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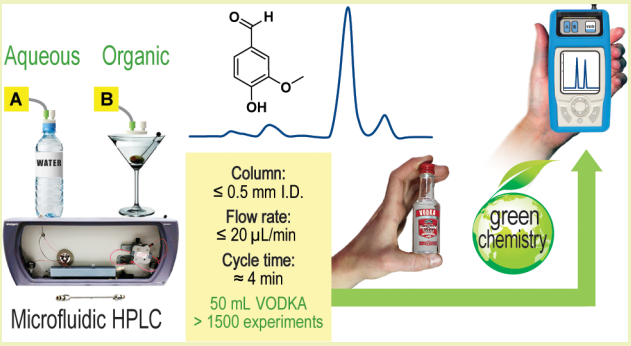
Cocktail Chromatography: Enabling the Migration of HPLC to Nonlaboratory Environments

Christopher J. Welch,* Timothy Nowak, Leo A. Joyce, and Erik L. Regalado*

Process & Analytical Chemistry, Merck Research Laboratories, Rahway, New Jersey 07065, United States

ABSTRACT: HPLC and HPLC-MS are powerful and wide ranging analytical tools employed in every aspect of modern chemical and biomedical research. The use of these tools is currently restricted to formal laboratory environments, partly due to cost and complexity but also owing to the special handling requirements for the solvents consumed and waste generated by these instruments. Ongoing innovations targeting the decrease in cost, size, and complexity of HPLC and HPLC-MS instrumentation raise the intriguing possibility that such tools may soon become both mobile and widespread in usage, breaking free of traditional laboratory boundaries. However, the dependence of these techniques on regulated organic solvents currently limits such mobility. In this study, we investigate the use of distilled alcohol spirits (cachaça, rum, vodka, aguardiente, etc.) as well as other household items typically available in a supermarket (vinegar, ammonia) as mobile phases and additives for carrying out HPLC and HPLC-MS experiments, showing that in many cases excellent analytical performance can be obtained using these low cost, universally available, green alternatives to the organic solvents typically used in such studies.



KEYWORDS: *Food chemistry, Green chromatography, Ethanol-based mobile phases, Microfluidic chromatography, High performance liquid chromatography*

INTRODUCTION

The technique of high performance liquid chromatography (HPLC) is a dominant method for analysis in modern chemical and biomedical research,[1−6] especially when coupled with mass spectrometry detection (HPLC-MS).[7−15] HPLC and HPLC-MS instruments line the laboratory benches wherever research into pharmaceuticals, biomedicine, agrochemistry, specialty chemicals, and food science is carried out. Currently, the use of these tools is restricted to formal laboratory environments, partly due to cost and complexity, but also owing to the special handling requirements for the solvents consumed and waste generated by these instruments (often mixtures of water with acetonitrile or methanol and small amounts of additives such as triflouoroacetic acid, ammonium formate, etc.). Innovations targeting decreased cost, size, and complexity of HPLC and MS instrumentation[16−28] raise the intriguing possibility that such tools may soon become both mobile and widespread in usage, breaking free of traditional laboratory boundaries. However, the dependence of these techniques on regulated organic solvents currently limits such mobility.

Figure 1 outlines the current state and several future scenarios for greener HPLC analysis. Current practice centers around a standard developed nearly 50 years ago: an HPLC column with an inner diameter of 4.6 mm and a length of 25 cm, operating at a flow rate of about 1.5 mL/min, and leading to the consumption of about a liter of solvent per instrument per day, with the concomitant production of an equivalent amount of waste (Figure 1a). Given the omnipresence of HPLC instrumentation within research laboratories, the cumulative solvent use and waste production of analytical HPLC has been identified as a significant target for green chemistry intervention.[29−37] Most HPLC analysis is carried out in the so-called reversed phase mode, with hydrophobic stationary phases and an eluent mixture of water and organic solvent, typically acetonitrile or methanol, containing small amounts of additives such as trifluoracetic acid or ammonium formate.[38,39] Consequently, the incoming stream of solvents and additives for HPLC analysis must be purchased from specialty chemical suppliers, shipped, stored, and inventoried under strict handling requirements. Typical purchase costs for methanol and acetonitrile HPLC-grade solvents are about $50−130/L. In addition, the controlled on-site storage and delivery of regulated solvents, as well as the collection and disposal of hazardous waste solvents, adds significantly to the overall cost of operating LC-MS instrumentation.

Replacement of acetonitrile or methanol with ethanol has been identified as a greener alternative for improving the solvent use profile of HPLC (Figure 1b). While chromatographic efficiency and overall performance typically suffers with the switch to ethanol, significant recent improvements in HPLC column and instrumentation technologies[40−44] mean

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Figure 1. Developing new green alternatives to perform liquid chromatography experiments is not only a way to protect the environment but is also a pathway to point of use applications beyond the traditional laboratory.

that there is often "performance to spare", making the use of ethanol-based mobile phases a viable option in some instances.[33] In addition to the green chemistry advantages of moving from a petrochemical-derived feedstock to a renewable resource, the price and availability of ethanol offers compelling incentives for change—especially given the volatile price fluctuations for acetonitrile. While HPLC-grade absolute ethanol typically costs about $120/L, azeotropically derived 95% alcohol (containing 5% water) is often quite suitable for chromatography and can cost as little as $8/L. Furthermore, distilled alcohol is available at a relatively inexpensive price from local markets in virtually all regions of the world, offering the possibility of local, sustainable production of a green, nontoxic eluent for HPLC-MS studies.

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a)

Mobile phase	Cost*	Vol (L)	$/L	EtOH (%)	$/L EtOH
Grain alcohol	15.99	0.75	21.32	94.5 ± 1.0	22.44
VODKA	11.99	0.75	15.99	39.1 ± 0.2	39.97
Cachaça	18.99	1	18.99	38.9 ± 0.4	47.48
Bacardi Rum	14.99	0.75	19.99	39.7 ± 0.9	49.97
Aguardente	18.99	1	18.99	45.1 ± 0.5	41.28
HPLC EtOH	422.9	4	105.73	>99.5	105.73

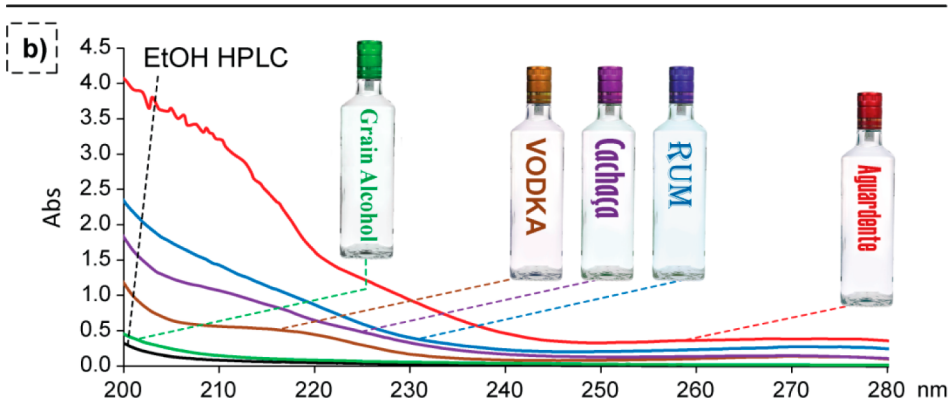

Figure 2. Ethanol composition, cost in $ (a) and UV spectra (b) of all mobile phases used in the study.*These spirits were purchased in a United States liquor store; costs can drastically change from store to store or country to country.

Use of microscale columns allows significant reduction in overall solvent use, either when conventional solvents are used or when alternative solvents such as ethanol are used (Figure 1c). The use of microscale columns has been shown to reduce solvent usage by several orders of magnitude[45−50] to only a few milliliters per instrument per day. Further reductions can easily bring the level of solvent consumption and waste generation to well below 1 mL per instrument per day. In addition to the trend toward narrower HPLC columns, recent improvements in stationary phase performance are enabling a move to shorter columns, with many separations and high-throughput analyzes now being possible with very short columns in the 1−5 cm range.[51−54] Together, these trends make possible the scenario depicted in Figure 1c, where a column, solvent reservoir, and waste reservoir can all fit into a prefabricated unit the size of an ink jet printer cartridge. Current technology allows for a fully functional LC-MS instrument the size of a carry-on suitcase, but there is clear possibility that hand-held instruments the size of a laptop computer or even a smart phone could one day be possible. While a contained solvent/waste reservoir offers an attractive option for increasing the mobility of HPLC-MS, the reliance on conventional organic solvents will limit the convenience and ease of use of dealing with such cartridges. Alternatively, the use of ethanol-based systems could allow greater freedom of use in nontraditional unregulated laboratory spaces, especially if all additives and components used for the assay are generally regarded as safe, perhaps even allowing for the spent cartridges to be disposed or recycled as common unregulated waste.

The availability of a miniaturized hand-held HPLC-MS instrument operating with nontoxic solvents and reagents could have a transformative effect on science, allowing application in environments far beyond the current range of use (Figure 1d). If cost and performance are sufficiently attractive, application in physician and dentist offices can be possible, helping to allow point of use assays to aid in rapid diagnosis of disease and monitoring of therapeutic drug levels. Similarly, point of use applications in the agricultural experimental station or even individual farms could allow monitoring of crop maturation, or levels of key pest or disease biomarkers, pesticide levels, or other factors relating to optimal timing of planting and harvest. Mobile use in law enforcement could allow for the possibility of immediate point of use forensics, allowing for applications in narcotics investigations or counterterrorism activities. The freedom from toxic and regulated chemicals means that the mobile HPLC-MS instrumentation could potentially be usable in pristine remote environments, enabling the search for natural products in remote jungle locations, islands, wetlands, etc. without fear of contamination. Similarly, environmental monitoring of point sources of pollution (oil spills, waste discharge outlets, etc.) could take place in real time or on an ongoing basis with the use of semipermanently deployed equipment. Military uses ranging from personal war gas detection units to deployed battlefield sensors could also be imagined. Minigreen LC-MS instrumentation would be well suited to classroom use, and recent experience has shown that high school students readily grasp the concepts of LC-MS operation and interpretation of results.[55,56] Such tools would increase the availability of this important equipment at the high school and possibly even the grade school level, leading to use in classroom investigations, science fair projects, etc. (clearly, safeguards surrounding the use of a potential intoxicant in the classroom would be required). Given recent interest in "molecular gastronomy" and mixology,[57−61] the use of minigreen LC-MS in restaurants and cocktail lounges can easily be imagined, and if the price point can be driven low enough, a big but not insurmountable challenge, the use of minigreen HPLC-MS in the home may eventually be possible.

In this study, we investigate the use of distilled alcohol spirits (cachaça, rum, vodka, aguardiente and grain alcohol) as well as other household items typically available in a supermarket (vinegar, ammonia) as mobile phases and additives for carrying

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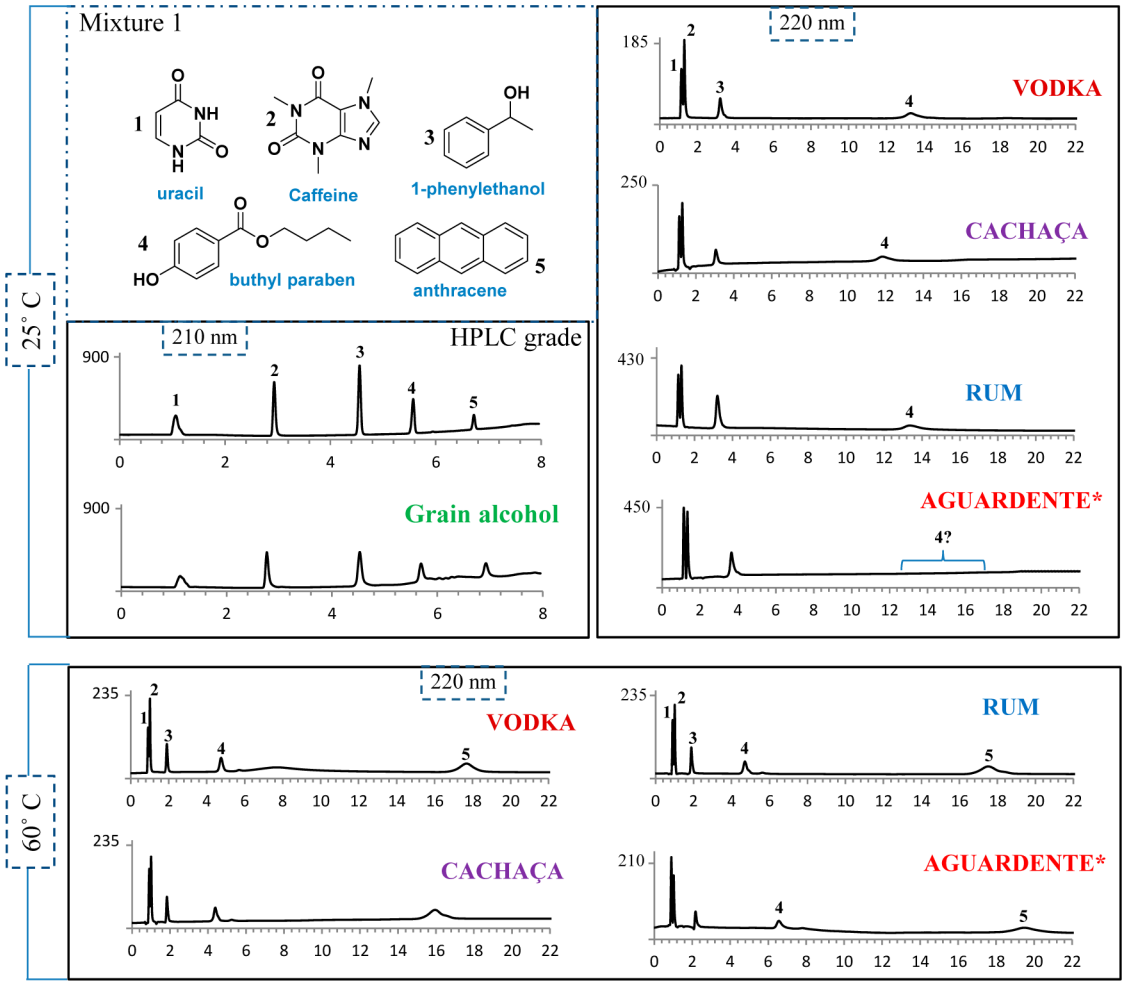

Figure 3. HPLC-DAD analysis of test mixture 1 using different spirit alcohol-based mobile phases and column temperatures. Column: Eclipse XDB-C8 (4.6 cm × 15 cm, 5 μm). A flow rate of 1.2 mL/min was used in experiments performed at 25 °C column temperature, while the flow rate was increased to 1.5 mL/min when working at 60 °C. Mixture 1: five components of a wide polarity spectrum from uracil commonly used as void marker in RP separations to antracene, an apolar compound strongly retained in RP mode.*All spirit alcohol-based mobile phase compositions were kept as listed in Figure 2 (≈ 40%), except for aguardente (38.3%) HPLC-grade ethanol was diluted to 95%.

out HPLC and HPLC-MS experiments, showing that in many cases excellent analytical performance can be obtained using these low cost, universally available, green alternatives to the solvents and chemicals typically used in such studies.

EXPERIMENTAL SECTION

Instrumentation. Reversed phase HPLC experiments were performed on two different instruments: ExpressLC-100 microfluidic HPLC instrument (Eksigent Technologies, Dublin, CA, U.S.A.), with a number of modifications that are described in detail in previous publication,[62] and an Agilent 1100 system (Agilent, Palo Alto, CA, U.S.A.). The Agilent stack comprised a G1312A binary pump, G1367A WPALS autosampler, G1315B diode array detector, and a 6120 quadrupole LC/MS detector with electrospray ionization in the positive mode. The system was controlled by Chemstation software, with the FIA mode enabled. UV spectra were measured on a Cary 300 Bio UV−visible spectrometer (Agilent, Palo Alto, CA, U.S.A.) by scanning 190 to 600 nm.

Beverage Mobile Phases, Chemicals, Reagents, and Stationary Phases. Ethanol (HPLC grade) was purchased from Acros Organics (Fair Lawn NJ, U.S.A.). Clear ammonia and distilled white vinegar were distributed by Wakefern Food Co. (Elizabeth, NJ, U.S.A.), orange juice, lemon, vitamins, pure vanilla extract, and whole and ground peppercorns were all purchased at a ShopRite store (Rahway, NJ, U.S.A.). Spirits used as mobile phase, including Everclear Grain Alcohol (Luxco, Inc., St. Louis, MO, U.S.A.), Aguardente Bagaceira (Caves do Solar de SãO Domingos, S.A., Anadia, Portugal), Cachaça 51 (Compahia Müller de Bebidas, Pirassunuga, Brasil), Bacardi Ron Carta Blanca (Bacardi Corporation, San Juan, Puerto Rico), and Smirnoff Vodka No. 21 (Smirnoff Co., Norwalk, CT, U.S.A.), were all purchased from a local liquor store (Rahway, NJ, U.S.A.) and directly used for analysis after filtration. Components of mixtures 1-3, hydrobenzoin, 4-flavanone, and omeprazole were all obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.) and Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A). Protocatechuic aldehyde, catechol, syringaldehyde, vanillin, guaiacol, propyl gallate, *p*-anisaldehyde, cinnamaldehyde, eugenol, and methyl salicylate were obtained from the Merck Building Block Collection (Newark, NJ, U.S.A.). Ultrapure water was obtained from a Milli-Q Gradient A10 from Millipore (Bedford, MA, U.S.A.). The 4.6 mm i.d. by 50 mm length, 2.5 μm XBridge phenyl column was purchased from Waters Corporation (Milford, MA, U.S.A.). The Eclipse XDB-C8 and XDB-C8 (4.6 cm × 15 cm, 5 μm) were purchased from Agilent. The AD-3R (4.6 mm × 100 mm, 3 μm) column was obtained from Chiral Technologies (West Chester, PA, U.S.A.).

Determination of Ethanol, Acetic Acid, and Ammonium Hydroxide. Determination of ethanol and acetic acid was performed on an Agilent 6890N GC-FID with an Agilent 7683B injector. The system was controlled by Empower 2 Chromatographic System (Waters, Milford, MA, U.S.A.). Ethanol samples were separated on an Agilent DB-624, 20 m × 0.180 mm, 1.0 μm film thickness column. Determination of acetic acid was performed on a DB-FFAP,

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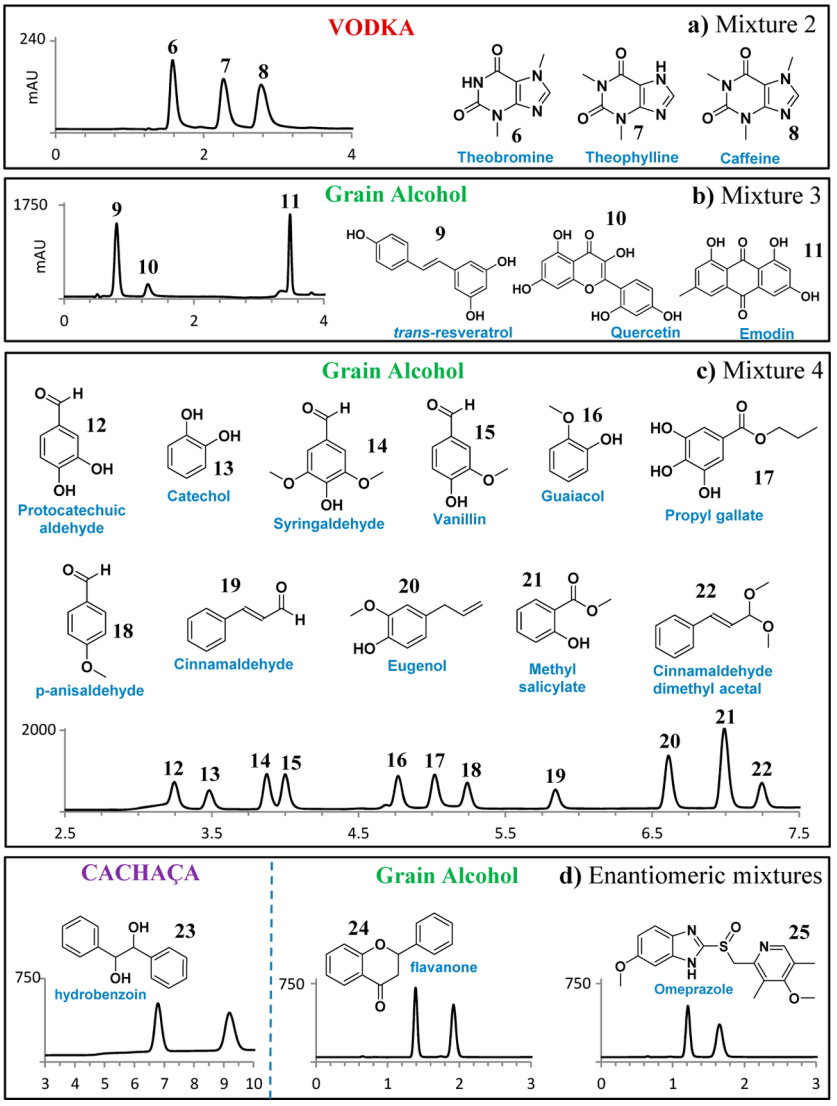

Figure 4. Reversed phase HPLC-UV analysis of several pharmaceutical and food-relevant compound mixtures using conventional HPLC instrumentation with different spirit alcohol-based mobile phases. (a) Column: Eclipse XDB-C8 (4.6 cm × 15 cm, 5 μm). Sample: equal volumes of 1 mg/mL component solutions in EtOH/H_2O; flow rate, 1.2 mL/min; detection, 210 nm; eluent A, H_2O; eluent B, vodka; mobile phase, 40% B isocratic. (b) Chromatographic conditions as described for mixture 1; detection, 220 nm; eluent A, H_2O; eluent B, grain alcohol; mobile phase gradient, 45% B for 2 min, 100% B at 4 min, keep 100% B for 3 min. (c) Column: Eclipse XDB-C18 (4.6 cm × 15 cm, 5 μm). Sample: equal volumes of 2 mg/mL component solutions in EtOH; flow rate, 1.2 mL/min; detection, 220 nm; eluent A, H_2O; eluent B, grain alcohol; mobile phase gradient, from 10 to 100% B in 8 min. (d) Enantioseparation of three different racemic mixtures. Column: AD-3R (4.6 mm × 100 mm, 3 μm); temperature, 40 °C; detection, 210 nm; eluent A, H_2O; eluent B, cachaça or grain alcohol; mobile phase, isocratic 100% B; flow rate, 1.2 mL/min or 1.8 mL/min, respectively.

30 m × 0.320 mm, 1.00 μm film column. Ammonia was determined using potentiometric titration measurements. The titrations were performed using a Metrohm LL Unitrode electrode with a Metrohm716 DMS Titrino automatic titrator with a 728 stirrer system (Herisau, SUI). The titrations were performed using the dynamic equivalence point titration calculated by the Tiamo Software.

Preparation of Buffer Solutions and Samples. Five mM ammonium acetate in H_2O (pH 4.7) and 5 mM ammonium acetate in alcoholic spirit solutions (pH 4.7): 8.333 mL clear ammonia (source of NH_4OH, 2.1%) and 5.884 mL distilled white vinegar (source of acetic acid, 5.1%) were dissolved in 1 L solvent. The pH was adjusted by adding vinegar.

A total of 100 mg black pepper was extracted with 4 mL EtOH; 2260 mg lemon slices, 5 mL orange juice, and 20 mg vitamin pill samples were extracted with 10, 15, and 10 mL of EtOH:H_2O (1:1), respectively. The suspensions were sonicated for 15 min in an ultrasonic bath and then centrifuged at 12000g for 5 min at 20 °C. The supernatants were filtered and transferred to HPLC vials for chromatographic analysis. The orange juice sample was diluted two times before the analysis. Twenty-five μL pure vanilla extract was extracted with 2.5 mL EtOH/H_2O.

HPLC-MS Conditions. HPLC separations were carried out on a 4.6 mm i.d. by 50 mm length, 2.5 μm Waters XBridge phenyl column at a flow rate of 1 mL/min. The LC eluents were 5 mM ammonium acetate in both aqueous and organic eluents (pH 4.7). The column and samples were maintained at a temperature of 30 °C. The misergrams were obtained from periodic sample injections (0.5 μL) every 1 min. The positive or negative ion ESI parameters were skimmer 45 V, desolvation gas (N_2), temperature 350 °C, and flow rate 12 L/min; the nebulizer was adjusted to 35 psig, fragmentor, and the capillary voltage to 150 and 3000 V, respectively.

RESULTS AND DISCUSSION

Distilled ethanol spirits are available worldwide in local markets, but the quality and purity can vary widely. In this study, vodka, caçacha, rum, aguardente, and grain alcohol were obtained from

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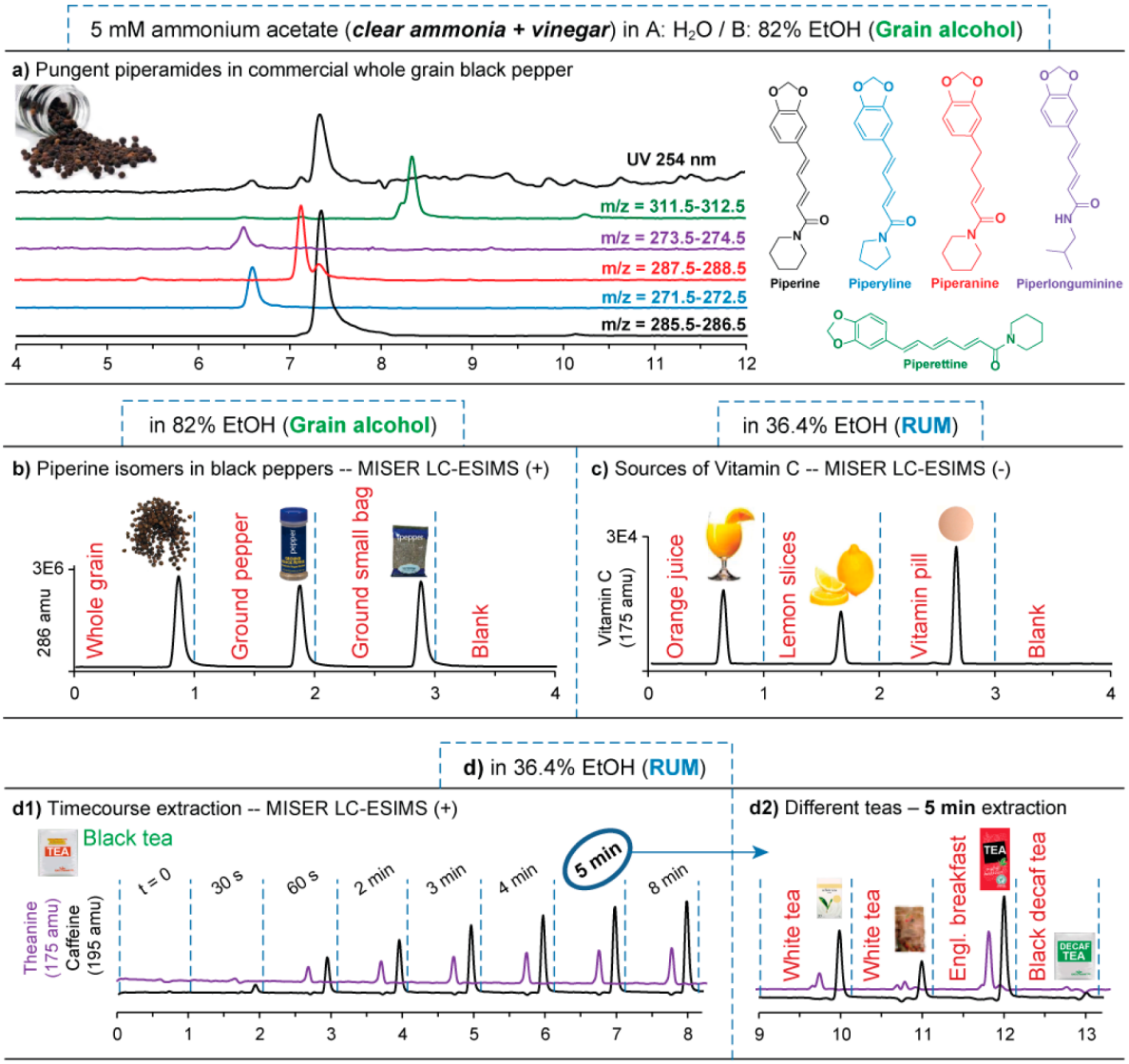

Figure 5. LC-MS analysis of several food-relevant mixtures using 5 mM ammonium acetate as mobile phase additive in a grain alcohol-based eluent and rum. Column: XBridge Phenyl (4.6 cm × 50 mm, 2.5 μm); flow rate, 1 mL/min; samples in EtOH/H_2O (see details on buffer and sample preparation in the Experimental section). (a) Conventional gradient separation of a black pepper extract. Eluent A, 5 mM ammonium acetate in H_2O (pH 4.7); eluent B, 5 mM ammonium acetate in 82% EtOH (grain alcohol, pH 4.7); mobile phase gradient, from 30 to 100% B in 12 min; detection, ESI (+), the indicated EIC at m/z values of each piperamides as illustrated in the same figure. (b–d) MISER LC-MS analysis allows simple visualization of relative amounts of piperine in several black peppers, vitamin C in different sources, and caffeine in a variety of teas. Detection: SIM ESI-MS($\pm$) at respective m/z values; 0.5 μL injection every 1 min. Isocratic mobile phases (pH 4.7): (b) 5 mM ammonium acetate in 82% EtOH (grain alcohol). (c and d) 5 mM ammonium acetate in 36.4% EtOH (rum).

a local liquor store and compared with HPLC-grade ethanol obtained from a chemical supply house (Figure 2). The cost and ethanol content varies considerably among these products, with grain alcohol (95%) being the best value at ~$22/liter of ethanol and rum (40%) being the worst value at ~$49/liter of ethanol (Figure 2a). Independent testing of alcohol content by gas chromatography (GC) generally substantiated label claims. Figure 2b illustrates the UV spectrum of the various distilled spirits compared with HPLC-grade ethanol. The large UV absorbance of aguardente in particular renders this spirit of limited value for investigations involving detection in the low UV range. On the other hand, the UV absorbance of rum, caçacha, and vodka are significantly better, and the UV absorbance of grain alcohol closely approaches that of HPLC-grade ethanol.

We next investigated the use of the various alcohol spirits as chromatographic eluents, focusing initially on HPLC analysis of a mixture of five standard compounds by gradient elution reversed phase chromatography with UV detection (Figure 3). Interestingly, the use of grain alcohol as an eluent shows performance that is very comparable to that achieved with HPLC-grade ethanol, even with UV detection at 210 nm. On the other hand, the relatively poor UV transparency of vodka, cachaça, rum, and aguardiente at low wavelengths required analysis at wavelengths ≥220 nm, while the relatively low alcohol percentage of these spirits (nominally 40–46%) resulted in very strong retention for the least polar component (5, anthracene). Performing the chromatographic separation at elevated temperature (60 °C) does enable elution of anthracene, albeit at an unacceptably long retention time of 16–20 min. While this shortcoming could potentially be mitigated by the use

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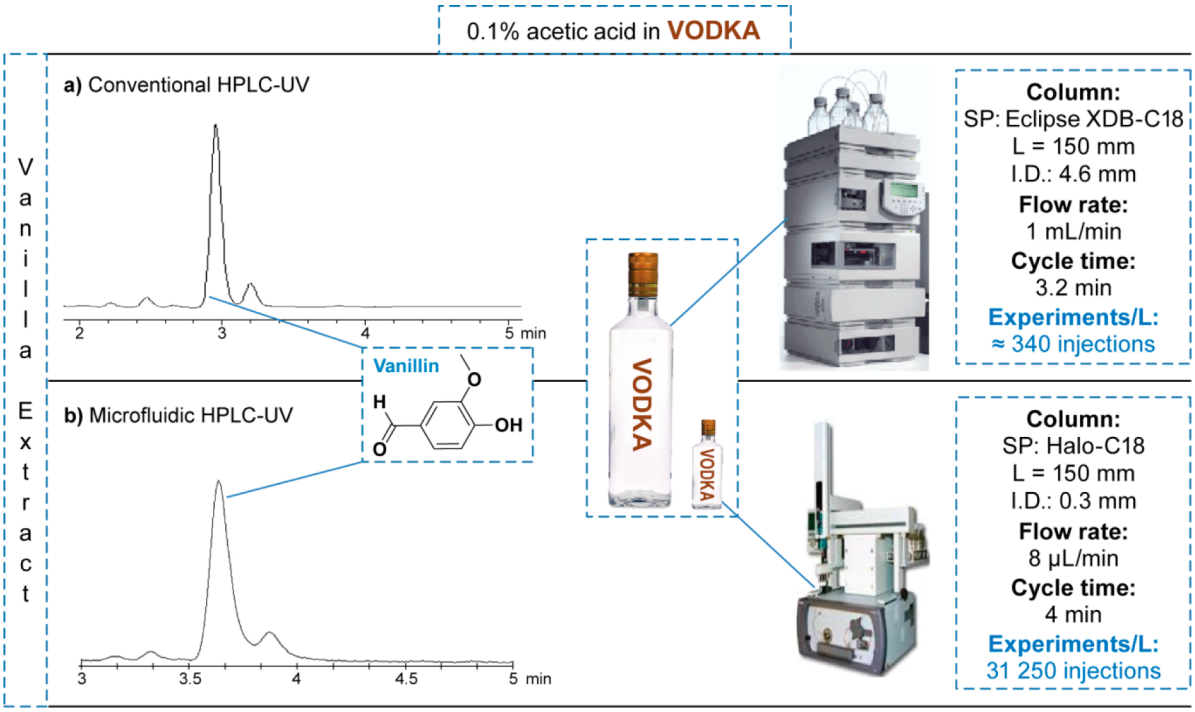

Figure 6. Chromatographic profiles of vanilla extract by two different LC-UV systems. (a) Conventional HPLC-UV analysis using 0.1% acetic acid in 30% EtOH (vodka) as mobile phase. (b) Eksigent Microfluidic HPLC-UV analysis using 0.1% acetic acid in vodka (40% EtOH) as mobile phase. UV Detection at 280 nm.

of a less hydrophobic stationary phase (e.g., C4), grain alcohol would seem to be the most generally suitable replacement for HPLC-grade ethanol, especially when the analysis of highly hydrophobic and strongly retained components is important. On the other hand, the use of vodka, cachaça, or rum may potentially be of service when investigating analytes that are not strongly retained in reversed phase mode.

A variety of examples of the use of distilled alcohol spirits as chromatographic eluents for HPLC studies are shown in Figure 4. Separation of the relatively nonpolar purine alkaloids (caffeine, theophiline, and theobromine) is easily accomplished using an isocratic vodka-based mobile phase (Figure 4a) with detection at 210 nm, while the separation of the flavonoids resveratrol, quercetin, and emodin requires gradient elution using the stronger grain alcohol eluent at 220 nm. Clearly, the need to perform analysis at slightly higher UV wavelength in gradient separations may present a problem for trace detection of some analytes with relatively poor UV chromophores; however, the potential strength of the approach is highlighted by the excellent separation of a mixture of 11 plant-derived simple natural products using a grain alcohol-based eluent, even with detection at 210 nm. In addition, the fast enantio-separation of natural products and pharmaceutically relevant compounds by chiral chromatography using cachaça- or grain alcohol-based mobile phases presented in Figure 4d clearly illustrates the potential for using spirit alcohol-based mobile phases for separation modes beyond simple reversed phase chromatography.

We next turned our attention to the use of HPLC-MS using spirit alcohol-based eluents. Mobile phases regularly used in LC-MS analysis contain additives to improve the MS ionization, for example, triflouoroacetic acid or ammonium salts, allowing detection in both negative and positive ion modes. Reasoning that the use of a sustainable nontoxic chromatographic eluent would be negated by the addition of regulated additives such as trifluoracetic acid, we investigated the use of LC-MS ionization-enhancing additives derived from easily obtainable vinegar (acetic acid) and ammonia cleaner (NH_4OH) obtained from a local supermarket. Exact measurement of concentration (849.7 mM for acetic acid and 599.3 mM for NH_4OH) by titration allowed mixing a cocktail containing 5 mM ammonium acetate, which afforded excellent ionization in the LC-MS studies highlighted in Figure 5. Examination of the ethanolic extract of whole grain black peppercorns using a grain alcohol-based eluent showed the presence of piperine as well as a number of congeners depicted in Figure 5a. MISER LC-MS is a simplified form of LC-MS that is well suited to fast analysis of the presence of a given component (in this case, piperine, m/z = 286 amu) within multiple samples. Repeated injections of different samples with continuous data recording provides a "misergram", a graphical depiction of piperine levels in the various commercial pepper extracts, allowing straightforward interpretation of results with an analysis time of about one sample per minute (Figure 5b).[63] Similarly, high-throughput MISER LC-MS using a rum-based eluent (Bacardi) containing 5 mM ammonium acetate derived from household vinegar and ammonia cleaning solution can be used for the analysis of vitamin C in various sources (Figure 5c) or the simultaneous measurement of caffeine and theanine present in brewed teas (Figure 5d).

While these results are impressive, requiring, for example, only 1 min per sample for the LC-MS analysis of piperine, caffeine, theanine, or vitamin C using inexpensive rum or grain alcohol, each analysis still consumes about 1 mL of spirits (cost ~$0.02) and generates an equivalent amount of waste. Microcolumn HPLC and HPLC analysis provides an attractive alternative to conventional approaches, with the typical column diameter being reduced from 4.6 to 0.5 mm or even smaller,

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and typical flow rate being reduced from 1 to 1.5 mL/min to 4−20 μL/min. Figure 6b shows an example of the use of microflow chromatography using spirit alcohol-derived mobile phases. Comparable chromatographic performance is obtained relative to the conventional-sized experiment (Figure 6a), with considerable savings in eluent consumption. As a case in point, the high-throughput analysis of vanillin in a vanilla extract requires only 32 μL of vodka per analysis, corresponding to a solvent cost of less than 0.08 cents per assay. At this rate, a single "airline serving" of vodka or rum would be sufficient for carrying out about 1560 vanilla assays, certainly good news to workers involved in vanilla production who might be interested in bringing scientific analysis and measurement technologies to their production site. Given such costs, the incorporation of spirit alcohol eluents in cartridge chromatography can easily be imagined. While the equipment of today is still relatively large (Figure 6b), recent advances in microfluidic chromatography show considerable progress on miniaturization,[18−20] making the idea of a hand-held device with disposable cartridges at least a conceptual possibility.

CONCLUSIONS

Ethanol-based mobile phases for HPLC offer a green and sustainable alternative to petrochemical-derived eluents such as acetonitrile. The somewhat reduced chromatographic performance obtained with ethanol-based eluents can often be compensated with modern HPLC column and instrumentation technology, making general analysis of pharmaceutical and food mixtures possible. In this study, we demonstrate that commonly available distilled alcohol spirits (cachaça, rum, vodka, and grain alcohol) as well as other household items typically available in a supermarket (vinegar, ammonia) can be used as chromatographic mobile phases and additives, providing a low cost, high performance, environmentally sustainable approach to modern HPLC and HPLC-MS analysis that does not rely on the use of hazardous organic solvents or the generation of regulated waste. Miniaturization of the approach using microscale columns (e.g., 300 μm diameter) operating at reduced flow rates (e.g., 20 μL/min) further reduces the environmental footprint, allowing more than 1500 analyses to be performed using a single airline serving of vodka.

AUTHOR INFORMATION

Corresponding Authors

*Tel.: +1 732 594 0032. E-mail: christopher_welch@merck.com (C.J.W.).

*Tel.: +1 732 594 5452. E-mail: erik.regalado@merck.com (E.L.R.).

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. These authors contributed equally: C.J.W and E.L.R.

Notes

The authors declare no competing financial interest.

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DEDICATION

Dedicated to our friend, Prof. Koji Nakanishi, on the occasion of his 90th birthday.

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