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Rapid, accurate and routine HPLC method for large-scale screening of pro-vitamin A carotenoids in oilseeds

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Running title: HPLC detection of carotenoids in seeds of oilseeds

ABSTRACT Plant carotenoids, the precursors of vitamin A display several important biological functions as antioxidants and anti-carcinogens. The oilseed crops, owing to their high oil content, form a good matrix for the bioavailability of β-carotene, thereby providing potential targets for biofortification to combat vitamin A deficiency (VAD). However, the screening and characterization of these crops, that otherwise contain very low levels of pro-vitamin A carotenoids has been difficult owing to their poor recovery and strong binding to the oil matrix. Here, we report a rapid method for high volume HPLC analysis involving the extraction and determination of β-carotene in four oilseed crops (peanut, soybean, sunflower and mustard). This included a comprehensive study of the factors that potentially influence the qualitative and quantitative yields of β-carotene in these crops. This is the first crop-independent HPLC method for the quantification of pro-vitamin A carotenoids that shows excellent recovery and reproducibility (>90 percentage recovery in oil) using small tissue sample and is capable of processing up to 30 samples per day. The protocol is sensitive, and enables better detection and separation of individual carotenoids by reducing artefacts during extraction, purification and chromatography that can be used for routine screening of oilseeds.

Keywords β-carotene; Biofortification; High Performance Liquid Chromatography; Mustard; Oilseeds; Peanut; Pro-vitamin A carotenoids; Soybean; Sunflower

Abbreviations

BHT Butylated hydroxyltoluene
DE Diethyl ether
HPLC High-performance liquid chromatography
PE Petrolium ether
PDA Photodiode array detector
PTFE Polytetrafluoroethylene
THF Tetrahydrofuran
VAD Vitamin A deficiency

Introduction

Micronutrient malnutrition is a rapidly growing public health problem affecting over 40% of the world population. Vitamin A deficiency (VAD) alone causes up to 2 million deaths annually in children aged between 1 and 4 years (Humphrey et al., 1992). Clearly, VAD is a major food-related primary health problem among populations of the developing world, and fortifying crops with pro-vitamin A or β-carotene to address VAD has high potential in the long-term. There is considerable interest in the development of food products rich in pro-vitamin A carotenoids for potential and beneficial effects on human health over the alternative dietary supplements (Cooper 2004). More recently, enhancement of micronutrient density of plant foods through agricultural practices, especially biotechnological tools referred to as "Biofortification" is considered as a potential strategy to alleviate VAD and to improve the nutritional content of staple food crops to benefit global health (Bouis 2003). With an established link between carotenoid intake from food and health, there is an obvious need for a reliable method for routine use that is rapid, simple and accurate for routine determination of pro-vitamin A carotenoid content of food crops.

Oilseeds are important crops for low-income families in the semi-arid tropics as they contribute 40% of the total calories in their diets (Graham and Vance 2003). Globally, oilseeds are being modified for high nutrition, oil quality and composition, besides enhanced quality of the meal for use as animal feed. Nevertheless, carotenoids are present in very small quantity in these crops that otherwise serve as important sources of nutrition. Moreover, since vegetable oil appears to be a suitable carrier for fortification with β-carotene, oilseed crops have a potential for biofortification with β-carotene, aimed at higher bioavailability and bioefficacy (Shewmaker et al. 1999). Hence, it is of critical importance to have an accurate method for routine use to provide rapid and reproducible results on the extraction and determination of β-carotene and other pro-vitamin A carotenoids in these crops.

Although, much work has been done in optimizing methods for the extraction and estimation of carotenoids from fruits and vegetables, little attention has been paid to the development of improved methods for oilseeds. So far, in most of the studies with the oilseeds, the concentrations of pro-vitamin A carotenoids, specifically β-carotene are either not detectable or the results are not consistent (Pattee et al. 1967; Siong et al. 1995; Yu et al. 2008). This is mainly due to a dilution effect by the oil. Since, the carotenoids are

stored in the fatty acid matrix of oilseeds, their extraction is problematic. Moreover, owing to hydrophobic nature of carotenoids, they are transported by lipoproteins and their distribution linked to the lipid profile (Broszeit et al. 1997). Their insolubility in water and rather poor solubility in several organic solvents demands attention on the selection of extraction keeping in mind limitations on the composition of HPLC mobile phases. Besides, since a complete recovery of carotenoids from oil matrix is troublesome due to their strong binding to the oil matrix, the extraction step has to be repeated several times to obtain sufficient recovery of these pigments. Nevertheless, the methods available and recommended for the extraction of carotenes in oilseed crops are labour-intensive, use toxic solvents, and require saponification for longer periods of time (Pattee et al. 1967; Ping and Gwendolone 2006).

Keeping in view these challenges in estimation of carotenoids in the oilseeds, the present study was aimed to standardize and develop an efficient and high throughput extraction method for these crops. Here we report the results of a comprehensive study on the important factors that potentially influence qualitative and quantitative yields of β-carotene, the major pro-vitamin A carotenoid in various oilseed crops like peanut, soybean, mustard and sunflower with the aim of developing a simple, accurate and rapid method suitable for large-scale screening for pro-vitamin A carotenoids.

Material and methods

Reagents and materials

The β-carotene standard used in this study was purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.). Other reagents including methanol, acetonitrile, chloroform, n-Hexane, petroleum ether (PE), diethyl ether (DE), acetone, ethanol, sodium chloride, potassium hydroxide, tri-ethyl amine and butylated hydroxyltoluene (BHT) were of analytical or HPLC grade.

Plant Material

Mature freshly harvested seeds of mustard (var. Pusa Mustard 21), soybean (var. Pusa 9712) and sunflower (var. Morden) were procured from the Directorate of Oilseed Research, Hyderabad, India, while the seeds of peanut (var. JL 24) were procured from the Peanut Breeding Unit of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India**.** Freshly harvested seeds were stored at 4 ^{0}C prior to use.

Standard β-carotene preparation

Solution of β-carotene standard (5 mg/100 ml) was freshly prepared in n-hexane and stored in an amber coloured volumetric flask prior to use. β-carotene (1 ml) from this stock solution was further diluted with n-hexane to yield a final concentration of 1 µg/ml.

Sample Extraction

Freshly harvested seeds (200-300 mg) were ground with a mortar and pestle in 8 ml of pre-warmed extraction medium containing absolute ethanol, 0.5% BHT. To this 2 ml of 2:1 ratio of hexane and acetone was added. The extract was subjected to 10 min incubation under dark at room temperature (25-30 ºC), subjected to centrifugation at 5000 rpm for 10 min at 4ºC, and the supernatant transferred to a fresh tube. To the supernatant, an equal amount of 15% alkaline methanol KOH containing 0.5% BHT was added and incubated at 80^0C for 15 min in a rotary water bath and chilled on ice for 5-10 min. To the saponified extract, 4 ml of distilled water and 3 ml of 2:1 PE:DE containing 0.5% BHT was added to achieve better phase separation. This was subjected to centrifugation at 5000 rpm for 10 min at 4ºC, followed by transfer of the upper coloured organic phase to a fresh tube. The left-over residue was again extracted twice with 4 ml of 2:1 PE:DE, the upper phases collected and pooled. Solvent evaporation was carried out in a vacuum drier at room temperature followed by the residual suspension in a mobile phase consisting of methanol:acetonitrile:chloroform (50:40:10) with 0.5% BHT (Fig. 1). The final carotenoid extract was filtered through a 0.45 µm PTFE syringe filter (Millipore®) into an HPLC sample vial.

The published extraction protocols employed for various crops such as mustard (Shewmaker et al. 1999; Yu et al. 2008) and maize (Kurilich and Juvik 1999) were also carried out for comparison among all the selected crops as described below.

Randomly selected seeds (100 mg) were extracted with hexane/acetone/ethanol (50/25/25 v/v) according to Shewmaker et al. (1999). The residue was back-extracted twice with extraction solvent. The extracts were combined and centrifuged again for better phase separation. The top layer, containing isoprenoids and hexane was removed and transferred to a new glass tube. The bottom layer was back-extracted twice with 2 ml hexane. All hexane extracted layers were dried under nitrogen. The residue was dissolved in 2 ml acetonitrile/methylene chloride/methanol (50/40/10 v/v) and centrifuged for 3 min.

Approximately 1 ml of supernatant was filtered through a 0.45 mm filter and collected in a brown auto-sampler vial and capped immediately.

In the second protocol, seeds (200 mg) were extracted according to Yu et al. (2008) using extraction solvent (hexane/acetone/ethanol, 50/25/25) and pulverised by rapidly shaking for 30 min in a scintillation vial containing a steel rod. The sample was centrifuged for 10 min at 1,800g and the supernatant collected. The pellet was washed with another 3 ml extraction solvent and the supernatant collected and pooled. The solvent was removed by evaporation at room temperature under a stream of nitrogen. Triacyl glycerides were saponified in the residue by heating at 80 ^{0}C for 1 h in 5 ml methanolic-KOH (10% w/v KOH in methanol:water [80:20 v/v]). Carotenoids and aqueous compounds were partitioned using 2 ml H2O and 3 ml petroleum ether. The ether phase and two 3 ml ether washes were collected, pooled and the solvent evaporated at room temperature under a nitrogen gas stream. The residue was resuspended in 200 ul of acetonitrile/methylene chloride/methanol (50/40/10 v/v) with 0.5% (w/v) butylated hydroxytoluene and filltered through a 0.2um pore size nylon syringe filter into an HPLC sample vial.

The extraction procedure employed by Kurilich and Juvik (1999) is a modification of a method described by Weber (1987). 600 mg of tissue sample was extracted with ethanol containing 0.1% butylated hydroxytoulene (BHT) and incubated at 85 ^{o}C in water bath. Saponification was carried out with 80% potassium hydroxide (KOH) for 10 mins followed by partition using equal volume of cold deionized distilled water and n-Hexane. The upper layer was pippeted into a seperate test tube, and pellet was re-extracted twice more using hexane. The combined hexane fractions were washed with 3ml of deionized distilled water, vortexed and centrifuged for 10min prior to pipetting into another tube. The hexane fraction was dried down in a vacuum evoporator and reconstituted in 200ul of acetonitrile:methanol:methylene chloride.All operations were carried out under gold fluorescent lights to avoid degradation of carotenoids.

Chromatography

The extract was immediately analyzed using Waters Alliance 2695e Separation Module (Waters Corporation, Milford, MA, USA) equipped with a Waters 998 Photodiode Array detector (PDA). Aliquots of 20 µl were injected into Luna ODS2 reverse-phase C_{18} (5 µ, 4.6 mm × 250 mm) at 30 ^{0}C. Mobile phase consisted of methanol containing 0.5% triethylamine, acetonitrile and chloroform. Elution was done using

solution A containing methanol:acetonitrile:chloroform (50:40:10) and solution B containing methanol:acetonitrile:chloroform (35:35:30). The column was developed with solution A for the first 2 min, then a linear gradient with solution B was applied over a period of 8 min, following which solution A was pumped through the column for another 10 min. Flow rate of 1.2 ml min^{-1} was used for elution. Compounds present in the eluting sample were monitored at 450 nm using a PDA. Peaks were identified by their retention time and absorption spectra were compared to those of known standards (Sigma Chemicals). β-carotene was quantified using peak areas of the authentic standard.

Analytical evaluation and statistical analysis

Four experiments were performed to test the accuracy and reproducibility of this procedure. Samples were spiked with known amount of β-carotene standard which were then extracted and chromatographically run to obtain recovery values. Four separate extractions of the same sample were run to evaluate the sample variability. Sample concentrations were calculated by comparing peak area of samples to peak area of the standards. Recovery values for β-carotene were obtained by comparing the concentration of spiked to un-spiked samples. Means were calculated to estimate reproducibility between separate extractions of the same sample. Analysis of variance was done to test for significant differences in concentrations of β-carotene between the samples analyzed with the reported protocols.

Results and discussion

Recent human intervention studies have shown that presence of carotenoids in the oil may potentially improve their bioefficacy (van Lieshout et al. 2001). Hence, pro-vitamin A enrichment of oilseeds using conventional as well as transgenic options would have a significant impact on the nourishment and nutrient interactions involving other micronutrients under multiple commodity diets by playing a major role in the bioavailability and metabolic efficiency. So far, most of the reported extraction protocols for the carotenoids have generally been too complicated for implementation in a large-scale screening programs (Barua and Olson 1998). This is particularly due to the large volumes of toxic and inflammable organic solvents commonly used for extraction, followed by problematic need to dry-down and concentrate extracts of these light and oxidation-sensitive compounds (Hart and Scott 1995). There have been essentially no

systematic investigations into the quantitative recovery of β-carotene from oilseed crops, although, these crops contribute a major role in our daily dietary routine.

Quantity of the tissue sample

The present study was aimed at efficient recovery of β-carotene from oilseed crops using a small quantity of the sample. The comparative analysis of all the previously reported carotenoid extraction protocols for oilseeds with the current protocol has shown its effectiveness with smaller quantities of seed tissues, i.e., 100-300 mg when compared to 500 mg-10 g seed sample used in other studies (Weber 1987; Siong et al. 1995). This has comparative advantage, especially when dealing with limited quantity of seeds) in the primary generations (e.g., pre-breeding material, early generation transgenics, etc.). In order to carry out non-destructive estimations in peanut, the distal cut half of the peanut seed were used for the extraction and profiling of carotenoids, while the proximal half near the embryo axis containing half cotyledon and intact embryo was used for recovery of the plants following seed germination and advancement of generation. Similarly with other crops (sunflower, mustard and soybean) having harder seed coats, 100-150 mg of seed sample was used for carotenoid profiling.

Extraction and saponification

In the present study, the extraction of carotenoids from oilseed samples involved grinding of the seed tissues using pre-warmed absolute ethanol in an extraction solvent consisting of high polar:non-polar solvent ratios. This resulted in dissociation of the fatty acid matrix, thereby maximizing the release of carotenoids present in the oil matrix into the solvent phase. In contrast, all the previously reported protocols involved the use of a single extraction solution which did not release the fatty acid matrix properly, thereby interfering with the partition and quantification step. Several researchers have recommended including saponification (alkaline hydrolysis) step in the analysis of carotenoids of plant tissues mainly to simplify chromatographic profiles by removing potentially interfering compounds such as chlorophyll degradation products, chlorophyll-esters and unwanted lipids (Granado et al. 2001; Schierle et al. 2004). For this, the hexane extraction method has been reported for the separation of oily particles, following addition of an inorganic salt such as sodium chloride to the sample in a separatory funnel (Shewmaker et al. 1999). However, this method is too long due to the formation of emulsion of the sample that takes as long as 1 h to break-up. Furthermore, it is not

possible to separate the oily particles and fat-soluble vitamins by the hexane extraction method, thereby making it unsuitable for estimation of β-carotene in the emulsified products. Interestingly, no differences were observed in the recovery values by replacing hexane and acetone (50:25) with tetrahydrofuran (THF; data not shown). However, we chose to avoid inclusion of THF due to its role in the production of peroxides that could lead to structural breakdown of carotenoids. Moreover, the maximum volume of THF extract that could be directly injected onto HPLC columns was only 10 µl without leading to peak distortion that significantly restricts detection sensitivities (Davey et al. 2006).

Since, the oilseeds are rich source of xanthophylls like lutein, a saponification step was included for the removal of esterified xanthophylls for accurate quantification of the pro-vitamin A carotenoids. Although, saponification is considered as the most crucial step for increasing extraction efficiencies and is useful for quantitative measurements of total carotenoids, in the absence of optimal conditions it is less successful for determining individual carotenoids due to their degradation, producing artefacts, besides isomerization at higher temperatures, long duration exposure to light during the extraction procedures (Kimura et al. 1990). Hence, post-extraction, the optimal composition of alkaline methanolic KOH for the saponification was standardized to allow minimum time and low temperature (15 min at 80 ^{0}C) to minimize the degradation of carotenoids present in the sample. Although, the saponification step was not carried out by Shewmaker et al. (1999), all others reported carrying out saponification along with the extraction, which might have resulted in lower recovery of the carotenoids. Our results emphasize the usefulness of saponification following extraction, which increased the recovery of carotenoids by maximizing the degradation of fatty acids, while minimizing the carotenoids degradation in all the tested oilseed crops.

Similarly, the mobile phase composition including methanol:acetonitrile:chloroform (50:40:10) increased the solubility and eliminated the appearance of unwanted peaks that were visibly interfering with the desirable peaks in HPLC chromatograms of the previous reports. Since we used 0.5% triethylamine, the peaks obtained were sharper than those reported earlier. The results are in line with a previous report on the use of tetraethylamine to initiate better peak separation and composition (Davey et al. 2006).

Comparative analysis of oilseed samples using different HPLC protocols

Since, a major source of error in HPLC analysis of carotenoids is reportedly due to difference in sample preparation methods, a comparative analysis of HPLC data using different solvent combinations, i.e., polar to non-polar ratios and retention times reported in different protocols was carried out, which resulted in significant differences in the yield of carotenoids.

β-carotene extraction from the seed samples

Since, the selected oilseeds contain very low levels of β-carotene (especially sunflower, soybean and peanut), its detection and quantification was performed by using different protocols to reassess the reproducibility and efficiency of the protocol (Fig. 2). Interestingly, the data showed statistically significant differences in the recovery of carotenoids in the seed samples with our protocol when compared to other published protocols. In the case of peanut, while no β-carotene was detected using the protocols of Shewmaker et al. (1999) and Kurilich and Juvik (1999), the protocol by Yu et al. (2008) and our current protocol could detect β-carotene which is naturally present in trace amounts in this legume. The β-carotene recovery and quantification using our protocol was significantly higher (15.23 µg/100 g) than that reported by Pattee et al., (1969) which detected merely 3 µg/100 g of β-carotene in peanut seeds (Fig. 3). Similarly, in sunflower which also contains low amount of β-carotene, 12.71 µg/100 g β-carotene was detected using our protocol which was significantly higher than the other protocols (Fig. 4) that either did not detect any β-carotene peak (Shewmaker et al. 1999, Kurilich and Juvik 1999) or recovered very low content (6.19 µg/100 g; Yu et al. 2008).

Although, in soybean no β-carotene peak was detected with the protocol of Kurilich and Juvik (1999), our protocol recovered 20.19 µg/100 g of β-carotene which is much higher (Fig. 5) than that reported previously (5-11 µg/100 g) by Siong et al. (1995). In the case of mustard, while β-carotene recovery ranged from 12.6-45.2 µg/100 g using all other protocols, significantly higher β-carotene recovery of 71.9 µg/100 g was obtained using our protocol (Fig. 6).

These results clearly indicated the merit of our protocol for efficient recovery of β-carotene in a range of tested oilseed crops which otherwise naturally contain very low levels of β-carotene (especially sunflower, soybean and peanut). Here, we report for the first time, the extraction of β-carotene in oilseed crops with a recovery of > 90% which

was highest reported so far in these crops (Shewmaker et al. 1999; Yu et al. 2008; Kurilich and Juvik 1999). The detection sensitivity was significantly higher where the β-carotene levels as low as 60 ng/100 mg could be detected using this protocol. Moreover, there were clear differences in the HPLC profiles of other associated carotenoids including lutein, zeaxanthin and β-cryptoxanthin having much sharper and separable peaks of individual carotenoids using our protocol, when compared to the ones reported previously (Figs 4-7).

3.5 β-carotene recovery in the spiked seed samples

The extraction and saponification steps were very crucial for accurate analysis and quantification of the total carotenoids and β-carotene present in the spiked samples of the oilseeds (Fig. 7). In mustard, our protocol resulted in a recovery of 79.91% of β-carotene, while the protocols of Shewmaker et al. (1999) and Yu et al. (2008) that used hexane:acetone:ethanol (50:25:25 v/v) as the extraction solvent resulted in significantly lower β-carotene recovery of 4.89% and 12.52%, respectively, besides a very poor separation of lutein and zeaxanthin. Interestingly, the method of Kurilich and Juvik (1999) resulted in β- carotene recovery of 88.64% which was statistically higher than that from our current protocol; however, the peaks for lutein, zeaxanthin and β-cryptoxanthin did not separate well with the former.

In the case of sunflower, while there was no recovery of β-carotene using the protocol of Shewmaker et al. (1999) the recovery values using the other two protocols (Kurilich and Juvik 1999; Yu et al. 2008) were 31.9% and 87.4%, respectively. Here, again our protocol resulted in β-carotene recovery of 91.7% which, although was statistically at par with the protocol of Kurilich and Juvik (1999), had clear chromatographic differences with respect to peak sharpness that were much clearer and narrow without any interferences.

Similarly, in the case of peanut and soybean, the current protocol had an edge over the rest with respect to the recovery of β-carotene. In peanut, while there was no β-carotene recovery using the protocol of Shewmaker et al. (1999), a recovery of 13.8% and 31.2% was obtained with the protocols of Yu et al. (2008) and Kurilich and Juvik (1999), respectively. Consistently, our protocol with peanut showed highest recovery of β-carotene (86.4%) which was significantly higher that that by all the other three protocols. Similarly, with soybean also, while the recovery percentage ranged from 6.18-67 % using other three protocols, highest recovery of 81.49% was recorded with our protocol which was statistically significant.

In conclusion, we report a method that does not seem suffer from the shortcoming found in the methods reported earlier, especially with regard to the following advantages:

- The protocol is novel and robust for problematic oilseed crops, that is highly effective with minimal tissue (100-300 mg).
- Highest percentage recovery of β-carotene (80-92%) in the major oil seed crops with great deal of reliability, accuracy and precision.
- Highly efficient protocol for crops where levels of β-carotene levels are either too low or are undetectable.
- High throughput analysis (extraction, partition and HPLC analysis) of the samples in very less time is possible.

This protocol has a potential to be not only used for large scale screening of carotenoids, but also for its use in evaluation of biofortified oilseed technologies.

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Legends to figures

Fig. 1 Flow-chart showing various steps involved in the protocol for efficient extraction and recovery of pro-vitamin A carotenoids in oilseeds using HPLC

Fig. 2 Quantification of β-carotene in the selected oilseeds and the relative rankings based on the detection sensitivities using various protocols. * denotes significance at $p<0.01$ level

Fig. 3 Representative HPLC chromatograms of peanut seed samples using the four protocols. (a) Shewmaker et al., (b) Yu et al., (c) Kurilich and Juvik (d) Current protocol. Indicative peak numbers of the potential markers 1: lutein and zeaxanthin, 2: β-cryptoxanthin, 3: β-carotene

Fig. 4 Representative HPLC chromatograms of sunflower seed samples using the four protocols. (a) Shewmaker et al., (b) Yu et al., (c) Kurilich and Juvik (d) Current protocol. Indicative peak numbers of the potential markers 1: lutein and zeaxanthin, 2: β-cryptoxanthin, 3: β-carotene

Fig. 5 Representative HPLC chromatograms of soybean seed samples using the four protocols. (a) Shewmaker et al., (b) Yu et al., (c) Kurilich and Juvik (d) Current protocol. Indicative peak numbers of the potential markers 1: lutein and zeaxanthin, 2: β-cryptoxanthin, 3: β-carotene

Fig. 6 Representative HPLC chromatograms of mustard seed samples using the four protocols. (a) Shewmaker et al., (b) Yu et al., (c) Kurilich and Juvik (d) Current protocol. Indicative peak numbers of the potential markers 1: lutein and zeaxanthin, 2: β-cryptoxanthin, 3: β-carotene

Fig. 7 Comparison of the extraction methods for β-carotene recovery in the spiked samples of the four oilseed crops. * denotes significance at $p<0.01$ level

Fig. 1

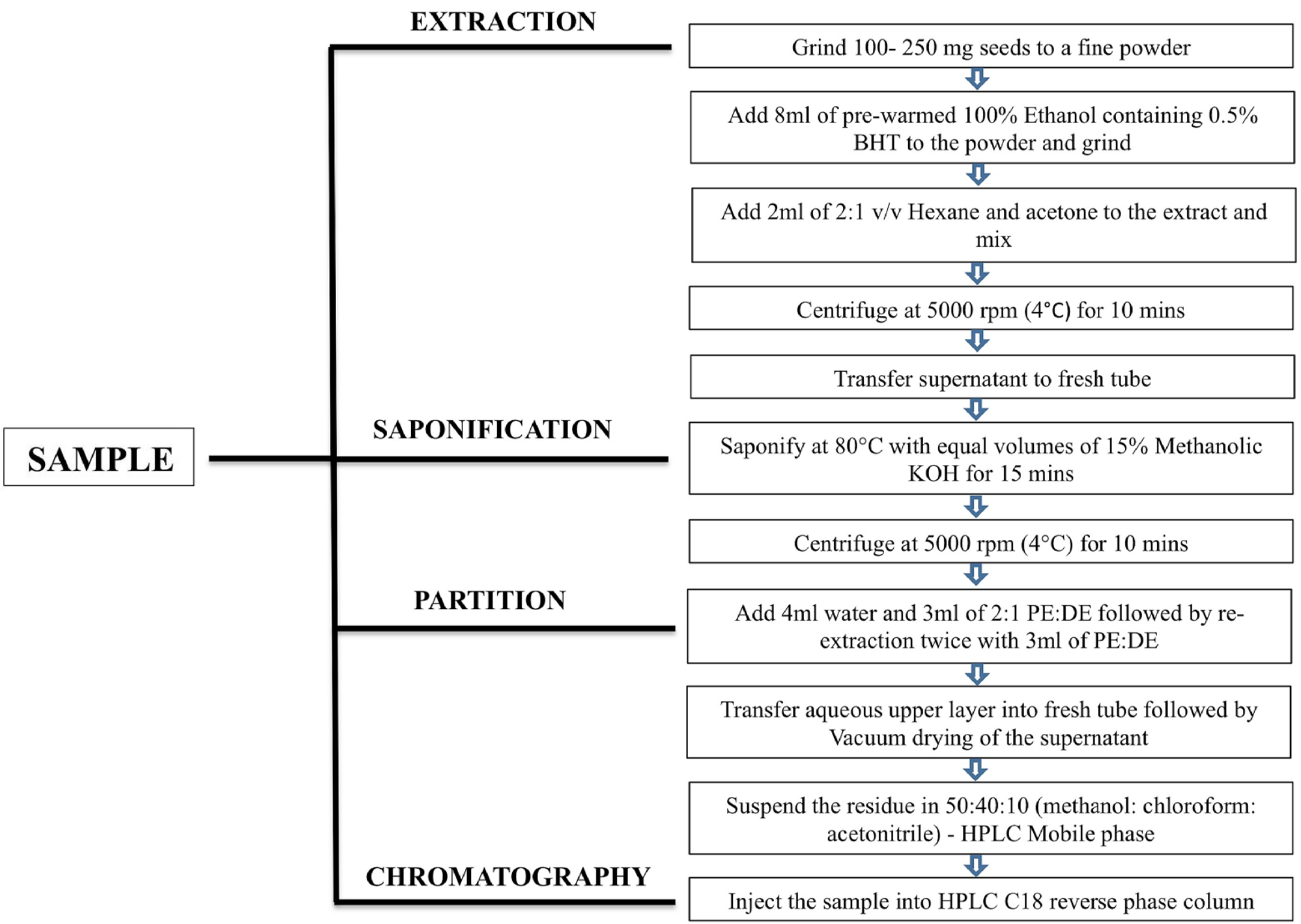

SAMPLE
EXTRACTION
Grind 100- 250 mg seeds to a fine powder
Add 8ml of pre-warmed 100% Ethanol containing 0.5% BHT to the powder and grind
Add 2ml of 2:1 v/v Hexane and acetone to the extract and mix
Centrifuge at 5000 rpm (4°C) for 10 mins
Transfer supernatant to fresh tube
SAPONIFICATION
Saponify at 80°C with equal volumes of 15% Methanolic KOH for 15 mins
Centrifuge at 5000 rpm (4°C) for 10 mins
PARTITION
Add 4ml water and 3ml of 2:1 PE:DE followed by re-extraction twice with 3ml of PE:DE
Transfer aqueous upper layer into fresh tube followed by Vacuum drying of the supernatant
Suspend the residue in 50:40:10 (methanol: chloroform: acetonitrile) - HPLC Mobile phase
CHROMATOGRAPHY
Inject the sample into HPLC C18 reverse phase column

Fig. 2

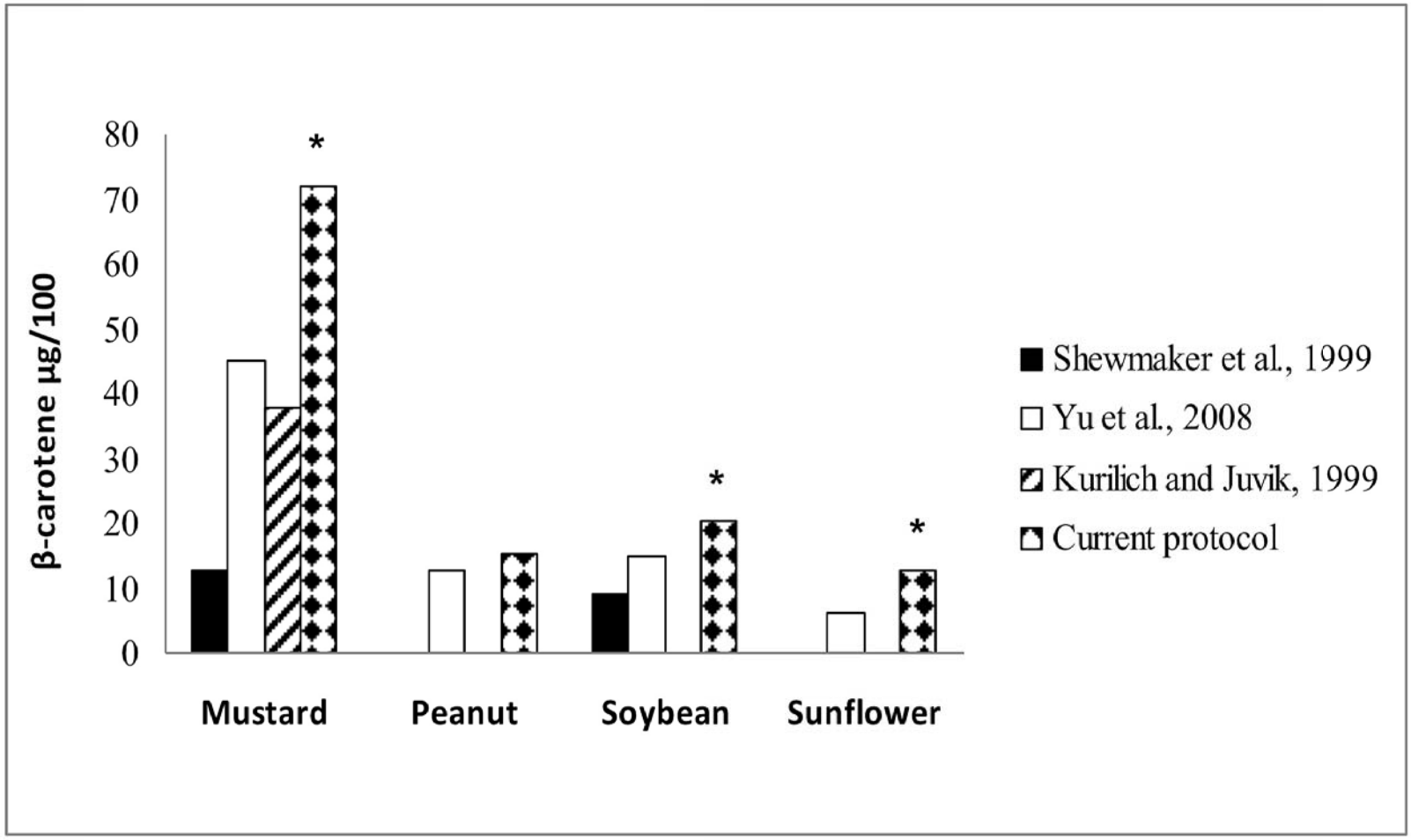
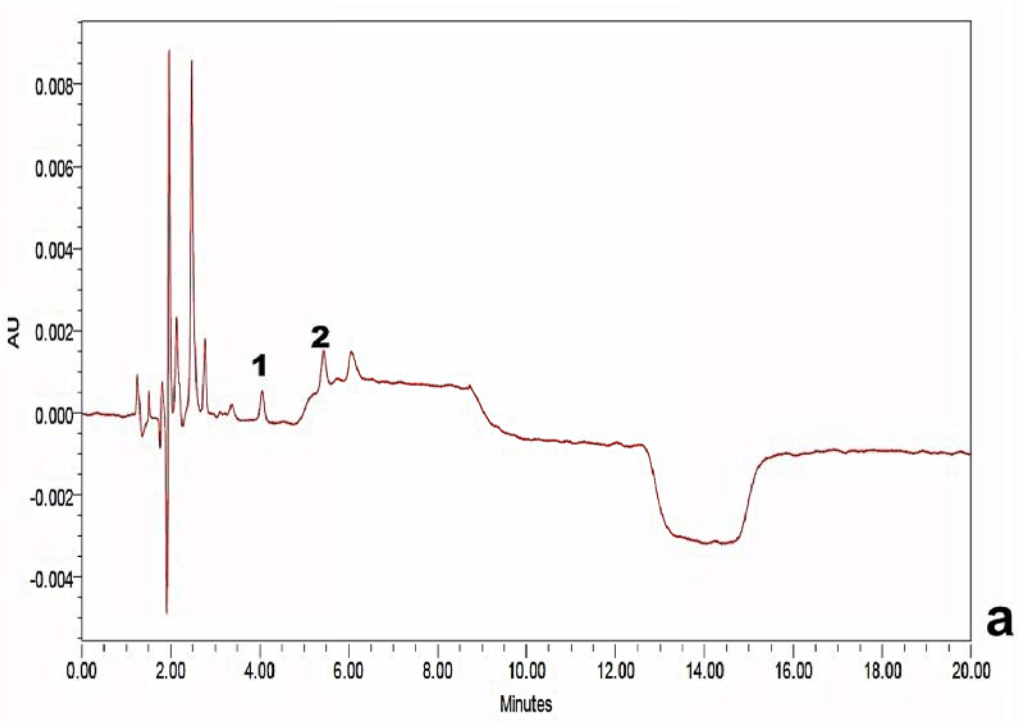

β-carotene µg/100
80
70
60
50
40
30
20
10
0
*
*
*
Mustard
Peanut
Soybean
Sunflower
Shewmaker et al., 1999
Yu et al., 2008
Kurilich and Juvik, 1999
Current protocol

Fig. 3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0.006
0.004
0.002
0.000
-0.002
-0.004
1
2
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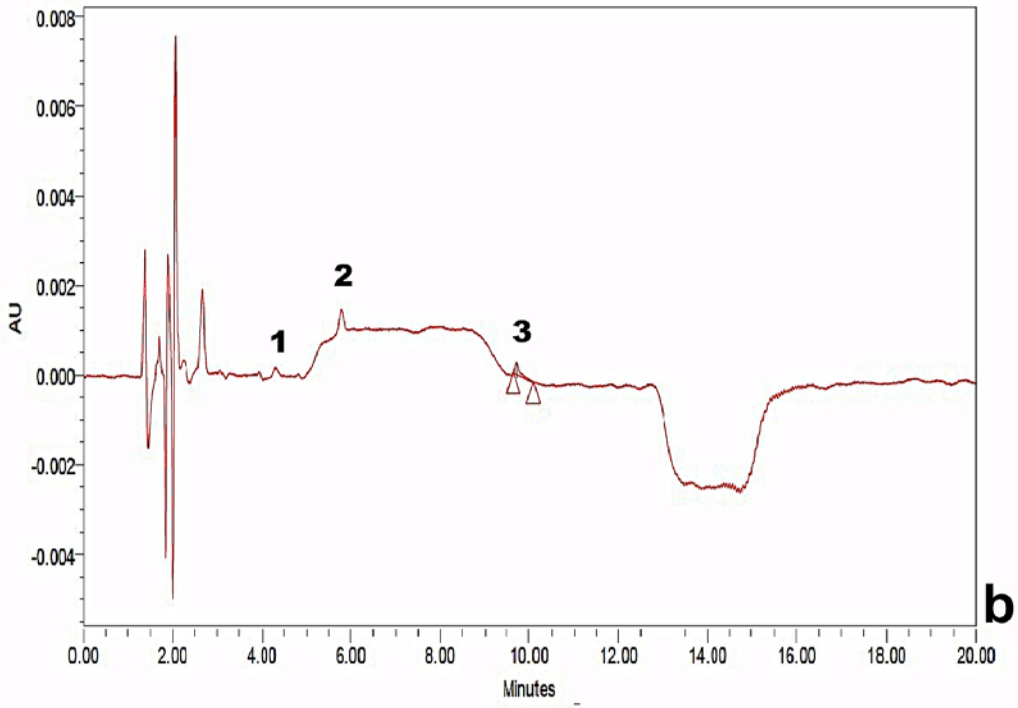
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

a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0.006
0.004
0.002
0.000
-0.002
-0.004
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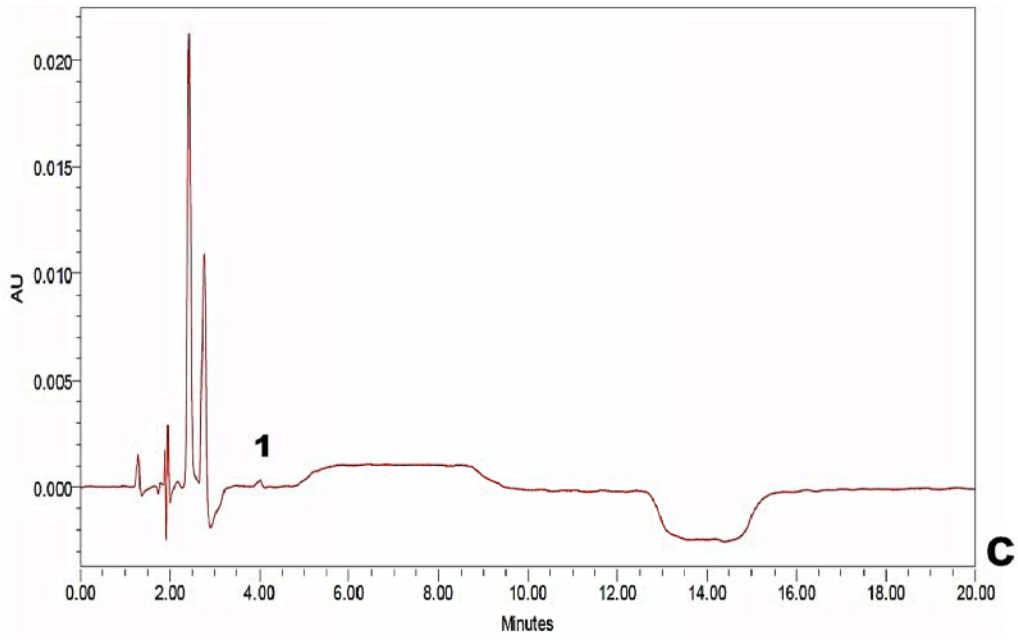
2
3
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

b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0.010
0.005
0.000
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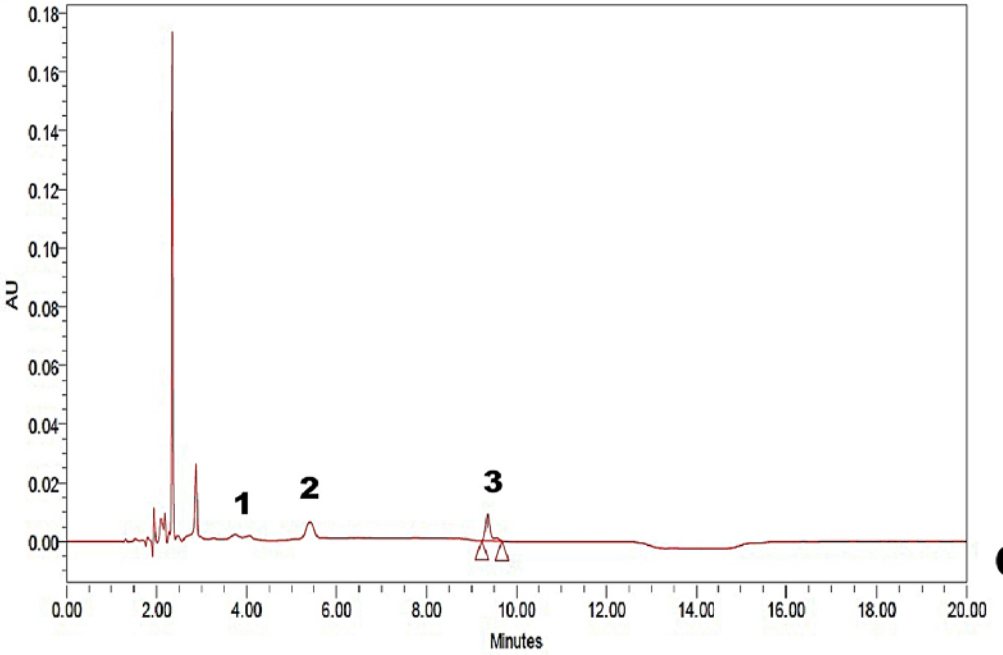
12.00
14.00
16.00
18.00
20.00
Minutes

c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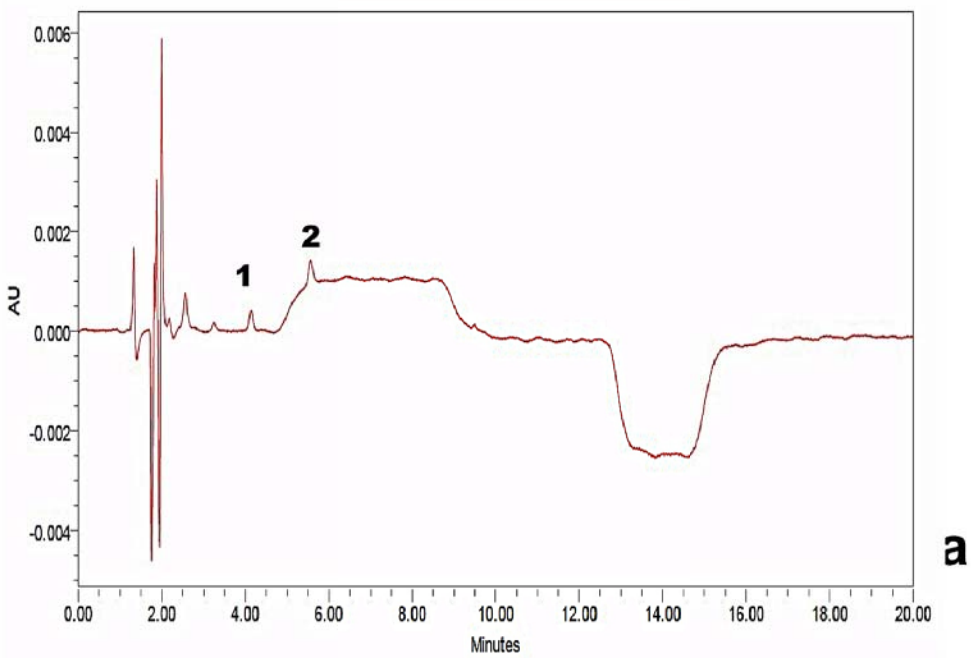
0.10
0.08
0.06
0.04
0.02
0.00
1
2
3
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

d

Fig. 4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0.004
0.002
0.000
-0.002
-0.004
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2
0.00
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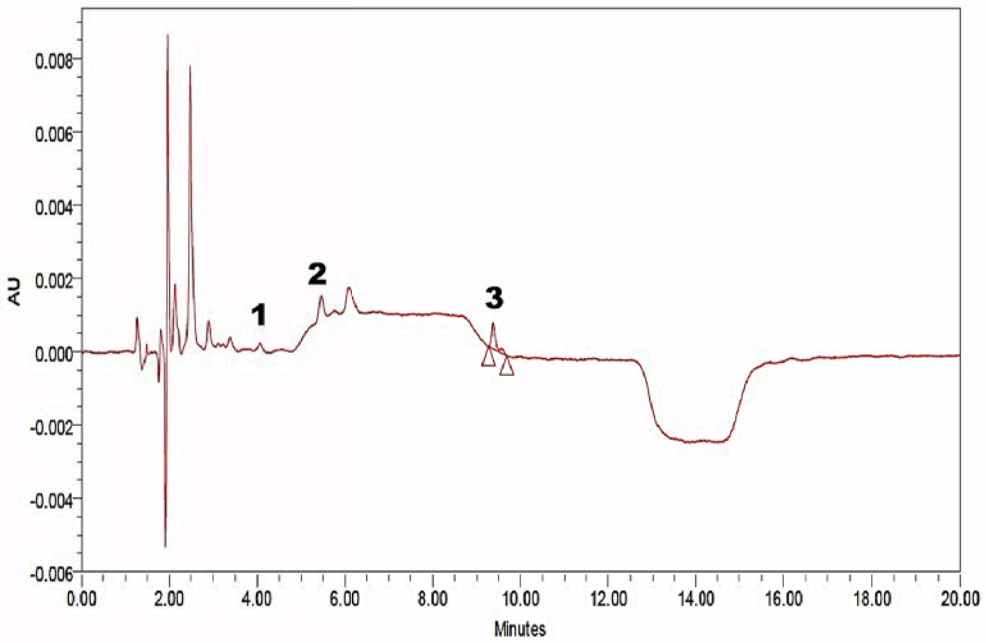
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

a


AU
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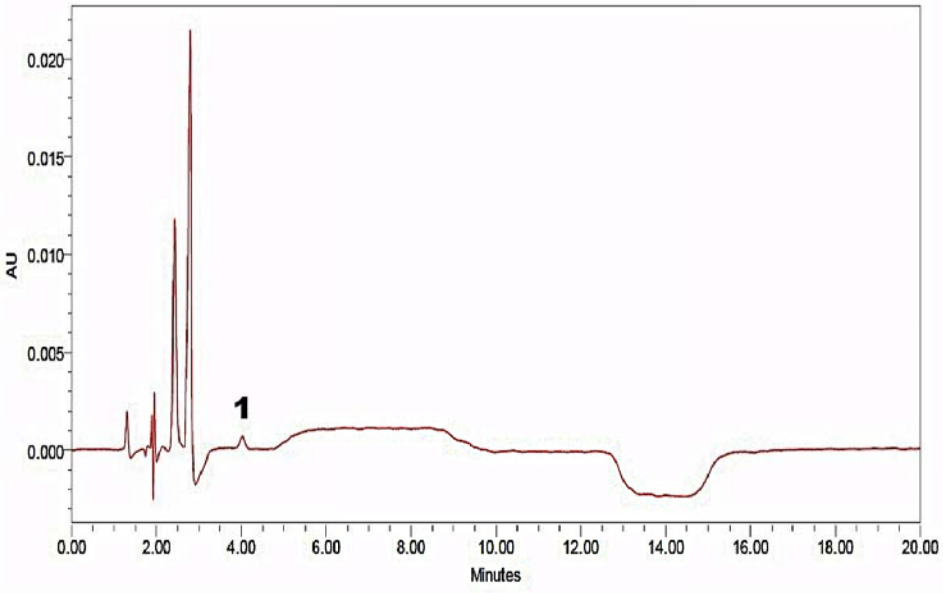
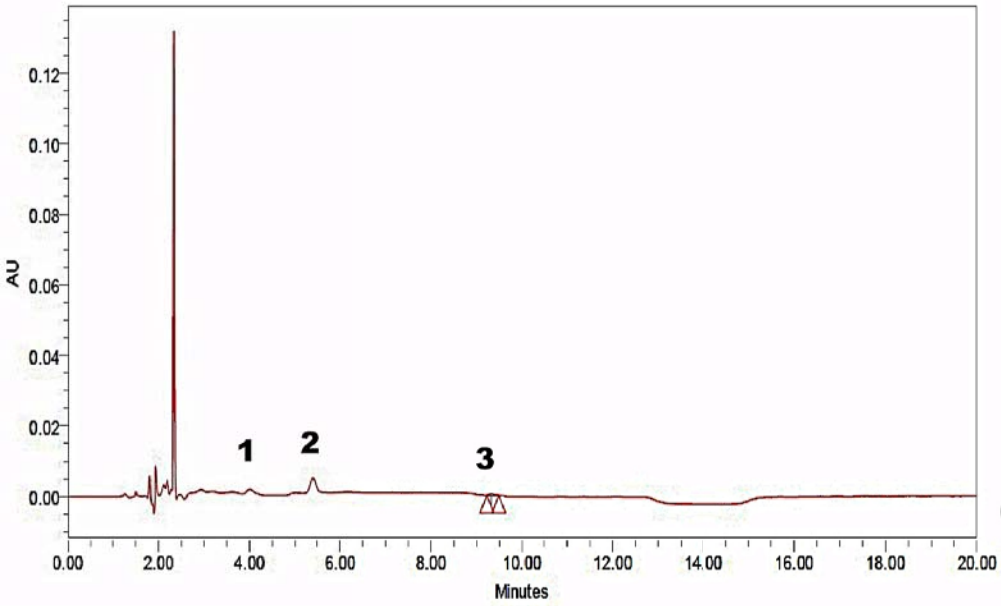
0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
1
2
3
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

b


AU
0.020
0.015
0.010
0.005
0.000
1
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

c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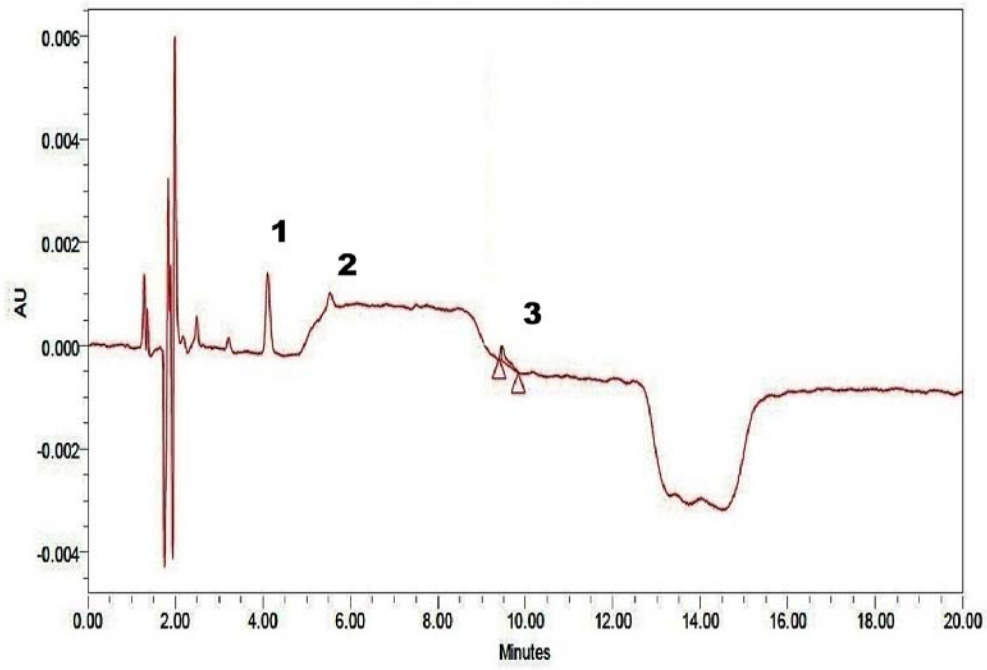
0.10
0.08
0.06
0.04
0.02
0.00
1
2
3
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

d

Fig. 5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0.002
0.000
-0.002
-0.004
1
2
3
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2.00
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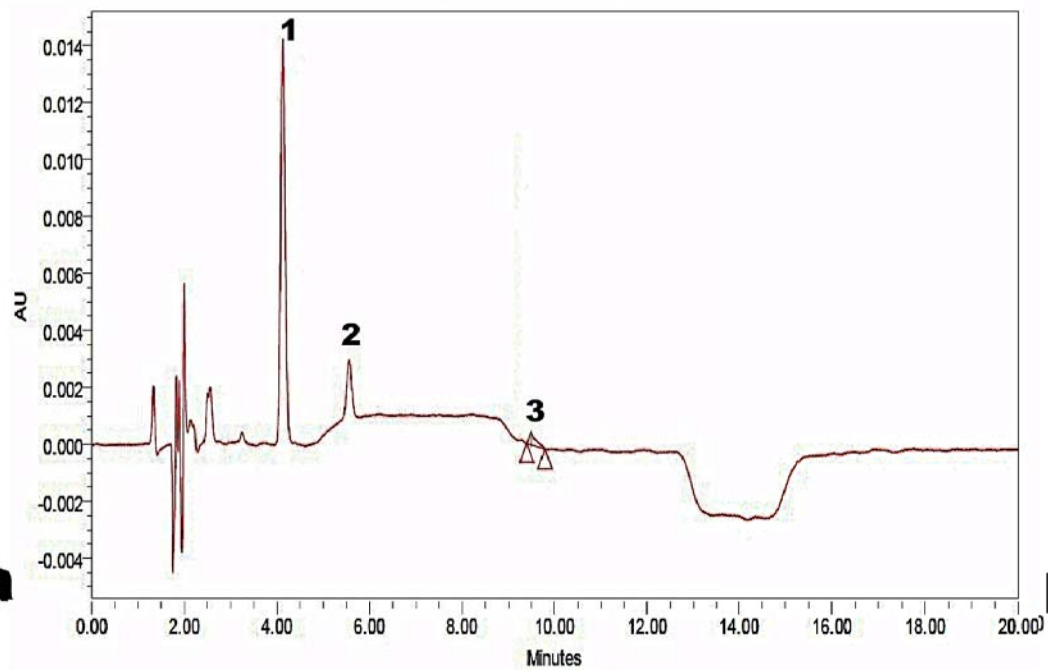
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

a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0.006
0.004
0.002
0.000
-0.002
-0.004
1
2
3
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2.00
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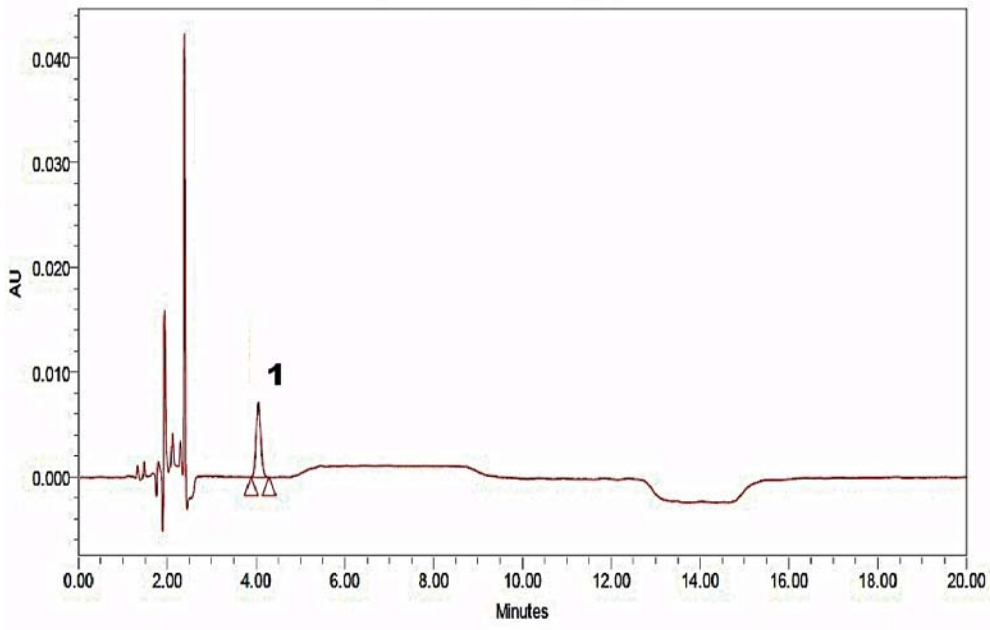
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

b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0.030
0.020
0.010
0.000
1
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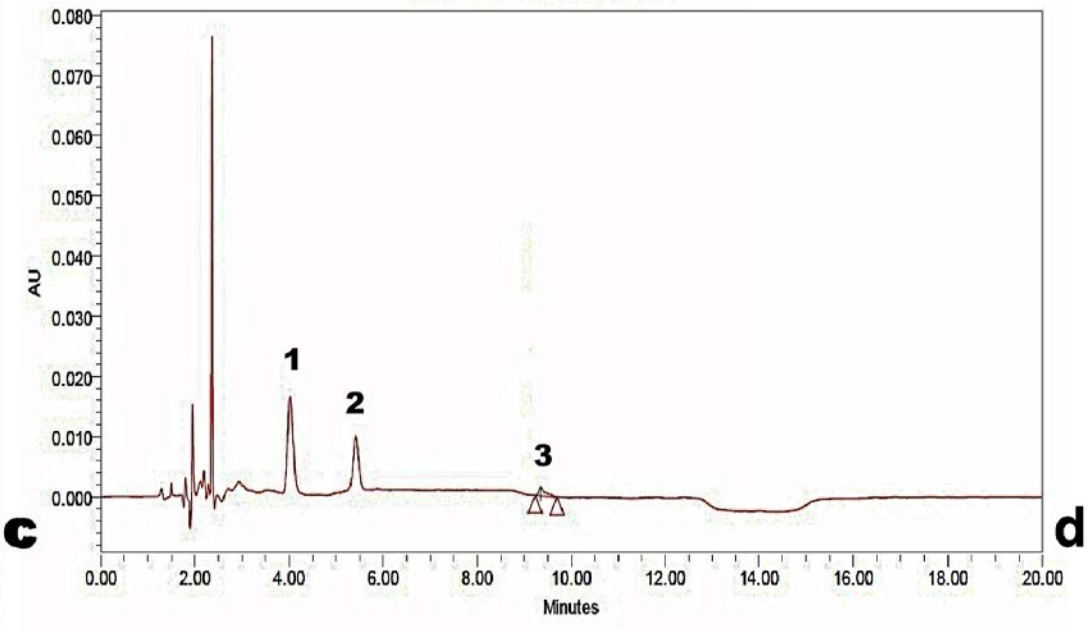
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

c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0.040
0.030
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0.000
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2
3
0.00
2.00
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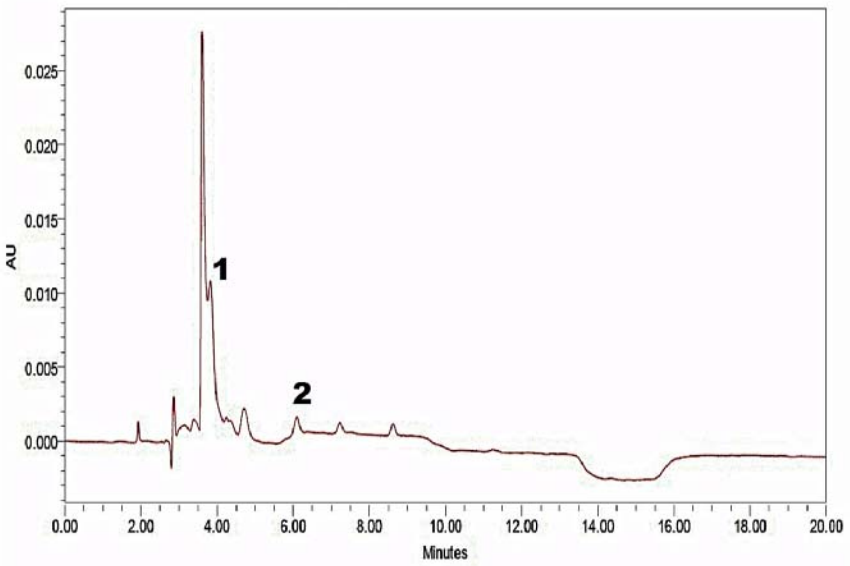
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

d

Fig. 6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0.010
0.005
0.000
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2
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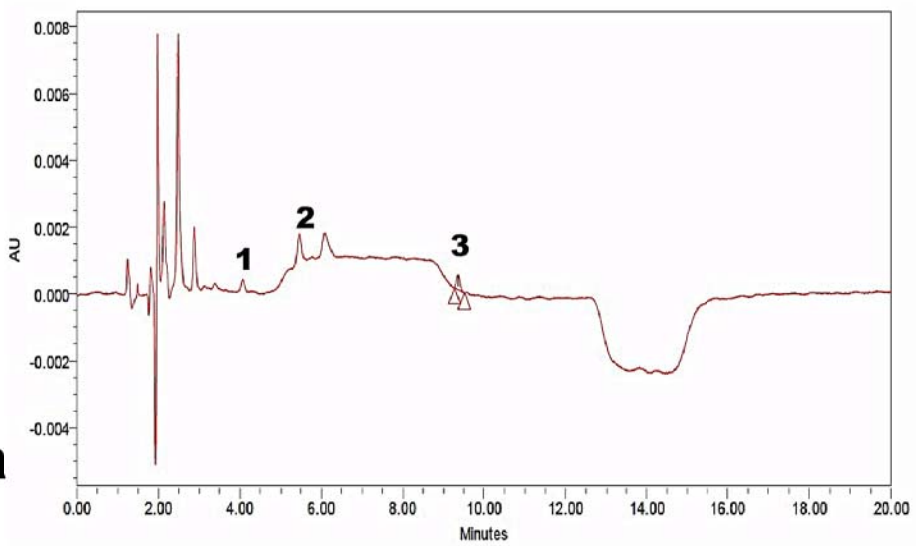
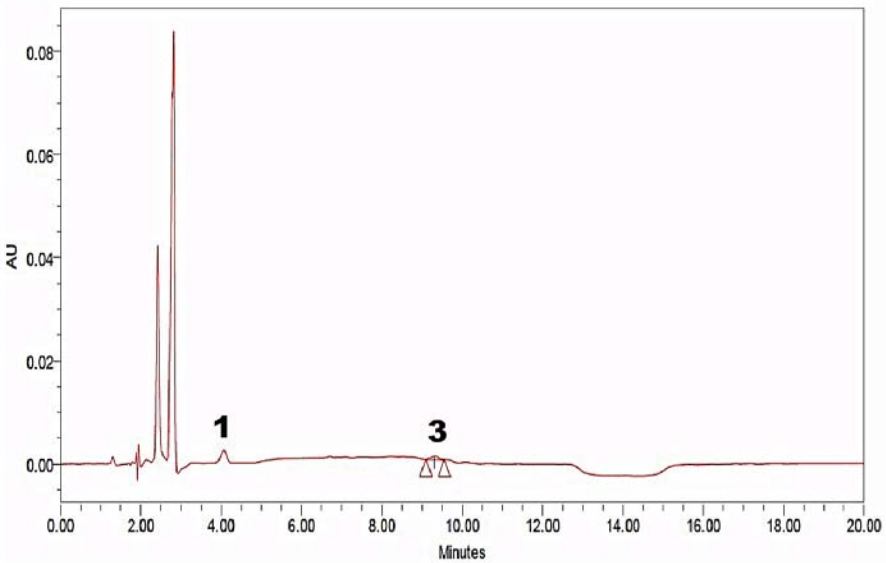
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

a


AU
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
1
2
3
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

b


AU
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0.06
0.04
0.02
0.00
1
3
0.00
2.00
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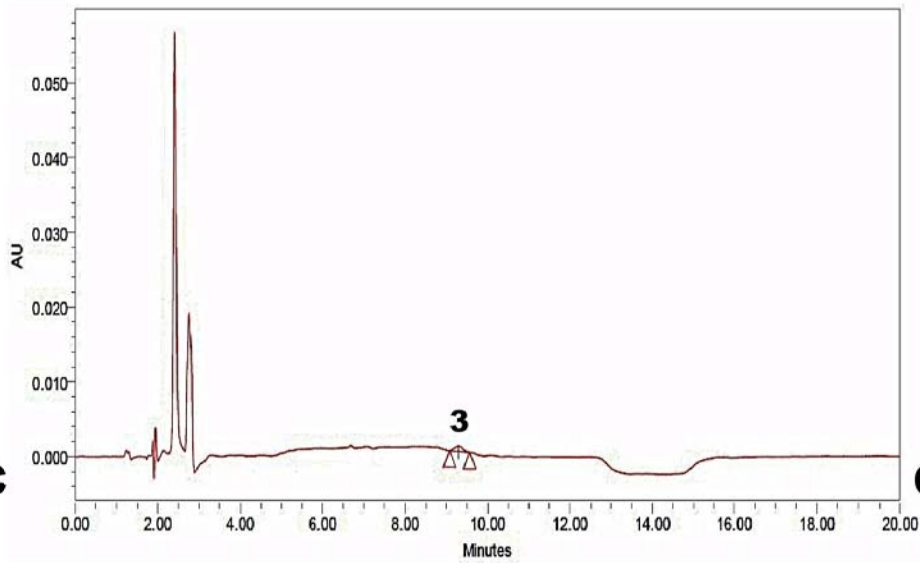
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

c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0.040
0.030
0.020
0.010
0.000
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0.00
2.00
4.00
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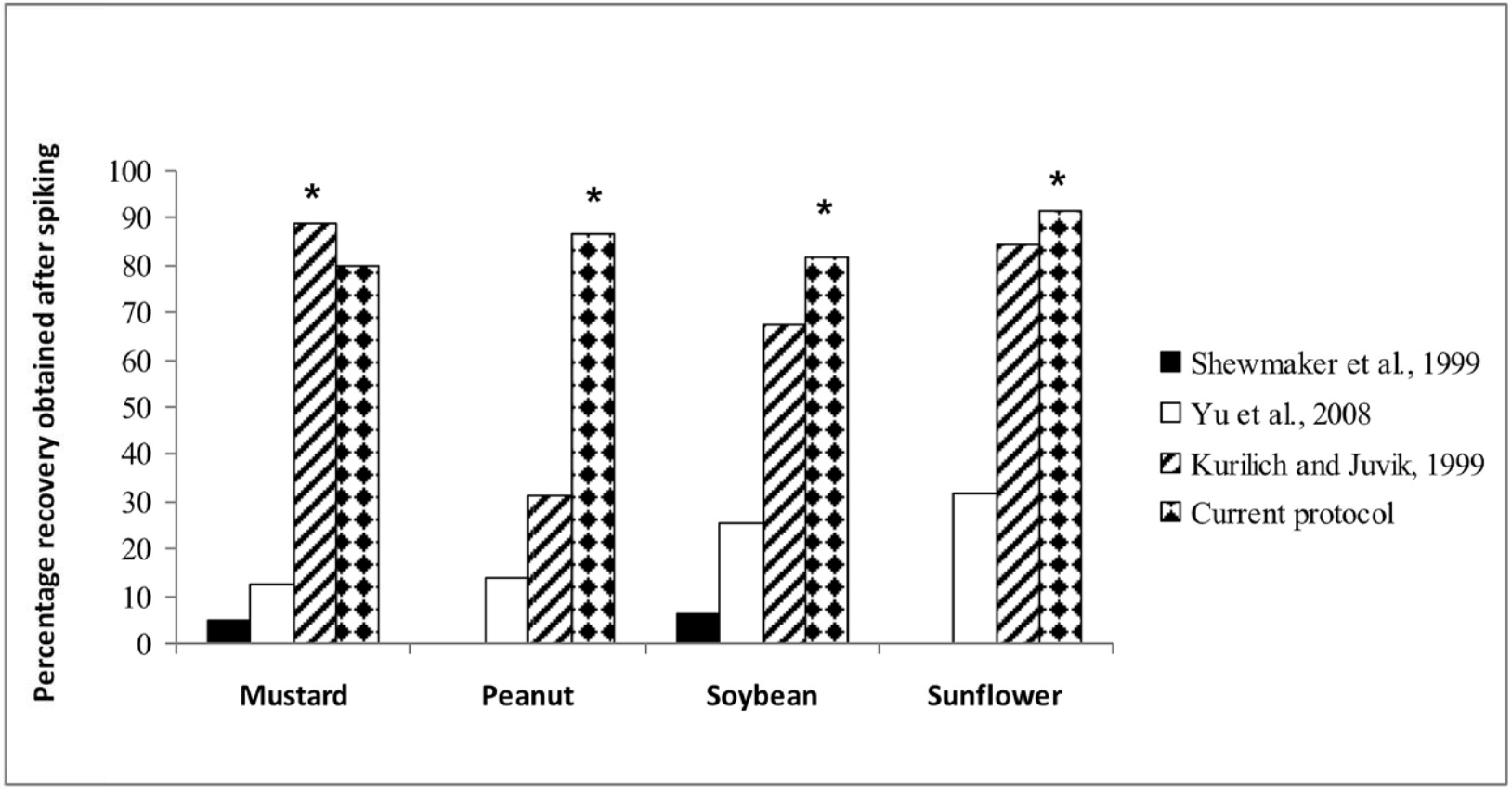
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

d

Fig. 7


Percentage recovery obtained after spiking
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
Mustard
Peanut
Soybean
Sunflower
Shewmaker et al., 1999
Yu et al., 2008
Kurilich and Juvik, 1999
Current protocol