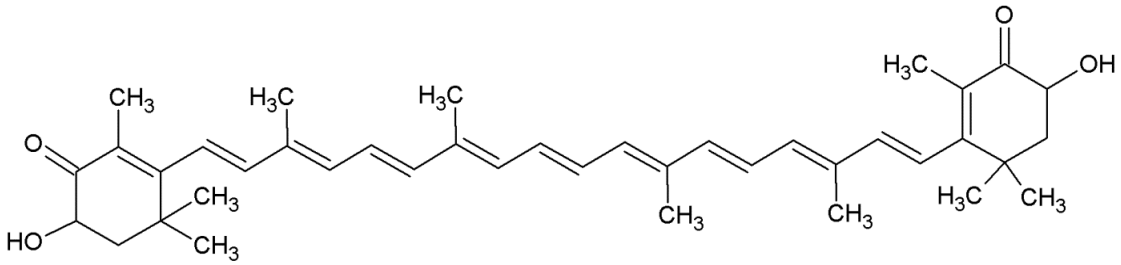
Supplementary data

A) Chemical formulae of the used commercially available carotenoid standards: beta-carotene, canthaxanthin, astaxanthin.



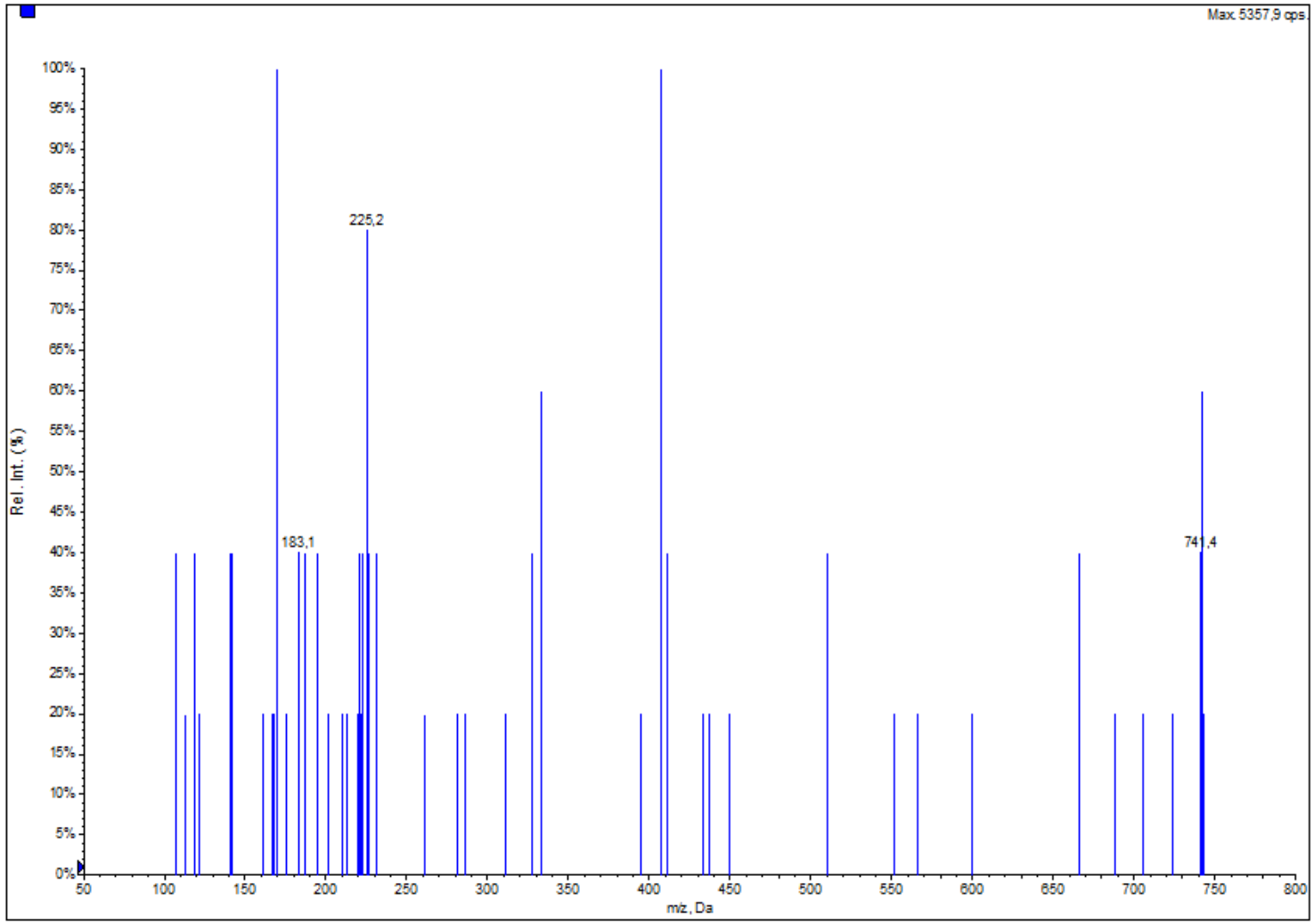


B) The seven fragmentation mass spectra from the seven BR chromatographic peaks Regarding the lower intensities of the fragmentation mass spectra from the seven BR chromatographic peaks, this issue should be commented as:

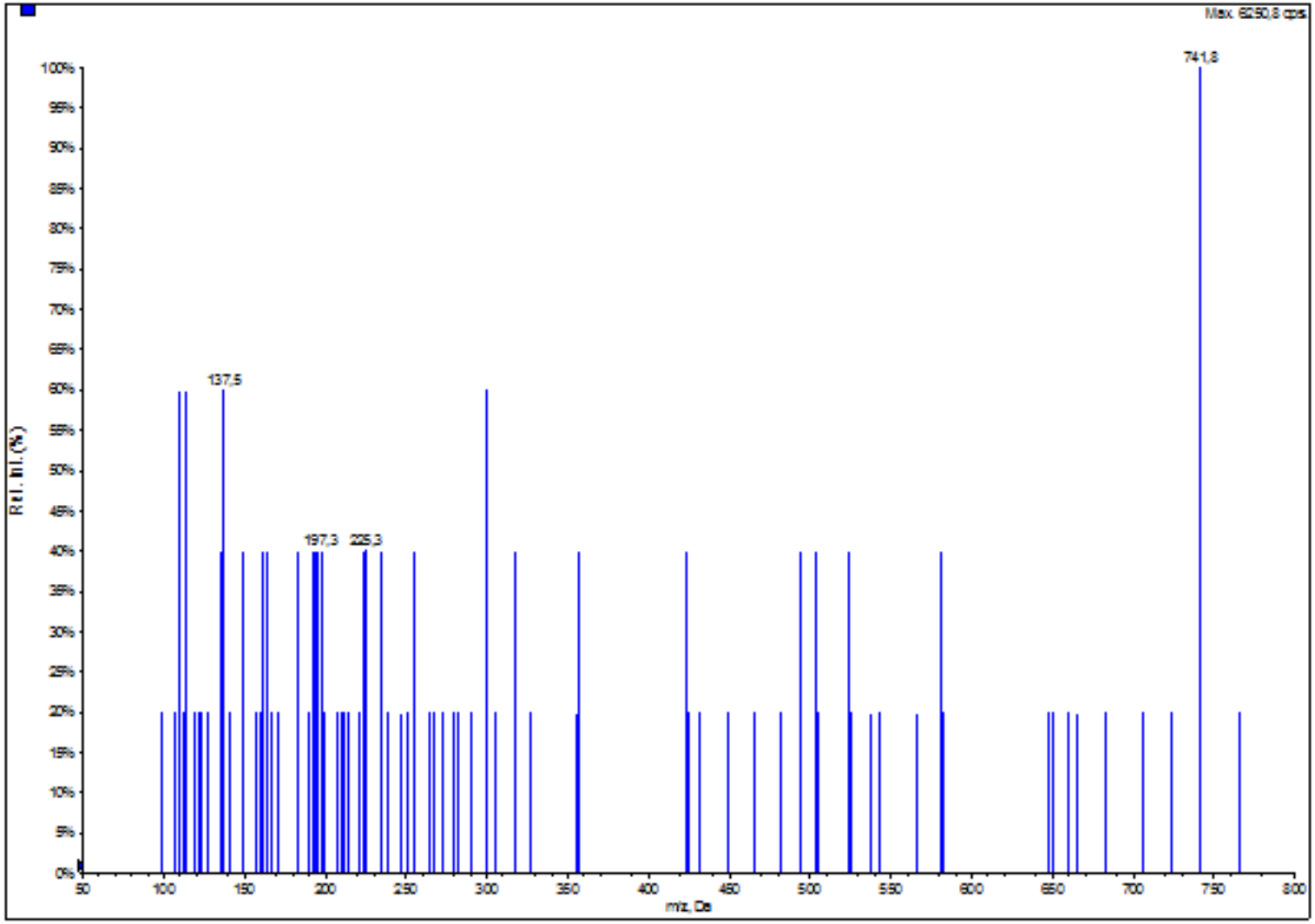
i) Due to precipitations, more concentrated sample extract could not be achieved.

ii) The maximum injection volume was injected to the applied C_{30} column.

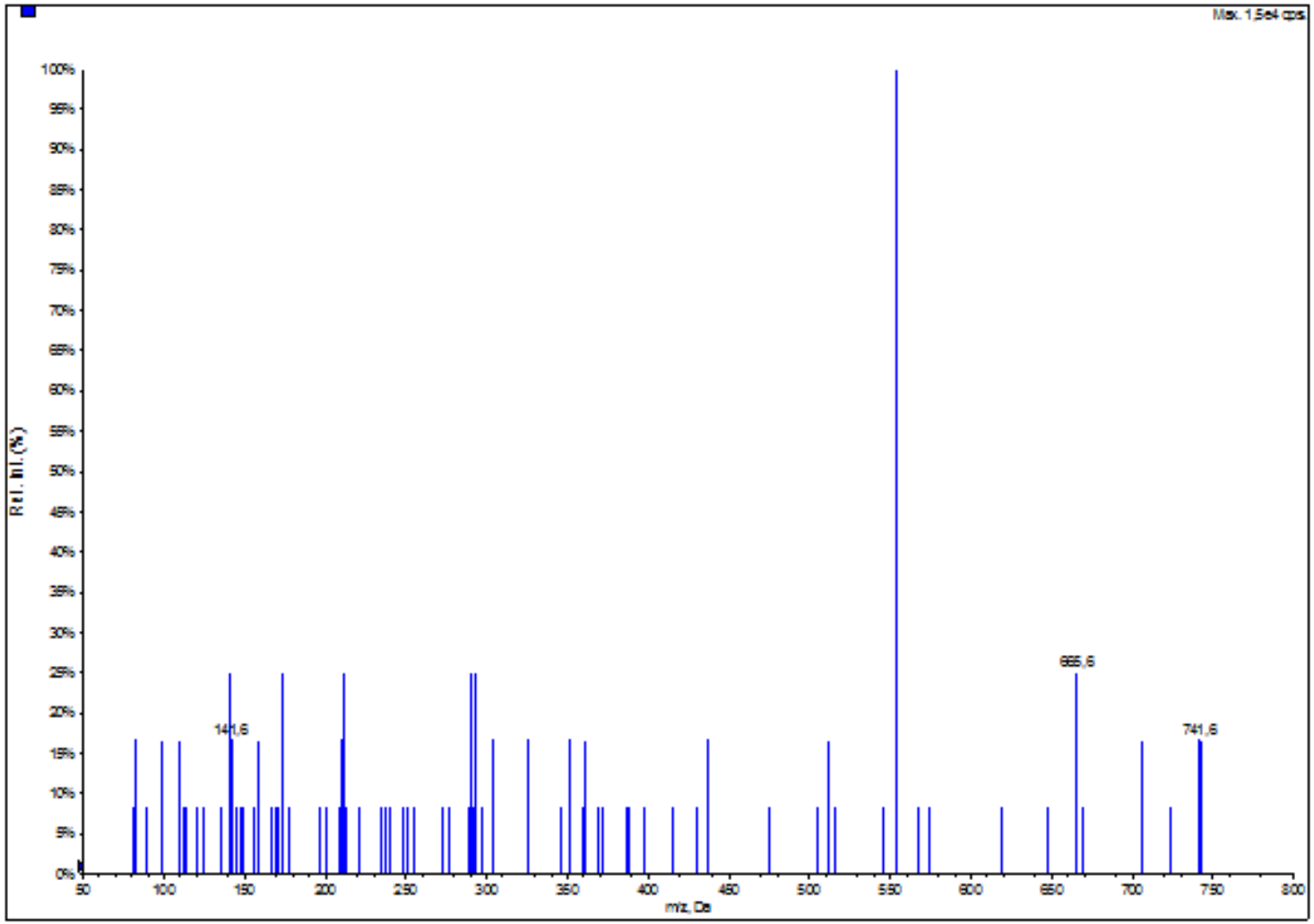
Peak 1


Max 5357,9 cps.
Rel. Int. (%)
100%
95%
90%
85%
80%
75%
70%
65%
60%
55%
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
225,2
183,1
741,4
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
m/z, Da

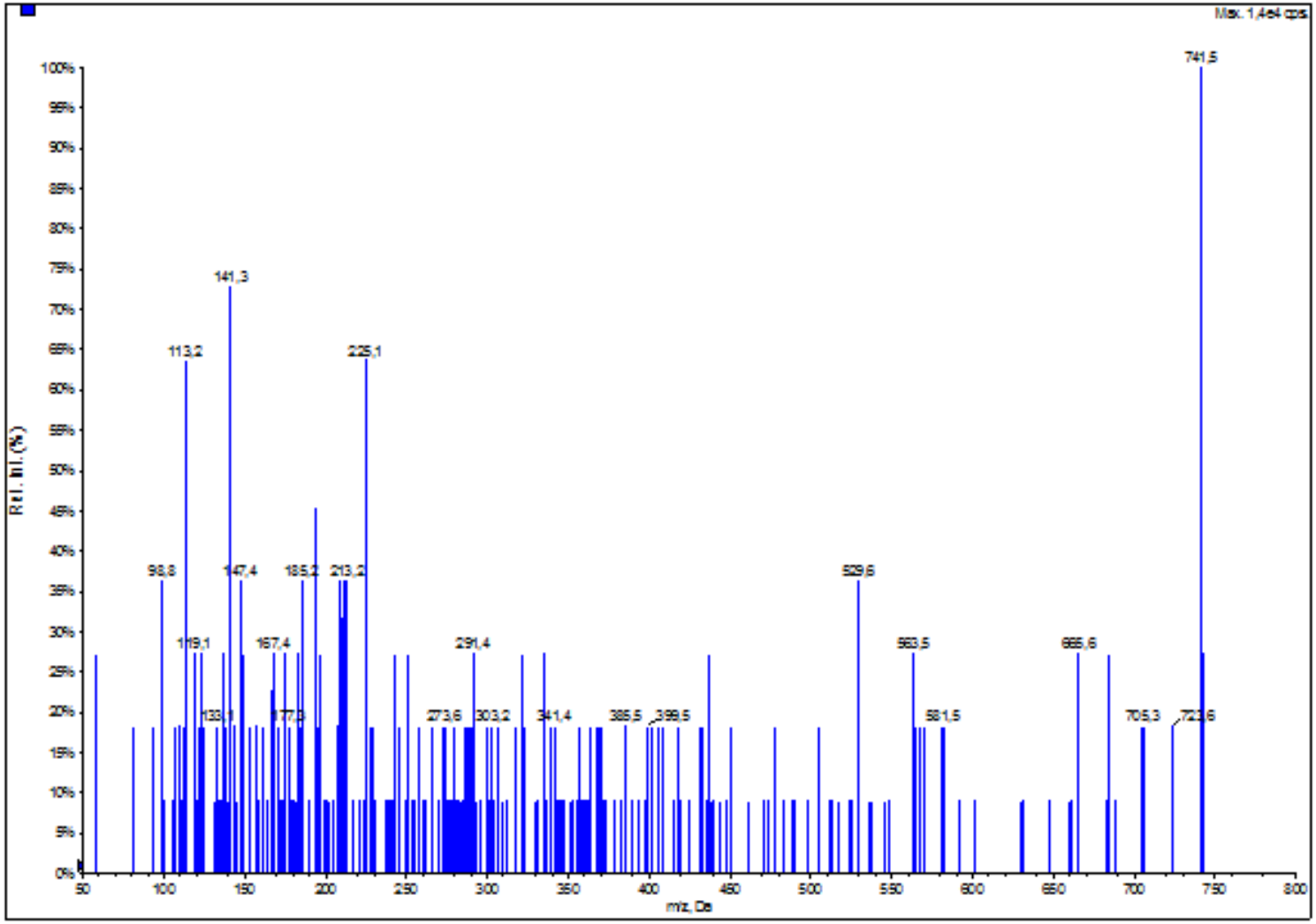
Peak 2



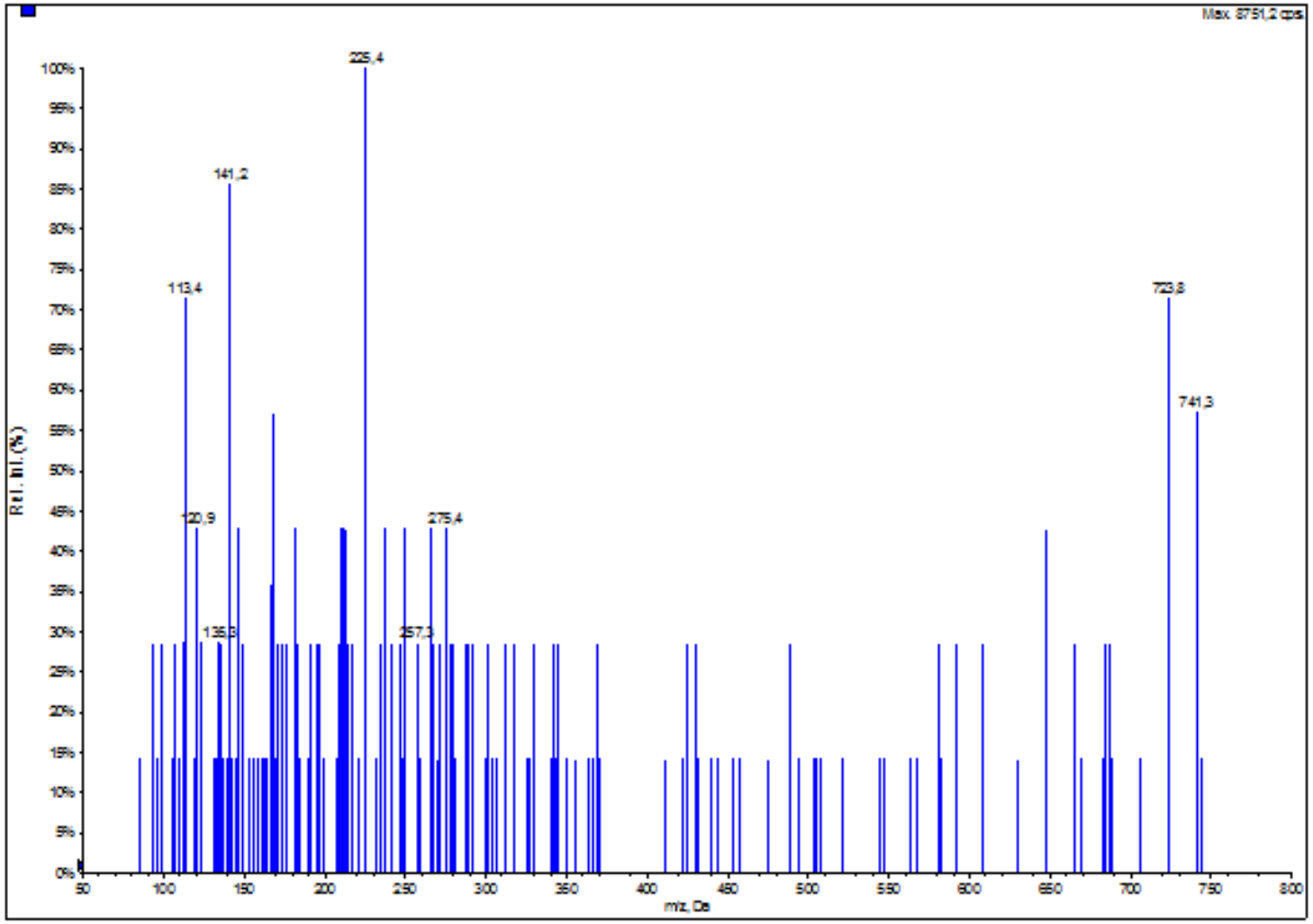
Peak 3


Max. 1,5e4 cps
Rel. Int.(%)
100%
95%
90%
85%
80%
75%
70%
65%
60%
55%
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
141,6
665,6
741,6
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
m/z, Da

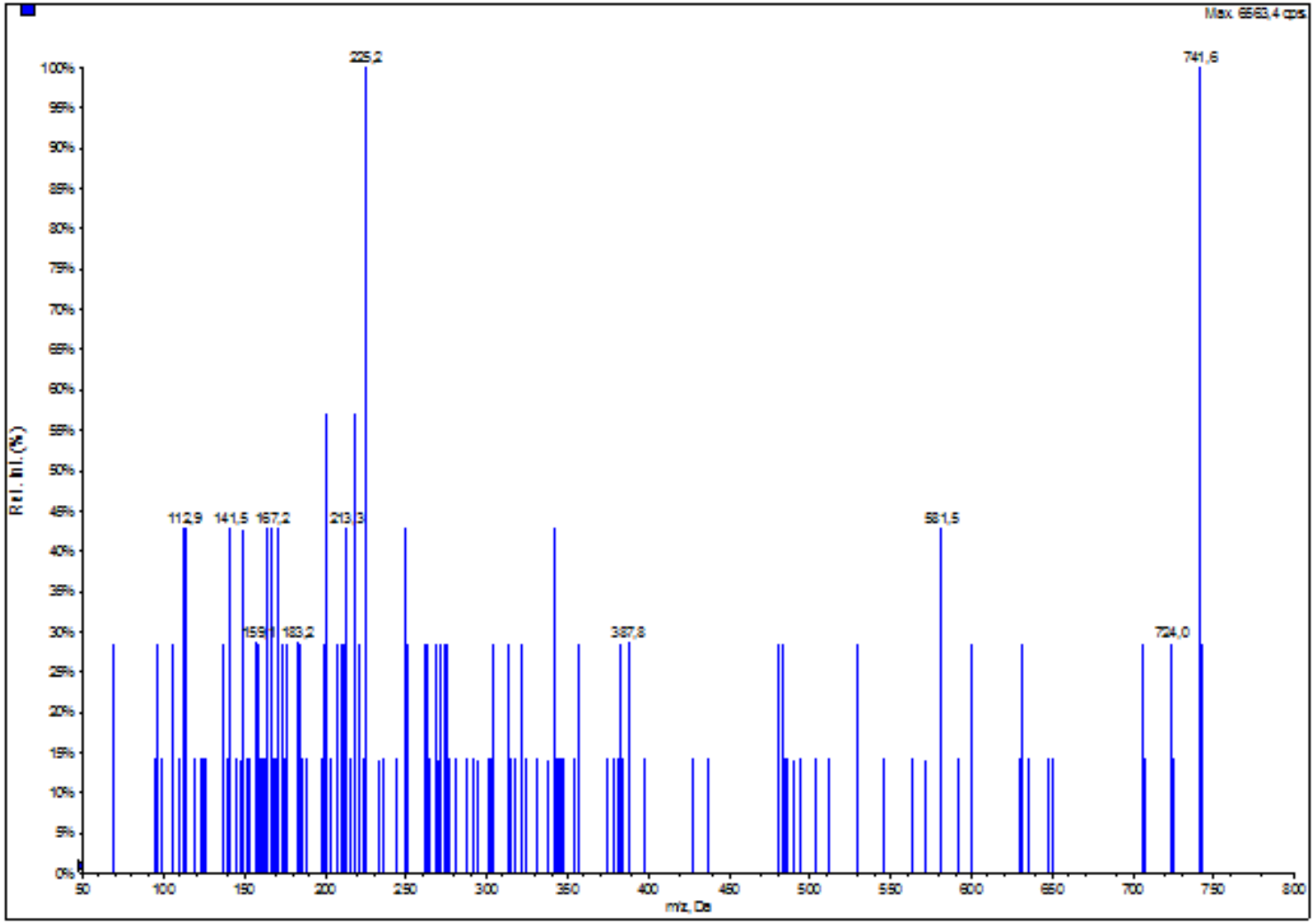
Peak 4


Max. 1,4e4 cps.
Rel. Int.(%)
m/z, Da
741,5
141,3
113,2
225,1
98,8
147,4
185,2
213,2
529,6
119,1
167,4
291,4
563,5
665,6
133,1
177,0
273,6
303,2
341,4
385,5
399,5
581,5
705,3
723,6

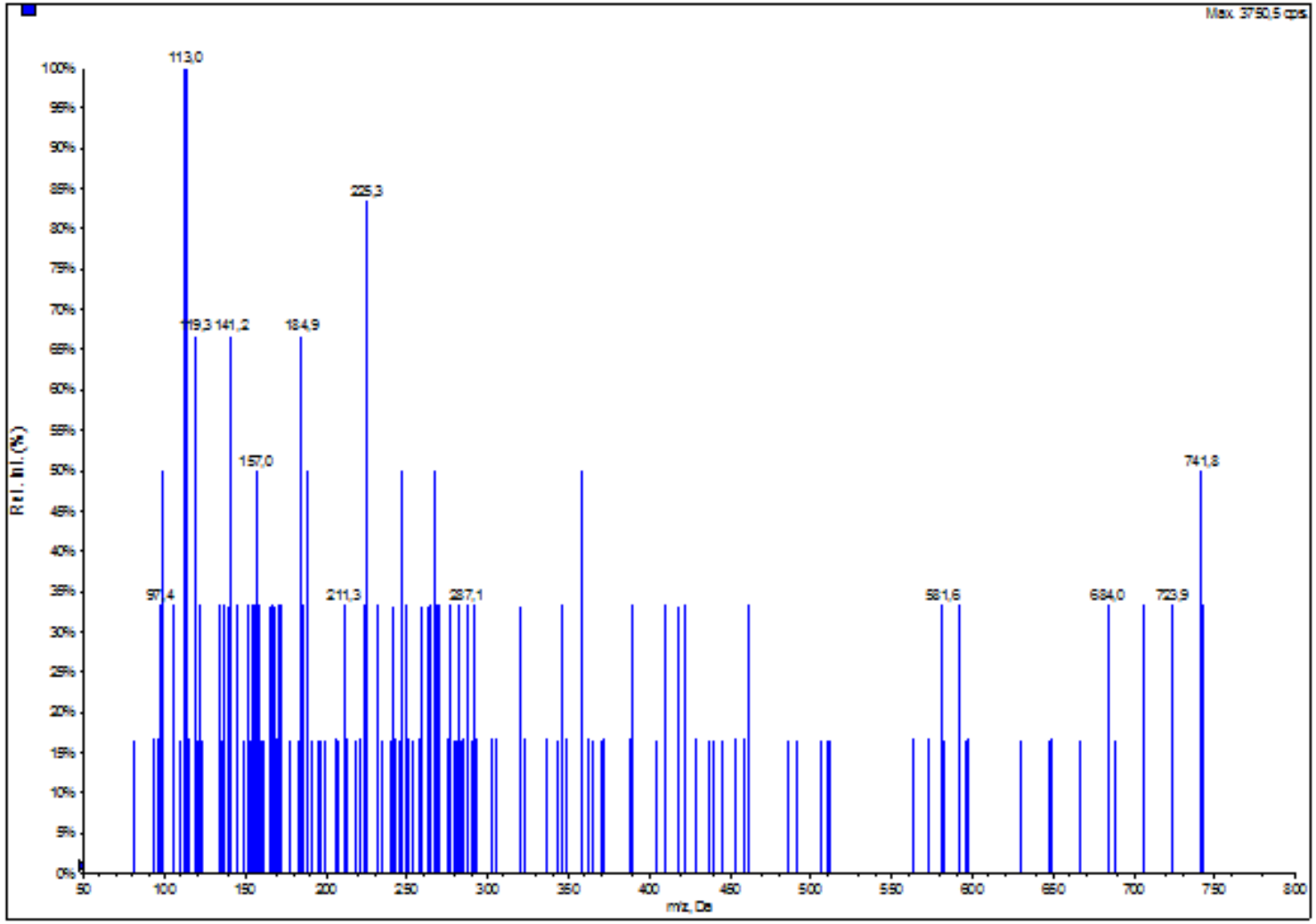
Peak 5


Max. 8751,2 cps.
225,4
141,2
113,4
723,8
741,3
120,9
275,4
135,3
257,1
Rel. Int.(%)
m/z, Da

Peak 6


Max. 6663,4 cps.
Rel. Int.(%)
m/z, Da
225,2
741,6
112,9
141,5
167,2
213,3
581,5
159,1
183,2
387,8
724,0

Peak 7


Max. 3750,5 cps.
Rel. Int.(%)
m/z, Da
113,0
225,3
119,3
141,2
184,9
157,0
741,8
97,4
211,3
287,1
581,6
684,0
723,9

C) Summary of identified polar lipids in the HFX strain samples. Depending on the polarities of the compounds, eluent A and/or acetone were used as the extraction solvents.

Ret. time Fig. 2&6	**Compound**	**m/z in positive ionization in acetone**	**m/z in negative ionization in acetone**	**m/z in positive ionization in Eluent A**	**m/z in ngative ionization in Eluent A**
17.5-18	diunsaturated $C_{20}C_{20}$ diether	-	-	667 (NH_4^+ adduct) 650	-
19.5-20	monounsaturated $C_{20}C_{20}$ diether	-	-	669 (NH_4^+ adduct) 652	-
22	Saturated $C_{20}C_{20}$ diether	-	-	671 (NH_4^+ adduct) 654	901 (PGP-Me $C_{20}C_{20}$)
22.5	diunsaturated $C_{20}C_{20}$ diether glycolipid	667-829-974	-	667-829-974	Mono-, di-, tri- etc. acetate adducts of glycolipids 713 Acetate adduct of $C_{20}C_{20}$ diether lipid
23.5	monounsaturated $C_{20}C_{20}$ diether glycolipid	669-831-976	-	669-831-976	
27.8	Saturated $C_{20}C_{20}$ diether glycolipid I	671-833-978	875-1018	671-833-978	
29.2	Saturated $C_{20}C_{20}$ diether glycolipid II	671-833-978		671-833-978	
33.5	Saturated	671-835-978		671-835-978	

	$C_{20}C_{20}$ diether glycolipid III				
35.5	Derivative of 4x unsaturated $C_{20}C_{20}$ diether	663	Acetate adducts of unsaturated lipids 709, 711, 713	-	-
37	Derivative of 3x unsaturated $C_{20}C_{20}$ diether	665		-	-
38.5	Derivative of diunsaturated $C_{20}C_{20}$ diether	667		-	-
41	Derivative of monounsaturated $C_{20}C_{20}$ diether	669		-	-
42.4	Derivative of saturated $C_{20}C_{20}$ diether (possible cardiolipin)	-	-	671	-

D) Summary of the identified polar lipids in the NGR strain samples. Depending on the polarities of the compounds, eluent A and/or acetone were used as the extraction solvents.

Ret. time (Fig. 2&6)	**Compound**	**m/z in positive ionization in acetone**	**m/z in negative ionization in acetone**	**m/z in positive ionization in Eluent A**	**m/z in ngative ionization in Eluent A**
17.5-18	diunsaturated $C_{20}C_{20}$ diether	667 (NH_4^+ adduct) 650	-	667 (NH_4^+ adduct) 650	-
19.5-20	monounsaturated $C_{20}C_{20}$ diether	669 (NH_4^+ adduct) 652	-	669 (NH_4^+ adduct) 652	-
22	Saturated $C_{20}C_{20}$ diether	671 (NH_4^+ adduct) 654	713 806 (PG-$C_{20}C_{20}$) 888 (PGP-$C_{20}C_{20}$)	671 (NH_4^+ adduct) 654	713 (acetate adduct)
23.5	3x unsaturated $C_{20}C_{25}$ diether	733 (NH_4^+ adduct) 716	-	733 (NH_4^+ adduct) 716	-
25.5	diunsaturated $C_{20}C_{25}$ diether	735 (NH_4^+ adduct) 718	-	735 (NH_4^+ adduct) 718	-
27.5	monounsaturated $C_{20}C_{25}$ diether	738 (NH_4^+ adduct) 721	-	738 (NH_4^+ adduct) 721	781 (acetate adduct)
29.5	Saturated $C_{20}C_{25}$ diether	-	783 970 (PGP-Me $C_{20}C_{25}$)	740 (NH_4^+ adduct) 723	783 (acetate adduct)

E) Monounsaturated and the saturated form of vitamin MK-8 in sample extracts with hexane and acetone. The NGR and HFX extracts were in accordance regarding these two vitamin compounds. The structural assessment with product ion scan was carried out with HFX sample extracts with acetone in positive ion mode.

Ret. time	**Compound**	**m/z in negative ionization in hexane**	**m/z in positive ionization in hexane**	**m/z in negative ionization in acetone**	**m/z in positive ionization in acetone**	**Fragments in positive mode**
43-44	Monounsaturated MK-8	716	**718**	716	735 (NH_4^+ adduct) **718**	188 quinone residue 532 quinone loss 594 isoprene unit losses (68) 526 458 390 322 254 monounsaturation: 188+66) 188
44-45	MK-8	718	**720**	718	737 (NH_4^+ adduct) **720**	188 quinone residue 534 quinone loss Less intensive peaks, isoprene unit losses