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Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 773–780

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Identification of N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-5-carboxamide as components in urine extracts of individuals consuming coffee

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Received 5 September 2001; accepted 3 June 2002

Abstract

Caffeine metabolites were extracted from urine samples collected 4 h after consumption of a cup of coffee and were separated by high-performance liquid chromatography (HPLC) on a C18 (5 μm) reverse-phase column using an acetonitrile (5%), acetic acid (0.05%) solution as the mobile phase. The elution profiles indicated the constant presence of a major and a minor components eluting between the caffeine metabolites 5-acetamido-6-formyl-3-methyluracil (AFMU) and 7-methylxanthine (7X) in an approximate nine. A procedure was developed for the isolation of the major component in an apparent pure form, and the yield was 10–20 mg from 400 ml of urine. The minor component was isolated in an apparent pure form by this procedure as well, and the yield was 0.5 mg from 200 ml of urine. The average ratio of the two components in urine, UV absorption and ^{1}H-NMR spectra of the two components, and ^{13}C-NMR spectrum, mass spectrum and elemental analysis of the major component identified the major and minor components as N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-5-carboxamide, respectively, two major metabolites of the vitamin niacin present in a significant amount in coffee beans. The two metabolites were present in the same average amount in urine extracts of individuals irregardless of coffee consumption. The findings are briefly discussed in relation to the nutritional sources of niacin and to current procedures for measuring amounts of the two metabolites in urine samples.

Keywords: Caffeine; High-performance liquid chromatography; NAT2 phenotyping; Niacin; N^1-methyl-2-pyridone-5-carboxamide; N^1-methyl-4-pyridone-5-carboxamide

1. Introduction

The activity of the polymorphic enzyme *N*-acetyltransferase-2 (NAT2) can be measured using caffeine as a probe drug [1–4]. Toward this end, urine is collected 4–5 h after consumption of a cup of coffee and acidified to pH 3.0. Caffeine

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00384-9

metabolites are then extracted and separated by high-performance liquid chromatography (HPLC) on a reverse-phase column, and the ratio of 5-acetamido-6-formylamino-3-methyluracil(AFMU) and 1-methylxanthine (1X) is measured. We have previously developed a competitive enzyme-linked immunosorbent assay (ELISA) for NAT2 phenotyping by measuring the ratio of 5-acetamido-6-amino-3-methyluracil (AAMU) and 1X after transformation of AFMU into AAMU [5,6]. In validating the method of ELISA by HPLC on a reverse-phase column, it was noticed that the elution profiles of caffeine metabolite urine extracts showed the constant presence of a major and a minor components eluting between AFMU and 7-methylxanthine (7X) in an approximate ratio of 9. It is likely that the presence of the major component has been observed in previous studies on NAT2 phenotyping using caffeine as a probe drug as it is present in a prominent amount. For the following reasons, an investigation was undertaken to identify these two compoents. (a) The observation of the constant presence of the two components in an approximate constant ratio among urine extracts was intriguing and the approximate constant ratio suggested that the two components are related both structurally and metabolically. (b) Very little is known about ultraviolet (UV) absorbing components that can be extracted besides those of caffeine metabolites. Such knowledge could be useful in the interpretation of elution profiles and to define eluting conditions for the separation and determination of caffeine metabolites. This article shows that the major and minor components are N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-5-carboxamide, respectively, two major metabolites of the vitamin niacin present in a significant amount in coffee beans [7–9].

2. Experimental

2.1. Materials

Acetonitrile HPLC grade was obtained from Fisher Scientific; Dowex 1X8-400 basic anion exchange resin (chloride form), Supelco LC-18 SPE tubes 6 ml (1 g), Supelco VisiTM-1SPE single sample processor, TLC plates silica gel on aluminium (200 μm, 2.25 μm, 60 Á) with a fluorescent indicator were obtained from Sigma-Aldrich; silica gel 60 (0.04–0.063 mm; 230–400 mesh ASTM) from EM Science (Gibbstown, NJ) was purchased from VWR (Montreal, Que., Canada). Other reagents used were ACS grade.

2.2. Isolation of the major component eluting between AFMU and 7X

Four hundred milliliters (400 ml) of urine was lyophilized and the residue was suspended in 300 ml of methanol. After stirring for 15 min, the suspension was filtered by gravity through a Whatman Number 1 paper (15 cm). The residue was suspended in 300 ml of methanol, stirred for 15 min and filtered as above. After pooling the filtrates, the solvent was evaporated at 40 °C under reduced pressure. The residue was solubilized with an acetic acid (0.05%) solution to a final volume of 15 ml, and the solution was transferred into a 1-l erlenmeyer flask containing 9 g of ammonium sulfate and stirred for 15 min. Thirty volumes of an isopropanol (5%), chloroform (95%) solution (v/v) (450 ml) was added and, after covering the flask, the suspension was stirred for 30 min. The suspension was transferred into 30 ml corex tubes using a 500 ml separatory funnel and the tubes were centrifuged at $500 \times g$, 2 min, 24 °C with a tabletop centrifuge (IEC CentraB centrifuge model). Tube contents were filtered by gravity through a Whatman Number 1 paper (15 cm). The filtrate was dried over solid sodium sulfate, filtered as above, and the organic solvents were evaporated at 40 °C under reduced pressure. The residue was suspended in 1 ml of methanol and the suspension was vortexed vigourously. The suspension was diluted with 1 ml of a methanol (5%), chloroform (95%) solution (v/v) and the major component was purified by flash chromatography (2.54×21.6 cm column) using a methanol (5%), chloroform (95%) solution (v/v) as the mobile phase. The first 250 ml of eluate was collected in a 500 ml flask and discarded. Subsequently, fractions of 10–12 ml were collected (70 fractions). Fractions containing components absorbing UV light were identified by pipeting

1–2 μl of each fraction on a TLC silica gel plate and illuminating the plate with a short wavelength UV lamp. Several of UV light absorbing fractions were then analyzed by HPLC after evaporating 10 μl-aliquots and dissolving the residues with 50 μl of an acetic acid (0.05%) solution. Fractions containing the major component free of the minor component were pooled and the solvent was evaporated. The residue was dissolved with 2 ml of an ammonium hydroxide solution, pH 10 and applied on a 2–3 ml Dowex anion exchange resin column equilibrated with an ammonium hydroxide solution, pH 10. The column was then washed with the equilibrating buffer. The eluate collected during the application of the sample and washing of the column was evaporated at 40 °C under reduced pressure. The residue was dissolved with 1–2 ml of an acetic acid (0.05%) solution and the solution was applied on a 6 ml Supelco LC-18 SPE column equilibrated with an acetic acid (0.05%) solution: a Supelco VisiTM-1SPE single sample processor was used for the elution of the column. The column was then washed with the equilibrating buffer. Fractions of approximately 1 ml were collected. Fractions with the desired absorption spectrum were pooled and evaporated at 40 °C under reduced pressure. The product was then dissolved with a small volume of methanol and the solution transferred in a vial. After evaporating the methanol at 24 °C under a stream of nitrogen gas and drying over night with a lyophilizer, the product was stored under vacuum in a dessicator.

2.3. Urine samples

Urine samples used for analytical purposes (10–15 ml) were obtained from healthy donors with and without the habit of consuming coffee. They were immediately acidified to pH 3.0 with a HCl (1 N) solution and stored as 1 ml aliquots at −20 °C. Caffeine metabolites were extracted from these urine samples by a procedure previously described with the modification that 20 μl of the internal standard acetaminophen solution (0.5 mg/ml) was added per 200 μl of urine [2]. Urine samples used for the isolation of the minor and major components (400–500 ml) were obtained from a single healthy donor consuming daily coffee and who had a relatively high amount of the major component. These urine samples were stored as 40 ml aliquots at −20 °C until isolation.

2.4. High-performance liquid chromatography

HPLC was performed using a Spectra-Physics pump (P100) model, a Rheodyne 7123 syringe loading sample injector model with a 20 μl loop, a Spectra-Physics detector (UV 100) model and a Spectra-Physics DataJet integrator model. Caffeine metabolites were separated on a Luna C18 (2) 5 μ column (250 × 4.6 mm) (Phenomenex, Torrance, CA, USA). Samples of 20 μl were injected. The column was eluted isocratically at a flow rate of 1 ml/min with an acetonitrile (5%), acetic acid (0.05%) solution (v/v) [3]. The eluate was monitored at 280 nm.

2.5. Spectra

Absorption spectra of the minor and major components were determined with a Shimadzu spectrophotometer UV160U model. The reference and sample cell holders were at 24 °C. The ^{1}H-NMR of the minor and major components and the ^{13}C-NMR spectrum of the major component were recorded with 300 and 75 MHZ Varian Mercury spectrophotometers, respectively (Varian Inc., Palo Alto, CA, USA), using deuteromethanol or deuterodimethylsulfoxide as the solvent. The mass spectrum of the major component was performed at the Biomedical Mass Spectrometry Unit of McGill University with a Quattro II (Mieromass, Manchester, UK) triple quadrupole mass spectrophotometer using electrospray in positive mode. The sample was dissolved in 100 μl of an acetonitrile (80%), formic acid (0.1%) solution (v/v) and was introduced by direct infusion using a 25 μl Hamilton syringe. Capillary voltage was set at 3.8 kV, cone voltage at 20 V and the source temperature was kept at 80°C.

2.6. Elemental analysis

Elemental analysis of the major component was performed by Guelph Chemical Laboratories Ltd. (Guelph, Ont., Canada).

3. Results and discussion

Caffeine metabolites were extracted from urine samples collected 4 h after a consumption of a coffee cup, and caffeine metabolite extracts were analyzed by HPLC on a C18 (5 μm) reverse-phase column (250 × 4.6 mm) using an acetonitrile (5%), acetic acid (0.05%) solution (v/v) as the mobile phase. The elution profiles of the different extracts indicated the constant presence of a major component and a minor one eluting between AFMU and 7X (Fig. 1, retention times of 6.59 and 7.03 min). The average peak ratio of the major and minor components was 9.35 ± 1.05 ($n = 11$; range, 8.07–10.95) among urine samples. The relatively small coefficient of variation (11.2%) suggested that the two components are both structurally and metabolically related to each other. The major and minor components were also found to be present in relatively significant amounts and at a similar ratio in all urine extracts of individuals not consuming coffee but consuming caffeine under the form of a soft drink or a cup of tea: 9.55 ± 0.77 ($n = 12$; range, 8.22–10.60).

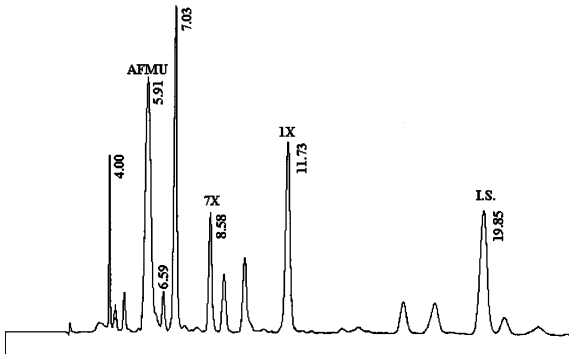

Fig. 1. The elution profile obtained of an extract of caffeine metabolites from urine of an individual consuming daily coffee on a C18 (5 μm) reverse-phase column. The column was eluted isocratically with an acetonitrile (5%) and acetic acid (0.05%) solution (v/v). The elution profiles of all the urine extracts of individuals consuming daily coffee indicated the constant presence of a minor and a major components eluting between the caffeine metabolites 5-acetamido-6-formylamino-3-methyluracil (AFMU) and 7X in a ratio of approximately 9 (retention times of 6.59 and 7.03 min). The elution profile shown indicates also that the individual is a fast NAT2 *N*-acetylator since the peak area ratio of AFMU and 1X is 1.7 and that the antimode peak area ratio is 1.0. The peak I.S. corresponds to that of the internal standard acetaminophen. For convenience, only a portion of the elution profile of the urine extract is shown.

The basic scheme developed for the isolation of the major component was as follows. The first step involved a lyophilization of a large volume of urine (400 ml) followed by an extraction of the residue twice with a large volume of methanol. After evaporation of methanol and solubilization of the residue with a small volume of an acetic acid (0.05%) solution, the minor and major components and caffeine metabolites were extracted with 30 volumes of an isopropanol (5%), chloroform (95%) solution (v/v). After evaporation of the organic solvents, the major component was then separated from the minor component by flash chromatography using a solution of low polarity as the eluent (methanol (5%), chloroform (95%) solution (v/v)). The isolated major component was then further purified by successive chromatographies on a Dowex anion exchange resin column and on a SPE C18 reverse-phase column. The rationale of the scheme of isolation was the following. (a) Large volumes of urine were found to be required for the isolation of the major component in sufficient quantities for identification. Thus, it was not feasible to scale up proportionally the usual step of extraction for this component and caffeine metabolites (30 volumes of an isopropanol (5%), chloroform (95%) solution (v/v)). (b) The major component appeared soluble in methanol, and AFMU readily transforms into AAMU in methanol, facilitating the isolation of the major component since AAMU is strongly retained on the silica gel column during flash chromatography [10]. (c) The major component isolated by flash chromatography using a mobile phase of low polarity was greater than 95–100% pure with respect to the minor component, as assessed by HPLC on a C18 (5 μm) reverse-phase column (data not shown). However, it was impure with respect to other components: components eluting after the major component on the C18 (5 μm) reverse-phase column and yellowish components were always present (data not shown). (d) The major component and a fraction of the yellowish components

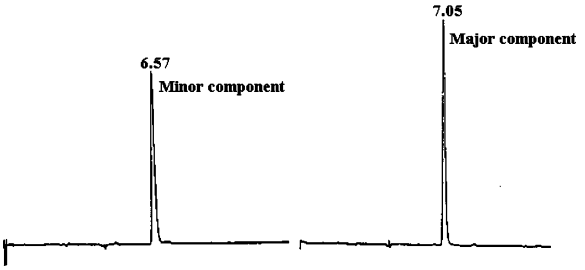

Fig. 2. Purity of the isolated minor and major components, as assessed by HPLC on a C18 (5 μm) reverse-phase column. The column was eluted isocratically with an acetonitrile (5%) and an acetic acid (0.05%) solution (v/v). The isolated minor and major components (retention times of 6.57 and 7.05 min) were 95–100% pure. See text for additional details.

were observed not to be retained on a Dowex anion exchange resin column eluted at pH 10 while components eluting after the major component on the C18 (5 μm) reverse-phase column and a fraction of the yellowish components were retained. (e) The yellowish components not retained on a Dowex anion exchange resin column were retained on SPE C18 reverse-phase column (6 ml) using an acetic acid (0.05%) solution as the eluent while the major component was not retained. (f) The isolated major component after chromatography on a SPE C18 reverse-phase column appeared to oxidize slowly, as indicated by the change of its color. This oxidation could be minimized by storage under vacuum, and oxidized products could be removed by chromatography on a SPE C18 reverse-phase column.

Between 10 and 20 mg of the major component were isolated from 400 ml of urine obtained from a healthy donor consuming daily coffee and producing a relatively high amount of this component. The isolated major component appeared 95–100% pure by HPLC on a C18 (5 μm) reverse-phase column (Fig. 2). The minor component could not be reliably isolated in an apparent pure form by the procedure used for the isolation of the major component. However, 0.5 mg of the minor component was eventually isolated from 200 ml of urine in an apparent pure form, which was sufficient for UV and ^{1}H-NMR spectroscopic analysis (Fig. 2). Like the major component, the minor component was not retained on a Dowex anion exchange resin column.

The observation that the minor and major components could be extracted from urine samples at pH 3 and were not retained on a Dowex anion exchange resin column indicated that these two components are neutral compounds. In an attempt to identify them unequivocally, spectroscopic methods and elemental analysis were employed. The data obtained did not allow their identification, although it provided useful structural information and confirmed that the two components are structurally and metabolically related. The ability to identify them unequivocally relied on the possibility that the major component could be N^1-methyl-2-pyridone-5-carboxamide. This neutral compound is present in urine and is referred as compound X in an article describing the isolation of AAMU from urine by a Dowex anion exchange chromatography [11]. A survey of the literature on N^1-methyl-2-pyridone-5-carboxamide in relation to our biochemical and structural data on the major and minor components allowed us to conclude unequivocally that the major and minor components are N^1-methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-5-carboxamide (4-Py), respectively, two major metabolites of the vitamin niacin (Fig. 3). The pieces of evidence are as follows. (a) The average ratio of the major and minor components in urine samples is 9.45 ± 0.89 ($n = 23$, including urine samples of individuals with and without the habit of consuming coffee), the same as that previously reported for 2-Py and 4-Py in urine samples [8]. (b) The UV absorption spectra of the major component and of 2-Py in water and in an HCl (1 N) solution share the same features: same appearances, an absorption maximum at 258 nm and a shoulder at 293 nm; a shift of the absorption spectrum toward lower absorbance values in an HCl (1 N) solution (Fig. 4) [7,8]. (c) The UV absorption spectra of the minor component and of 4-Py in water and an HCl (1 N) solution share the same features: the same appearance, an absorption maximum at 257 nm and a shoulder at 293 nm in water; the same appearance and an absorption maximum at 239.5 nm in an HCl (1 N) solution (Fig. 4) [7]. (d) The mass spectrum of the major component indicated a base mass unit of M + H of 153 and a 2M + H mass of 305, corre-

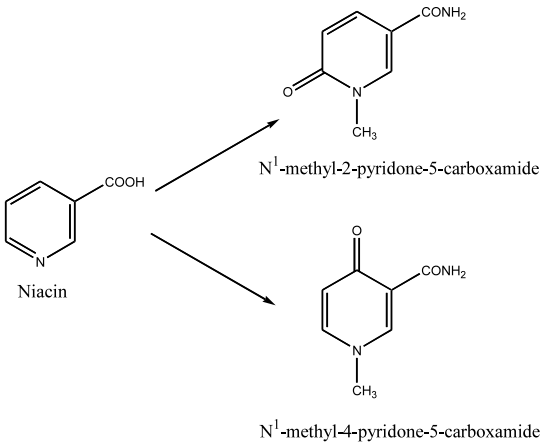

Fig. 3. Chemical structures of the vitamin niacin and of its two major metabolites N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-5-carboxamide. Formations of the two niacin metabolites from its precursor are catalyzed in man and rat by liver aldehyde oxidase, an enzyme of broad specificities. However, the formation of a fraction of N^1-methyl-2-pyridone-5-carboxamide in man is catalyzed by liver xanthine oxidase, an enzyme also implicated in the metabolism of caffeine [16].

sponding to an exact mass of 152, the formula weight of 2-Py (mass spectrum not shown). (e) The ^{1}H-NMR spectrum of the major component in deuteromethanol was the same as that of synthetic 2-Py [12]. The observed ^{1}H chemical shifts were: δ, 8.35 (d, $J = 2.4$, H-6); δ, 7.85 (dd, $J = 9.3$, 2.4, H-4); δ, 6.5 (d, $J = 9.3$, H-3); δ, 3.48 (s, CH3). The ^{1}H-NMR spectrum of the minor component in deuteromethanol was similar to that of 2-Py in the same solvent and consistent with the structure of 4-Py. The observed ^{1}H chemical shifts were: δ, 8.6 (d, $J = 2.1$, H-6); δ, 7.8 (dd, $J = 7.5$, 2.1, H-4); δ, 6.6 (d, $J = 7.5$, H-3); δ, 3.25 (s, CH3). (g) The ^{13}C-NMR spectrum of the major component in deuterodimethylsulfoxyde was in agreement with the structure of 2-Py. The spectrum indicated the presence of seven carbon atoms with the following chemical shifts: δ, 165.86 for the carbon atom of the carboxamide at C-5; δ, 162.52, 143.09, 138.74, 118.38 and 112.87 for C-2, C-6, C-3, C-4, C-5, respectively; δ, 39.67 for CH_3. (h) The elemental analysis of the C, H and N of the major component corresponded to that of 2-Py. Found: C, 55.79; H, 5.31; N, 18.17%. Expected: C, 55.26; H, 5.26; N, 18.42%.

Niacin is present many foods consumed and can be derived from the metabolism of the amino acid tryptophan. However, the coffee bean contains about 1% of trigonelline (*N*-methylbetaine of nicotinic acid), a percentage considerably higher than in foods such cereal grains, potatoes, beetroots and tomatoes [9]. Trigonelline is transformed into niacin upon roasting the coffee bean. Nicotinamide, the amide derivative of nicotinic acid and a moiety of the coenzymes nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP), is also produced upon roasting but in a ten times lower amount than that of niacin. The amount of niacin in coffee varies between 2 and 80 mg/100 g of coffee depending on the coffees geographical origin, the intensity of roasting and

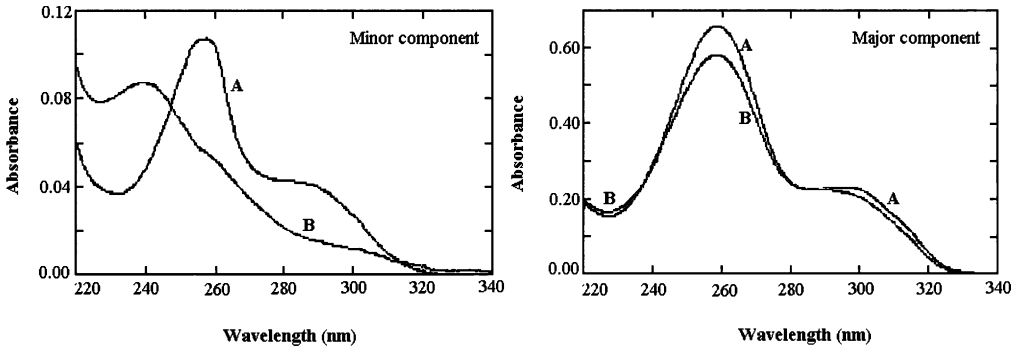

Fig. 4. Absorption spectra of the isolated minor and major components in water (A) and an HCl (1 N) solution (B). See text for additional details.

other modes of preparations of coffee (e.g. instant coffee) [9]. Thus, the question arised whether 2-Py and 4-Py in urine of individuals consuming daily coffee mainly derived from the metabolism of niacin in the consumed coffee. If the two metabolites were derived mainly from the metabolism of niacin in consumed coffee, the relative amounts of 2-Py and 4-Py in urine samples obtained from individuals not consuming coffee would be substantially less than in urine samples obtained from individuals consuming coffee daily. The relative amounts of 2-Py and 4-Py, as assessed by the peak area ratio of 2-Py and of the internal standard, were observed to vary broadly among individuals consuming and not consuming daily coffee. However, the average peak area ratio was the same for the two groups of individuals: 1.10 ± 0.51 ($n = 11$, range, 0.19–2.02) versus 1.01 ± 0.67 ($n = 12$; range, 0.27–2.42). This result indicates that the niacin metabolites 2-Py and 4-Py in the urine of individuals consuming daily coffee may not derive primarily from the metabolism of niacin in coffee.

Monitoring amounts of 2-Py and 4-Py in urine is of interest since these amounts mirror the niacin nutritional status [8]. Moreover, both niacin and nicotinamide are used for therapeutic purposes including as a radiosensitizer for tumour therapy, treatment of a variety of skin conditions, reducing hyperlipidemia and use as a possible means of preventing type 1 diabetes [13–16]. A method has been previously described for determining amounts of 2-Py and 4-Py in urine samples which involves an extraction of the two niacin metabolites from urine followed by an HPLC on a reverse-phase column [8,13]. However, the method has the disadvantage of involving a complex extraction scheme of the niacin metabolites. A more recent method has been described which has the advantage of involving a simpler extraction scheme but has the disadvantage of determining only amounts of 2-Py [14]. The apparent ability to extract both 2-Py and 4-Py from urine with an isopropanol (5%), chloroform (95%) solution (v/v) and to separate them by HPLC on a C18 (5 μm) reverse-phase column may provide a resolution of the disadvantages of the above methods.

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