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Identification of bioactive peptides in kashar cheese and its antioxidant activities

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Abstract

This study analyzed the peptide profile and antioxidant activity in commercially sourced Kashar cheese. The antioxidant activity of Kashar cheese was found to be 41.09 mM Trolox g−1. However, the antioxidant activity of its F3, F4, F5 and F14 fractions was found to be 920.726 mM Trolox g−1, 545.544 mM Trolox g−1 , 783.864 mM Trolox g−1, and 392.12 mM Trolox g−1, respectively. In Kashar cheese, the Tandem Mass Spectrometry (MS/MS) spectrum for the 875 g mol−1 m/z signal was matched to αs1-casein, and it showed that 1012 g mol−1 (875+137) histidine can be a part of 1140 (1012+128) glutamine amino acid. Peptide sequences were matched to 875:RPKHPIK-H-Q peptide 1012:RPKHPIK+H peptide and 1140:RPKHPIKH+Q peptide. It can be concluded that the peptide fractions of Kashar cheese demonstrated antioxidant activity.

Keywords: Bioactive peptides, kashar cheese, antioxidant activity

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Introduction

Kashar cheese is a type of cheese that is boiled in hot water and kneaded after its curd has become acidized to a specific level. It is included in the semi-hard "pasta filata" group (Ucuncu, 2008). Kashar cheese is the most widely produced and consumed type of cheese in Turkey after Beyaz cheese (Cetinkaya and Soyutemiz, 2006; Urkek, 2008).With its 80,000 tons of production per year, it is the most widely consumed type of cheese with a semi-hard characteristic in Turkey. In terms of its production and components, it resembles Caciocavallo, Provolone, Mozzarella and Kashkaval cheeses (Turhan and Oner, 2015).

Generally, Kashar cheese is produced from raw milk, and the ripening process is important in determining its features. Many biochemical events occur during ripening, and these biochemical events need to occur properly during the ripening period to deliver a cheese output with unique quality characteristics (Ozturk, 1993).

Proteolysis is the breakdown of the proteins, and this is the most complex biochemical event that occurs in cheese during the ripening period (Mc Sweeney, 2004). Proteolysis plays a vital role in the development of textural changes in cheese curd; it contributes to the flavour and it may also have an effect on the off-flavour (bitterness) of cheese through the formation of peptides and free amino acids (FAA) (Law and Goodenough, 1995). FAA are the final products of proteolysis, their concentrations depend on the cheese variety and they have been used as ripening indices (Mc Sweeney and Fox, 1997a; 1997b).

Bioactive peptides have been isolated from cheese, yoghurt and fermented foods. They are released from protein molecules by enzymatic hydrolysis (Korhonen, 2009).

There are no studies on the characterization of peptides in Kashar cheese, whereas there are many investigations on the specific activities of bioactive peptides in different cheeses, such as antimicrobial antioxidant, anticancer and antihypertensive activities (Saito et al., 2000, Gomez-Ruiz et al., 2006; Silva and Malcata, 2005; Ong et al., 2007). Mass spectrometry has been employed for characterizing the proteins of different milk species (Bernardi et al., 2015), and to identify peptides in artisan or industrial Manchego cheese and Cheddar cheese (Karametsi et al., 2014; Gomez Ruiz et al., 2004). Mass spectrometry has also been employed to identify peptides in cheese.

In this study, three commercially sourced Kashar cheeses were investigated to determine their peptides and the antioxidant properties of those peptides. Peptides were separated using High Performance Liquid Chromatography (HPLC) and identified by peptide sequencing after MALDI-TOF MS/MS fragmentation.

Materials and Methods

Cheese samples

The samples of cheese were provided by a producer using traditional techniques to produce Kashar cheese in the Kars region. The cheese samples were maintained under refrigeration at 4ºC for 90 days. Peptide analyzes were carried on day 1 and on day 90. The cheeses were sampled three times, and analyses were duplicated.

The Kjeldahl method was used to produce 12% (v/v) trichloroacetic acid-soluble nitrogen (TCA-SN) and 5% (v/v) phosphotungstic acid-soluble nitrogen (PTA-SN) (IDF 1993).

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The total free amino acid levels were determined by the previously published method of Folkertsma and Fox (1992).

Extraction of Kashar peptides

To obtain bioactive peptides, cheese samples were prepared according to Donkor et al. (2007). Sixty grams of grated (commercial Kashar cheeses A, B and C) cheese were homogenized in 180 mL of distilled water and then centrifuged at 100 rpm at a temperature of 40°C. After centrifuging at 4000 rpm and 4°C for 30 minutes, the supernatant was again centrifuged using the same conditions. Following this step, the centrifuged samples were filtered through a roughing filter and the cheese samples were lyophilized (freeze dried). These lyophilized samples were dissolved in 0.1% trifluoroacetic acid (TFA) at a rate of 0.2 g $5mL^{-1}$, centrifuged at 14000 rpm at a temperature of 4°C for 30 minutes and then filtered through a 0.45 µm diameter filter to prepare them for chromatographic analyses.

Separation of water soluble peptides by reversed-phase chromatography (RP-HPLC)

The analysis of water-soluble peptides in cheese was carried out with reversed-phase (Shimadzu LC-20 AT series) HPLC and a Zorbax 300 SB-C8 monomeric column (250 × 9.4 mm i.d., 6.5 µm particle size and 300 A^0 pore diameter, Agilent, Waldbronn, Germany). The samples were dissolved in 0.1% TFA at the rate of 0.2 g $5mL^{-1}$, and 750 µL were injected into the HPLC column by filtering it through a 0.45 µm diameter filter. The peptides were eluted over a linear gradient from 100 to 0% solvent A (0.1% trifluoroacetic acid in deionized water) in solvent B (0.1% trifluoroacetic acid in 90% (v/v) acetonitrile in deionized water) over 80 minutes.

Analysis of peptide sequence

The SDS-PAGE technique and molecular sizes of the peptides obtained were determined according to Sambrook and Russell (2006). SDS-Polyacrylamide Gel Electrophoresis was performed by using a Mini-Protean® Tetra Cell from the Bio Rad Company and the procedure was carried out first at 60 volts for 30 minutes and then a 100 volts for 2 hours. It was dyed with Jeller Coomassie Brilliant Blue G-250 (Mitra et al., 1994). The peptide mixture purified from salts with ZipTip was observed by mixing it with a matrix solution (Dai et al., 1999).

The MS/MS spectrums, created by the fragments of the signals with sufficient MS/MS intensity, were obtained by the collision-induced dissociation (CID) method using argon gas in the LIFT mode (Suckau et al., 2003).

Determination of antioxidant activity

The antioxidant activity of either WSE, or the isolated peptides from them, was assayed according to the method described by Re et al., (1999). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation (ABTS+) was produced by the reaction of 7 mM of ABTS stock solution with 2.45 mM of potassium persulfate (final concentration in 10 mL of water) and keeping the mixture in the dark at room temperature for 12 to 16 hours before use.

Scavenging of the ABTS+ radical was observed spectrophotometrically (UV-1601, Shimadzu, Kyoto, Japan) by monitoring the decrease in absorbance at 734 nm. A reading was taken 1 minute after initial mixing and then periodically up to 6 minutes. A solvent blank was run in each assay (negative control). All procedures were carried out in triplicate, and their average was used as a datum point. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of antioxidants, and of the Trolox standard. To calculate the Trolox equivalent antioxidant capacity (TEAC), the gradient of the plot of the percentage inhibition of absorbance versus the sample concentration was divided by the gradient of the plot for Trolox to give TEAC at a specific time.

Statistical analysis

SPSS software for Windows (SPSS Inc., Chicago IL, USA) version 18 was used for statistical analysis of the Kashar cheeses and the corresponding sample replications. The Duncan test was used for comparison of the means.

Results And Discussions

Proteolysis of Kashar cheese

The nitrogen fractions are important parameters in the determination of the extent of proteolysis. The average fractions of nitrogenous compounds in Kashar cheese are presented in Table 1. Nitrogenous compounds increased significantly ($P < 0.05$) during storage. The 12% (v/v) TCA-SN fractions contain small peptides which were free amino acids, between two to twenty amino acid residues, ammonia and other minor compounds. The 5% (v/v) PTA-SN indicated the levels of small peptides to be <600 to 700 g mol^{-1} (di-, tri- and tetra- peptides) and free amino acids. For the cheeses, the soluble nitrogen content was expected to increase with the time of cheese ripening.

Total free amino acids were determined by the spectrophotometric method. The free amino acid content (FAA) changed from 0.45 to 4.9 mg Lys 100 g^{-1} during ripening ($P < 0.05$). The trends in the levels of total FAA in the cheeses were in agreement with the PTA-SN values.

Table 1. Fraction of nitrogenous compounds in Kashar cheese

	WSN	TCA –SN 12%	PTA-SN 5%	RI %	FAA mg Lys/ 100g
1st day	0.133±0.04	0.122±0.03	0.025±0.03	7.18	0.45
3.month	1.260±0.004	0.434±0.003	0.048±0.001	52.86	4.9

WSN:Water soluble nitrogen; TCA-SN:trichloroacetic acid-soluble nitrogen; PTA-SN: phosphotungstic acid-soluble nitrogen; RI: Ripening index; FAA: Free Amino Acid content

Results for RP-HPLC of cheese samples

To monitor the changes in peptide profiles during the storage of the water soluble extract (WSE) of cheese, WSE from day 1 and day 90 of storage were analyzed by RP-HPLC. Figure 1 and Figure 2 show the RP-HPLC profiles obtained from day 1 and day 90 of the cheese WSE. The peak number and peak height provide important knowledge about the ripening of cheese and proteolysis. The chromatograms of the cheese samples had different peaks (Fig. 1–2). The peaks for month 3 of the Kashar cheese samples had the highest amount of peptide peaks. The peaks increased in height significantly, changing the chromatographic profile.

Antioxidant activity in Kashar cheese

Antioxidant activity was determined using the ABTS method. In the ABTS method, the ability of the sample to quench a radical is measured. Caseins potentially have a high content of antioxidative amino acids such as tyrosine, tryptophan and lysine. The ABTS method is a sensitive and appropriate method for the measurement of antioxidant activity in cheese (Apostolidis, Kwon and Shetty, 2007).

ABTS radical scavenging values, the reported % inhibition value and TEAC (free radical and superoxide anion scavenging activity) were noted. Antioxidant activity was found to be 41.09 mM g^{-1} Trolox in the Kashar cheese. In terms of antioxidant activity, a significant difference was observed between day 1 and day 90 of storage ($P < 0.05$).

Fractions were collected at 10-minute intervals, and their TEAC values were obtained from the capacity of each sample to scavenge ABTS to Trolox, and the results were given in mM g^{-1} of Trolox of protein. The 3rd, 4th, 5th and 14th fractions with high values (Fig.2) were lyophilized, and the TEAC values of the fractions were examined. The fractionated peptide extracts were analyzed for antioxidant activity. Three different ratios of peptide extract (30, 40 and 50 µL) to ABTS were examined to determine if the percentage of inhibition increased with higher concentrations of peptide. The inhibition value percentages of the fractions collected before lyophilization were examined, and the results are given in Figure 3. The 3rd fraction showed (920.72 mM Trolox g^{-1}) the highest activity. The antioxidant activity of the peptide fractions are shown in Figure 4. The changes in the antioxidant activity of fractions were related to the rate of proteolysis in all the samples of cheeses up to the third month of ripening. In this study, when the proteolysis was compared with antioxidant activity, it was observed that changes in the antioxidant activity correspond to the rate of creation of proteolysis in the Kashar cheeses.

Other researchers have showed that similar Kashar cheese (cheddar cheese) has antioxidant activity and their activity was dependent on the ripening stage of the cheese (Apostolidis et al., 2007; Gupta et al., 2009; Pritchard et al., 2010; Meira et al., 2012). Biochemical processes were occuring in proteins during cheese ripening by hydrolysis of casein, by rennet activity, by plasmin, lactic acid bacteria and nonstarter bacterial proteinases (Mc Sweeney, 2004; Sousa et al., 2001).

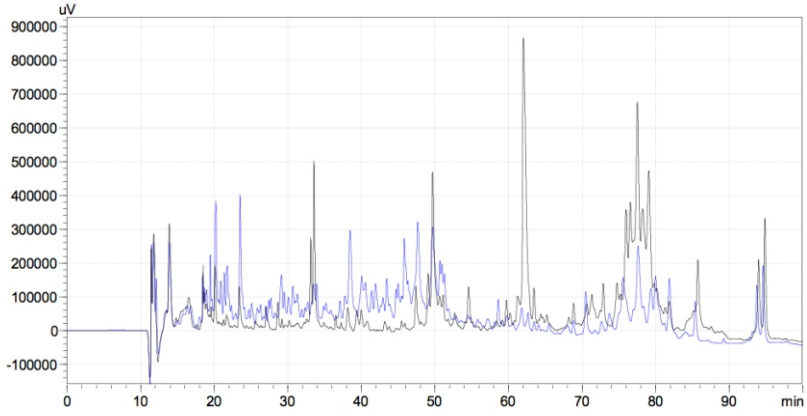

Figure 1. Shows the chromatogram of peptide in Kashar cheese 0- 3rd months. Blue peak: 0.month (1st day), Black peak: 3rd month of Kashar cheese

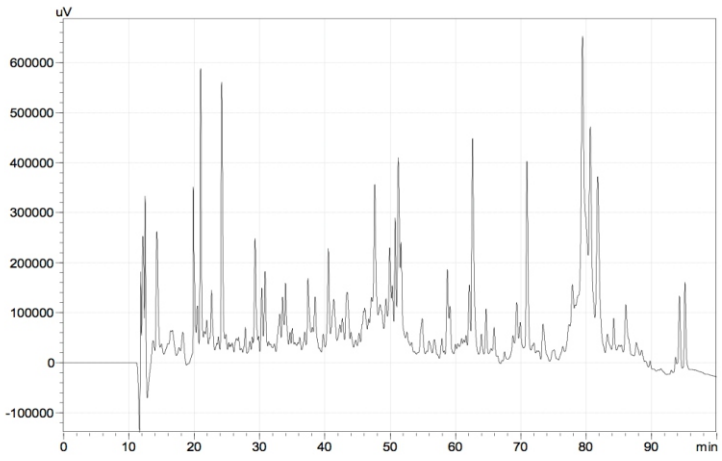

Figure 2. RP-HPLC chromotogram peaks of kashar cheese

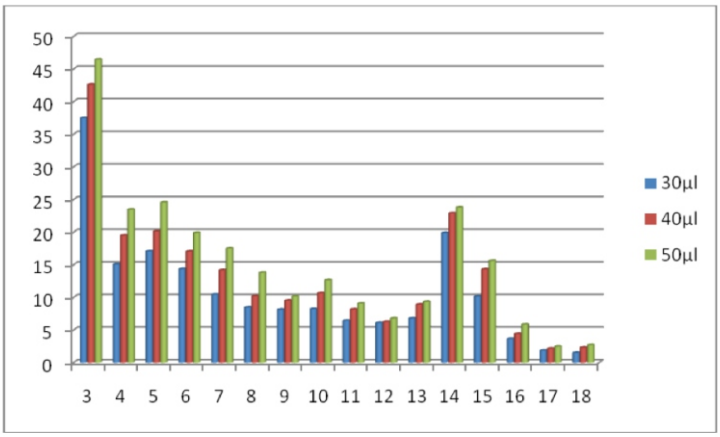

Figure 3. Inhibition ratio of water soluble extract of kashar cheese fraction collected by RP-HPLC

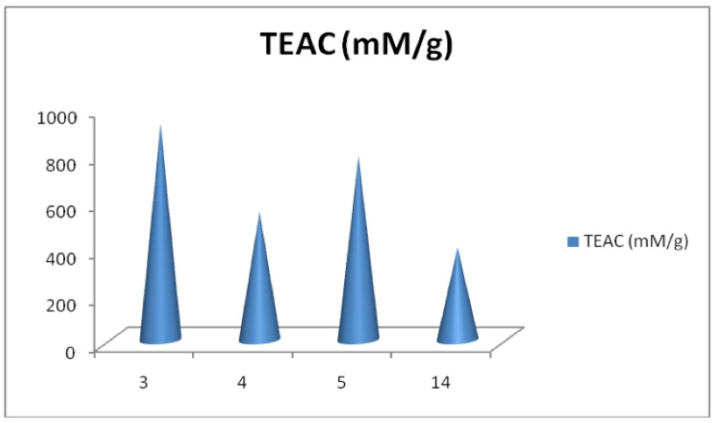

Figure 4. Antioxidant activity (TEAC) expressed as mM g^{-1} of Trolox of Kashar fractions which have higher inhibition activity

Peptide extraction of Kashar cheese and sequence analysis

After the chromatograms of the Kashar cheese had been taken, MALDI-TOF MS-MS was applied for peptide sequence analysis of the F3 and F5 fractions depending on the antioxidant activity results. Whether the samples contained peptides was determined by the one-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE), and MS spectrums were obtained by washing with ZipTip. Mascot database scanning was used. The MS/MS spectrum of 875 g mol^{-1} m/z signal matched up to α_{s1}-casein, and it showed that 1012 g mol^{-1} (875+137) histidine can be a part of 1140 (1012+128) glutamine amino acid. Peptide sequences were matched to 875:RPKHPIK-H-Q peptide 1012:RPKHPIK+H peptide and 1140:RPKHPIKH+Q peptide.

A similar case was observed in the 5th Kashar fraction for the 1535, 1664 and 1763 signals. The difference between the 1535 and 1407 signals corresponds to 128, which is the mass of lysine or glutamine amino acid; the difference between the 1664 and 1535 signals corresponds to 129, which is the mass of glutamic acid amino acid, and the 99-dalton difference between the 1763 and 1664 signals corresponds to the mass of valine amino acid. When the MS/MS spectrums of these signals were compared, the presence of some similarities strengthened the probability that one missing amino acid /extra peptide signal of the same peptide sequence was seen in the MS spectrum.

1D SDS-PAGE method was used Testing whether the samples contained protein was based on in-gel screening using the. For the peptide analysis, the highest number of peptide signals was observed with ZipTip, and the MS/MS spectrums were obtained by digesting those in sufficient amounts. Results within a confidence interval were not obtained from the Mascot database scanning, and since the sources showed similarity with that of the specimens (milk, α_{s1}-casein), the probability of matching within a confidence interval being true is in question. This similarity is the MS/MS spectrum of 875 g mol^{-1} m/z signal encountered in the Kashar 3 fraction samples. The 1012 g mol^{-1} (875+137) and 1140 (1012+128) g mol^{-1} m/z signals observed in the same spectrum can be an addition to the peptide N-terminal of histidine (137) amino acid and the continuing terminal of glutamine amino acid [18]. When it is compared by overlapping the digestion spectrums, the similarity shown strengthens this possibility (Figure 5).

875:RPKHPIK>gi|999048|gb|AAB34797.1| α_{s1}-casein A long form [ovine, skimmed milk, Peptide, 199 aa]
RPKHPIK-H
QGLSSEVLNENLLRFVVAPFPEVFRKENINELSKDIG
SESIEDQAMEDAKQMKAGSSSSSEEIVPNSAEQKYI
QKEDVP
SERYLGYLEQLLRLKKYNVPQLEIVPKSAEEQLHSM
KEGNPAHQKQPMIAVNQELAYFYPQLFRQFYQLDA
YPSGAWYYLPLGTQYTDAPSFSDIPNPIGSENSGKIT
MPLW
1012:RPKHPIK+H
1140:RPKHPIKH+Q

A similar case is observed in the 1535, 1664 and 1763 signals in the Kashar 5 fraction sample (Figure 6). The difference between the 1535 and 1407 signals corresponds to 128, which is the mass of either lysine or glutamine amino acid; the difference between the 1664 and 1535 signals corresponds to 129, which is the mass of glutamic acid amino acid, and the 99 g mol^{-1} difference between the 1763 and 1664 signals corresponds to the mass of valine amino acid. When the MS/MS spectrums of these signals are compared, the presence of some similarities strengthens the probability of observing one missing amino acid /extra peptide signal of the same peptide sequence in the MS spectrum. However, these results are empirical and not reliable since they do not match within a confidence interval for the database scan. Thus, they are only considered as a probability at this stage.

Proline and histidine showed lipoprotein peroxidation inhibiting peptide activity. The properties of these amino acids may be explained by phenolic and indol groups (Hernandez et al., 2005).

Conclusions

The results have shown that Kashar cheese and its peptide fractions demonstrated antioxidant activity. The Kashar cheese was matured for three months; therefore bioactive peptides may have been created during this time. It was found that Kashar cheese has some bioactive peptides. Further studies are now in progress to determine the peptides and identify the exact amino acid sequences conferring the bioactivities, which could enable the synthesis and purification of bioactive peptides for their application in food production and pharmaceuticals.

Acknowledgements

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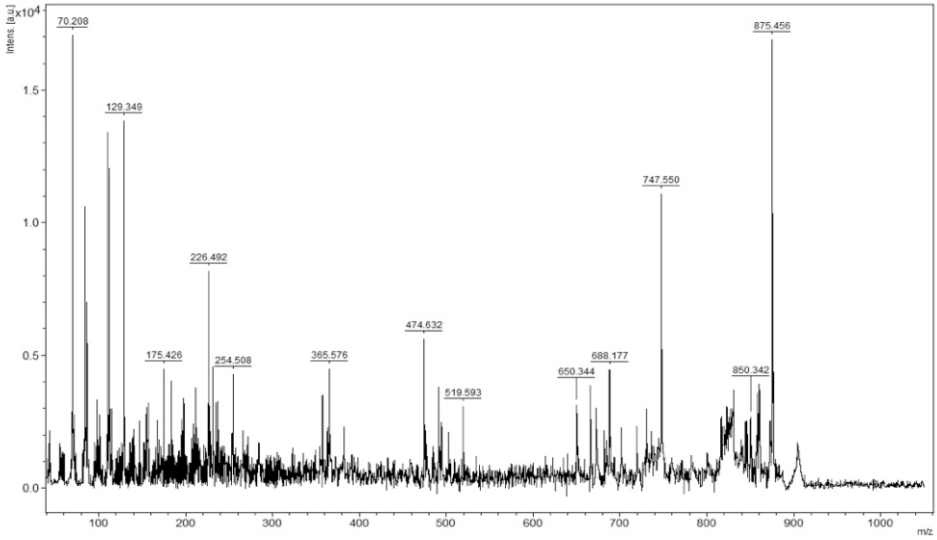

Figure 5. Kashar 3-875

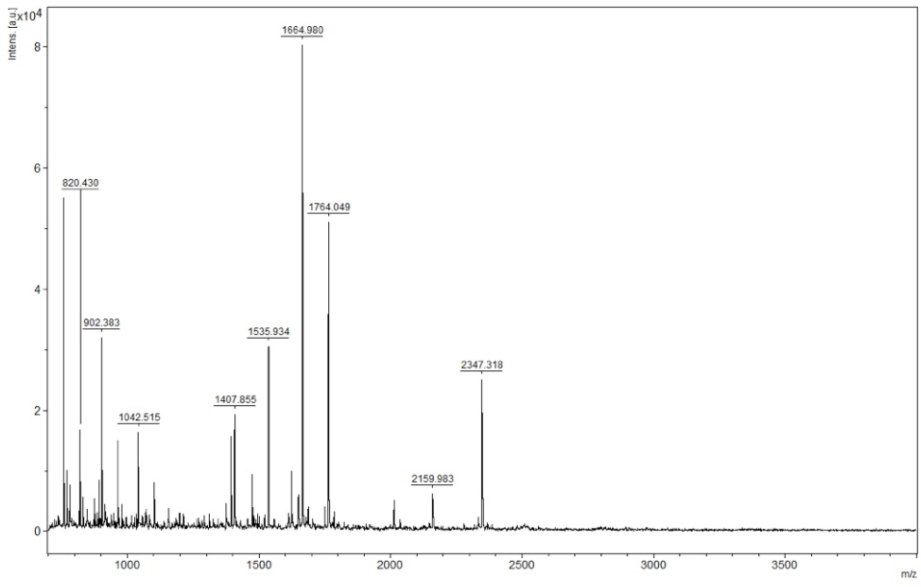

Figure 6. Kashar 5-Ziptip

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