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Optimization of extraction parameters for quantification of fermentation volatile by-products in industrial ethanol with solid-phase microextraction and gas chromatography

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Abstract

The rapid growth of the biofuels industry resulted in new research needs in chemical analysis. Methods for screening and quantification of impurities resulting from changes in feedstock, process and purification are needed. Direct sample injection methods are often not sensitive for lower concentrations. This research, developed an analytical method to simultaneously quantify fermentation volatile by-products in industrial corn-based ethanol. These include acetaldehyde, ethyl vinyl ether, 1,1-diethoxyethane, isoamyl alcohol, isoamyl acetate, styrene, 2-pentylfuran, ethyl hexanoate, ethyl octanoate, and ethyl decanoate. Headspace solid-phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS) and GC-FID were used. The effects of SPME coating, concentration, time, and salting out were tested. The optimized method used Carboxen/PDMS 85 μm coating with 10% (v/v) ethanol, 20 s headspace extraction, and no salt addition. The method had values of R^2 between 0.93 and >0.99 and relative standard deviations between 0.10 and 11.96%. The method detection limits were between 9.5×10^{-4} to 9.7×10^{-8} mol/L. This is one of the most comprehensive quantification methods for volatile impurities in raw ethanol to date. This new method was used to quantify 10 prevalent impurities in corn-based industrial ethanol.

Keywords

Industrial ethanol, biofuels, impurities, SPME, quantitation

Disciplines

Agriculture | Bioresource and Agricultural Engineering | Organic Chemistry

Comments

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Simultaneous quantification of fermentation volatile by-products in industrial ethanol with solid-phase microextraction and gas chromatography

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Abstract

Rapid growth of biofuels industry resulted in new research needs in chemical analysis. Methods for screening and quantification of impurities resulting from changes in feedstock, process, and purification are needed. Direct sample injection methods are often not sensitive for lower concentrations. In this research an analytical method to simultaneously quantify fermentation volatile by-products in industrial corn-based ethanol was developed. These include acetaldehyde, ethyl vinyl ether, 1,1-diethoxyethane, isoamyl alcohol, isoamyl acetate, styrene, 2-pentylfuran, ethyl hexanoate, ethyl octanoate, and ethyl decanoate. Headspace solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS) and GC-FID were used. The effects of SPME coating, concentration, time, and salt were tested. The optimized method used Carboxen/PDMS 85 μm coating with 10 % (v/v) ethanol, 20 s headspace extraction, and no salt addition. The method had R^2 between 0.93-1.00 and relative standard deviations (RSDs) between 0.10-11.96%. The method detection limits were between 9.5×10^{-4} to 9.7×10^{-8} mol/L. To our knowledge (supported by literature review in Supporting Information) this is the most comprehensive quantification method for volatile impurities in raw ethanol to date. The new method was used to quantify 10 prevalent impurities in corn-based industrial ethanol for the first time.

Keywords

Industrial ethanol; biofuels, impurities, SPME; quantitation

Introduction

Today, ethanol is mostly produced by alcoholic fermentation. In fermentation process, yeasts convert sugars into ethanol. However, no matter which yeast strain is used, the production of undesirable by-products is inevitable (Figure 1). Production of these by-products makes the purification step of ethanol more difficult. Specifically, the removal of volatile by-products is challenging due to the limitations of ordinary distillation. These volatile by-products of ethanol fermentation are mainly derived from starch and lignin. The starch-derived by-products include alcohols, acetaldehydes, esters, fatty acids, and ketones.(1) The lignin-derived by-products include cyclic- and hemicyclic compounds.(2, 3) The existence of these by-products in ethanol is undesirable since some of them are of concern due to toxicity and/or their aroma impact.(4-6) Thus,

there is a constant need for more comprehensive analytical methods capable of fast and efficient screening and quantification of impurities that result from changes of feedstock, process, and purification method.

Volatile by-products in liquid samples can be determined by various methods. Among these methods, gas chromatography (GC) and high performance liquid chromatography (HPLC) are the most common due to their high separation capacities and simultaneous identification by the combination with mass spectrometry (MS).⁽⁷⁻¹²⁾

The challenges of volatile by-product determination in ethanol samples are as follows:

- (1) concentrations of volatile by-products are relatively low;
- (2) industrial ethanol matrix is complex;
- (3) wide range of volatile compounds concentrations (from mg/L to ng/L level) (13);
- (4) high ethanol concentrations interfere with the extraction and separation.

Thus, advanced sampling and sample preparation techniques are required for industrial ethanol analysis. These advanced sample preparation techniques include liquid-liquid extraction,⁽¹⁴⁾ solid-phase extraction,⁽¹⁵⁾ supercritical fluid extraction,⁽¹⁶⁾ and ultrasound extraction.⁽¹⁷⁾ Although these specific applications were developed for ethanol analysis, they may still have several disadvantages such as cost-intensive equipment requirements, extensive sample preparation, use of solvents, and complicated procedures causing handling errors.

Solid-phase microextraction (SPME) can be used as an alternative technology that overcomes some disadvantages associated with other sampling and sample preparation techniques. It is a simple, reusable and solvent-free technique that combines sampling and sampling preparation into one step. ⁽¹⁸⁾ SPME concentrates analytes on its coating during extraction. This enables the quantification of a large number of compounds with low concentrations. Solid-phase microextraction has a wide range of application including field air analysis, ^(19, 20) livestock odor, ⁽²¹⁾ pharmaceutical research, ^(22, 23) food science, ^(24, 25) and other fields. Our group successfully developed a method and applied SPME for quantification of carbonyl compounds from ozonated suspension of food dyes. ⁽²⁶⁾

The extraction efficiency of SPME is affected by several parameters such as SPME fiber coating, mode of extraction (i.e., headspace vs. direct immersion), temperature, agitation speed, and pH.^(27,28) In addition, the presence of high concentration of ethanol interferes with extraction.⁽²⁹⁾ The optimal ethanol-water ratio for SPME needs to be examined. The addition of salting-out agent could potentially improve the extraction efficiency.

The goal of this study was to develop a new analytical method based on SPME for simultaneous analysis of volatile by-products and impurities in industrial ethanol with GC. The effects of following operating parameter were tested and optimized: comparison between direct injection and SPME, SPME coating selection, effect of ethanol concentration, extraction time selection, effect of salt addition, and determination of

linear range and method detection limits. The optimized method was applied to quantification of ten by-products in industrial ethanol produced from corn.

This is important to the industry because of the novelty of applying this methodology to industrial grade ethanol, and extending future applications to food-grade ethanol. This sample type has an organic carrier, ethanol, which contributes more than 99% of the volatile organic material, yet it is still possible to accurately determine hundreds of impurities. A review of current literature (SI Table 1) showing quantification of acetaldehyde, ethyl vinyl ether, 1, 1-diethoxyethane, isoamyl alcohol, isoamyl acetate, styrene, 2-pentylfuran, ethyl hexanoate, ethyl octanoate, and ethyl decanoate in various alcoholic beverages is provided in the supporting information file. To date, this report is the first to quantify *these* 10 prevalent volatile impurities in industrial grade ethanol. The extensive application of this newly developed method for screening, quantification, and testing of the effectiveness on these 10 impurities removal with a suite of chemical and physical methods (i.e. ozonation, stripping with gas, and activated carbon) is presented elsewhere. (30)

Materials and Methods

Ethanol sample. Seventy nine percent (v/v) industrial ethanol sample used in this study was provided by Grain Processing Corporation (Muscatine, IA). The sample was transferred to one gallon amber glass bottles (Iowa State University Chemistry Store, Ames, IA) from one gallon metal containers after shipping. All amber glass bottles were stored in the flammable-material storage at room temperature.

Reagents. Standard chemicals of acetaldehyde, 1, 1-diethoxyethane, ethyl vinyl ether, isoamyl alcohol, isoamyl acetate, styrene, 2-pentylfuran, ethyl hexanoate, ethyl octanoate, and ethyl decanoate were purchased from Sigma-Aldrich (Milwaukee, WI). Two hundred proof pure ethanol was purchased from Iowa State University Chemistry Store (Ames, IA).

Multidimensional GC-MS. Multidimensional GC-MS (MOCON Texas Laboratory, Round Rock, TX) was used for method development and quantitative analysis. The system was equipped with a non-polar precolumn and a polar analytical column connected in series as well as system automation and data acquisition software (MultiTrax™ V. 6.00, Microanalytics and ChemStation™, Agilent). Auto injection system, CTC PAL system auto sampler (LEAP Technologies, Carrboro, NC), was used for automated injection. The general run parameters used were as follows: injector, 260 °C; column, 40 °C initial, 6 min hold, 10 °C /min, 220 °C final, 4 min hold; carrier gas, GC-grade He. Mass to charge ratio (m/z) range was set between 29 and 280. Spectra were collected at 6 scans/s and electron multiplier voltage was set to 1500 V. The MS detector was auto-tuned weekly.

The identity of compounds was verified using (a) reference standards (Sigma-Aldrich, Fisher, Fluka) and matching their retention time on multidimensional GC capillary column and mass spectrums; (b) matching mass spectrums of unknown

compounds with BenchTop/PBM (Palisade Mass Spectrometry, Ithaca, NY, USA) MS library search system and spectrums of pure compounds.

GC-FID/PID. GC-FID/PID (MOCON Texas Laboratory, Round Rock, TX) was used for method development. The system was equipped with a non-polar column connected to PID and a polar column to FID in parallel as well as system automation and data acquisition software (MultiTrax™ V., Microanalytics and ChemStation™, Agilent). The general run parameters used were as follows: injector, 260 °C; FID, 280 °C, column, 40 °C initial, 6 min hold, 10 °C /min, 220 °C final, 4 min hold; carrier gas, GC-grade He.

Direct injection conditions. Direct sample injection in GC injector method was tested to compare the analytical efficiency with headspace SPME-based sampling/sample preparation/sample desorption. GC-MS was used for this analysis. For direct injection, splitless mode was chosen, and 1 µL of the ethanol sample was injected.

SPME conditions. All SPME fibers were conditioned before the first use according to the manufacturer's instruction. The SPME conditions for GC-MS with an auto sampler were as follows: Ten mL of 10 % diluted ethanol samples were transferred to 25 mL screw-capped amber vials with polytetrafluoroethylene (PTFE)-lined silicon septa. The vial was agitated for 10 min with 750 rpm at 40 °C before SPME extraction. The SPME fiber was inserted into the headspace of the vial through the septum on the screw cap. The SPME fiber was exposed in the headspace of the vial for 20 s. With each extraction, the SPME fiber was removed immediately from the vial and inserted into the GC injection port for the analysis.

The SPME conditions for GC-FID/PID were as follows: Twenty-five mL of diluted ethanol samples were transferred to 40 mL screw-capped amber vials with PTFE-lined silicon septa. The vial was agitated for 10 min with 850 rpm at 35 °C before SPME extraction. The SPME fiber was inserted into the headspace of the vial through the septum on the screw cap. The SPME fiber was exposed in the headspace of the vial for 30 s. With each extraction, the SPME fiber was removed immediately from the vial and inserted into the GC injection port for the analysis.

SPME fiber coating selection. Several different kinds of SPME fiber coatings are commercially available. The proper fiber coating selection is critical to maximize the extraction efficiencies of analytes. It is necessary to examine the extraction efficiencies of different fiber coatings for different kinds of samples. There are two modes of extraction depending on SPME coatings, absorption and adsorption. Poly(dimethylsiloxane) (PDMS) coating is non-polar absorptive coating, and polyacrylate (PA) coating is polar absorptive coating. Absorptive coatings do not show displacement of analyte. The equilibrium is fully governed by the chemical nature of the analyte. Therefore, absorptive coating is suitable for quantification. Divinylbenzene (DVB)/PDMS and Carboxen/PDMS coatings are adsorptive type coating. The extraction capacities are mainly governed by the pore distributions of porous particles (DVB and Carboxen). Adsorptive coatings typically exhibit more affinity to target analytes than absorptive coatings. Therefore, adsorptive SPME coating is suitable for the analysis of low concentration compounds.(31)

Four commercially available SPME fiber coatings, including Carboxen/PDMS 85 μm , PDMS 100 μm , PA 85 μm , and PDMS-DVB 65 μm (Supelco, Bellefonte, PA), were examined to select the most efficient fiber coating to extract volatile compounds from the ethanol samples. GC-MS was used for this analysis. The extraction efficiencies were compared based on the MS detector response of 10 target impurities (1, 1-diethoxyethane, ethyl vinyl ether, isoamyl alcohol, isoamyl acetate, styrene, 2-pentylfuran, ethyl hexanoate, ethyl octanoate, and ethyl decanoate). Each experiment was carried out three times and results were averaged.

Ethanol dilution ratio determination. Different ethanol concentrations were examined to investigate the effect of alcohol concentration on extraction efficiency on SPME fiber. GC-FID/PID-O was used for this analysis. The ethanol samples were prepared from the same stock of industrial ethanol sample (79 %, v/v) but with different diluted ethanol concentration (0.79 to 79 %, v/v). The extraction efficiencies were compared based on the FID detector response of 10 target compounds. Each experiment was carried out three times and results were averaged.

Extraction time determination. Different extraction times with a SPME fiber were examined to investigate the extraction tendencies for the target compounds. GC-MS was used for this analysis. The extraction times between 10 to 20 s were investigated, and a proper extraction time was selected according to the fiber coating type (absorption or adsorption). Ten % (v/v) ethanol samples were used, diluted from stock ethanol. The extraction efficiencies were compared based on the MS detector response of 10 target compounds. Each experiment was carried out three times and results were averaged.

Effect of salt addition. Different amounts of salt addition were examined to investigate the effect of the salting-out agent on the SPME headspace extraction efficiency on the target compounds. The salt addition to aqueous solution generally results in increases in the fiber/matrix distribution constant of neutral organic matter, by driving the equilibrium of dissolved analytes into the headspace of the sample. It is expected that the amount of the extracted compounds will increase with an increase in the salt concentration. However, the amount of the extracted compound decreases when the analytes are in dissociated form in the solution. (30) GC-FID/PID was used for this analysis. Ten % (v/v) ethanol samples were used, diluted from stock. Sodium chloride (Sigma-Aldrich, Milwaukee, WI) concentrations between 0 to 20 % (w/w) were investigated. The extraction efficiencies were compared based on the FID detector response of 10 target compounds. Each experiment was carried out three times and results were averaged.

Quantification. Calibration curves were calculated based on MS detector response to known concentrations of each compound. GC-MS was used for this analysis. The standard ethanol solutions were prepared from the standard chemicals purchased from Sigma-Aldrich (Milwaukee, WI) and Iowa State University Chemistry Store (Ames, IA). Ten % (v/v) ethanol concentration was selected. Five to seven different concentrations were examined for each target compound. The target compound concentrations between 1.76×10^{-6} to 8.52×10^{-2} mol/L were examined. Each experiment was carried out three times and results were averaged.

Method detection limits. Method detection limits (MDLs) of the MS detector were carried out for each target compound. MDLs were obtained based on the USEPA method with 99% confidential level. (32)

Results and Discussion

Direct injection vs. SPME. Direct injection and SPME extraction with Carboxen/PDMS 85 μm fiber were compared to select more efficient and comprehensive extraction technique of ethanol sample. Although SPME is an advanced pre-concentration technique, it is not clear which sample introduction method, direct injection or SPME, is suitable for the analysis of ethanol sample. It is expected that a high concentration of ethanol will result in a large, split chromatographic peak that will co-elute with, and therefore hide, other compounds of interest with retention time close to ethanol. Ethanol may also have other adverse effects on the chromatography. The comparison of the amount of analyte injected by these two methods is necessary.

Non-diluted 79% (v/v) industrial ethanol sample was analyzed with direct injection method, and diluted 10 % (v/v) industrial ethanol sample was analyzed with SPME. Figure 2 represents the comparison of direct injection and SPME based on the total ion chromatogram obtained from GC-MS. While direct injection provided bigger peaks for low retention time compounds including acetaldehyde, ethanol, isobutyl alcohol, and isoamyl alcohol, SPME provided bigger peaks of high retention time compounds such as ethyl octanoate, ethyl decanoate, and ethyl dodecanoate. In addition, the use of SPME allowed for separation of compounds with close GC column retention times. The peaks of ethanol and 1, 1-diethoxyethane and the peaks of isoamyl alcohol and isoamyl acetate were better resolved when using SPME. With direct injection, these peaks were co-eluted due to high concentration of ethanol and isoamyl alcohol. The total number of peaks resulting from SPME and direct injection for sample introduction was 14 and 4, respectively.

Based on this comparison between direct injection and SPME, SPME was more effective for the extraction of volatile by-products in the industrial ethanol sample in terms of number of compounds detectable and improved chromatographic peak resolution between high concentration analytes and others. Therefore, SPME was selected for the subsequent experiments.

SPME fiber coating selection. Four commercially available SPME fiber coatings, Carboxen/PDMS 85 μm , PDMS 100 μm , PA 85 μm , and PDMS/DVB 65 μm (Supelco, Bellefonte, PA), were examined to select the most efficient fiber coating to extract volatile compounds from the ethanol samples. GC-MS was used for this analysis. Figure 3 represents the effect of the fiber coating on extraction efficiency of volatile compounds in the 10% (v/v) diluted industrial ethanol sample.

The two adsorption type fiber coatings, Carboxen/PDMS and PDMS-DVB, had bigger extraction capacity, seen as total MS detector response, on the target compounds comparing to the two absorptive type fiber coatings, PA and PDMS. The two adsorptive coating had similar extraction capacities. Carboxen/PDMS 85 μm coating was more

efficient in extracting acetaldehyde and ethyl vinyl ether. PDMS-DVB 65 μm coating was more efficient in extracting ethyl octanoate and ethyl decanoate. The critical point is that Carboxen/PDMS also adsorbed ethyl octanoate and ethyl decanoate, while PDMS-DVB did not adsorb acetaldehyde and ethyl vinyl ether. Thus, Carboxen/PDMS was the only one coating which could extract all of the selected ten volatile by-products from the ethanol sample among the four different fiber coatings.

Based on this comparison of extraction capacities of four commercially available SPME fiber coatings, Carboxen/PDMS 85 μm coating was the most effective for extraction of volatile by-products in the industrial ethanol sample in terms of extraction efficiency. Therefore, Carboxen/PDMS 85 μm coating was selected for the subsequent experiments.

Effects of ethanol to water dilution ratio. Different ethanol concentrations were examined to investigate the effect of alcohol concentration on extraction efficiency on SPME fiber. GC-FID/PID was used for this analysis. Ethanol competes with higher molecular weight compounds for the adsorption sites on SPME coating. It is expected that the effect of high concentration of ethanol can be decreased with dilution of ethanol. Dilution of ethanol could result in less competition of adsorption on SPME coating between ethanol and other components. However, it is also expected that the dilution of ethanol sample simply results in the dilution to low concentrations of other components, resulting in decreases in extraction efficiencies of these components. Therefore, optimal ethanol concentration, where the total extraction efficiencies of volatile compounds in the ethanol sample are maximized, needs to be investigated.

Figure 4 represents the effect of ethanol concentration on extraction efficiency of volatile by-products in the industrial ethanol sample. The ethanol concentrations between 0.79 to 79%, (v/v) were examined. The extraction efficiencies of all target compounds, except acetaldehyde and ethyl vinyl ether had a bimodal trend, i.e. they initially increased with the dilution of ethanol between $\sim 10\%$ to 40% v/v. Then, the extraction efficiencies decreased with further dilution. The extractions of the low molecular weight compounds, acetaldehyde and ethyl vinyl ether, did not show this bimodal trend, i.e., the absolute amounts extracted decreased with ethanol dilution. The extraction efficiencies were simply decreasing for all target compounds with dilutions below $\sim 10\%$. This can be accounted for by considering that these low molecular weight compounds are less affected by competitive adsorption with ethanol. On the other hand, the extraction efficiencies of the high molecular weight compounds, ethyl octanoate and ethyl decanoate, were more affected by ethanol concentration. These high molecular weight compounds were not detected by FID when the ethanol concentration was higher than 20% (v/v). This can be due to these high molecular weight compounds being more slowly adsorbed and more affected by competition with ethanol.

Based on this comparison of extraction efficiencies of the volatile by-products in different ethanol concentrations, 10% (v/v) ethanol concentration was the most effective for extraction of the volatile by-products in the industrial ethanol sample in terms of maximum extraction capacities. Therefore, 10% (v/v) ethanol concentration was selected for the subsequent experiments.

Extraction time determination. The extraction time was determined by exposing Carboxen/PDMS 85 μm fiber to the headspace of vials for 10, 13, 16, and 20 s at constant agitation, 750 rpm, and the constant temperature, 40 $^{\circ}\text{C}$. GC-MS was used for this analysis. It is important to determine an extraction time within the linear range of the relationship between extraction time and mass extracted. Carboxen/PDMS, adsorptive type, fiber shows displacement of compounds after a time. There is a minimum effect of the displacement in the first linear range between extraction time and amount.(28)

As shown in Figure 5, all compounds showed high linearity in the extraction time ranging from 10 to 20 s, and longer extraction provided more mass extracted of the target compounds. The coefficient of correlation, R^2 , of each analyte ranged between 0.92 to 1.00, and the range of RSD was 0.19 to 13.53%. Based on the effect of the extraction time on the mass extracted of the target compounds, 20 s extraction was the most effective and proper for quantification of all 10 volatile by-products in ethanol sample. Therefore, 20 s extraction time was used for the subsequent analysis.

Salt addition effect. Different concentrations of salt addition were examined to investigate the effect of the salting-out agent on the headspace SPME efficiency for the target compounds. GC-FID/PID was used for this analysis. Salt concentrations between 0 to 20% (w/w) were examined. The salt addition to aqueous solution generally results in increases in the fiber/matrix distribution constant of neutral organic matter. Thus, it is expected that the amount of the extracted compounds increase with an increase in the salt concentration. However, the amount of the extracted compound decreases when the analytes are in dissociated form in the solution.(27) The salting-out effects were expected at the 10% dilution, (v/v), using HS_SPME, resulting in a more aqueous solution. Also, the generation of new volatile/semi-volatile by-products derived from the added salt need to be considered.

Figure 6 represents effect of sodium chloride addition on extraction efficiency of volatile by-products in the industrial ethanol sample. No new by-products derived from salt addition was observed. The extracted mass of all analytes were increased with an increase in the salt concentration until 10% (w/w) sodium chloride addition. The extracted amounts of ethyl octanoate and ethyl decanoate started decreased with higher salt concentration while the extraction amounts of all the other compounds kept increased.

Based on the effect of the salt addition on the mass extracted of the target compounds on the SPME coating, salt addition was not effective in terms of comprehensive and effective extraction of volatile by-products in the industrial ethanol sample. An increase in the concentration of salt resulted in increased extraction amounts of impurities in ethanol sample except for the ester compounds. Increased salt addition had an inverse effect on the mass extracted of ester compounds. Also shown in Figure 5, SPME has an advantage on extraction efficacy for high molecular weight compounds, especially the esters in ethanol sample. Also, sample preparation accuracy improvement is expected by avoiding the extra sample preparation step of salt addition. Therefore, no salt addition was introduced for subsequent analysis for the industrial ethanol sample.

Calibration. Calibration curves depicting the MS detector response to the prepared standard solutions concentration were generated for each target compound. GC-MS was used for this analysis. Five to seven different concentrations were examined for each target compound. The target compound concentrations between 1.76×10^{-6} to 8.52×10^{-2} mol/L were examined. Each experiment was carried out three time and results were averaged. Determination of method detection limits (MDLs) for each target compounds was carried out. MDL was determined based on the US EPA method with 99% confidential level. Each experiment was replicated for n=14 times.(32)

Table 1 represents the equations for calibration curves, R^2 , the range of relative standard deviations (RSDs), and MDLs. All calibration curves had high correlation coefficient (0.93 to 1.00) and row RSD ranges (0.10 to 11.96%). MDLs ranged from 9.5×10^{-4} to 9.7×10^{-8} mol/L. Based on the calibration curves and MDLs, SPME and GC-MS were a proper and sensitive technique to quantify volatile compounds in ethanol sample.

Analysis of industrial ethanol. The headspace SPME method was applied to industrial ethanol samples. Ten volatile by-products were quantified including: one aldehyde (acetaldehyde), two condensation products of acetaldehyde and ethanol (ethyl vinyl ether and 1, 1 diethoxyethane), one alcohol (isoamyl alcohol), two cyclic/heterocyclic compounds (styrene and 2-pentylfuran), and four esters (isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate). GC-MS was used for this analysis.

Table 2 represents the concentrations of impurities in the industrial ethanol sample. Acetaldehyde and isoamyl alcohol were most abundant impurities in the ethanol sample. It may be that acetaldehyde is an intermediate compound of ethanol fermentation and isoamyl alcohol is one of the main by-products of ethanol fermentation. The next abundant impurities are ethyl vinyl ether and 1, 1-diethoxyethane. These are generated from acetaldehyde and ethanol. The next abundant impurities are esters, which are also by-products of ethanol fermentation. The last is styrene and 2-pentylfuran, which are lignin derived cyclic/heterocyclic compounds. The extensive application of newly developed method for screening, quantification, and testing of the effectiveness of removal of these 10 industrial ethanol impurities with a suite of chemical and physical methods (i.e. ozonation, stripping with gas, and activated carbon) is described elsewhere. (30).

In summary, a quantification method of volatile by-products in industrial ethanol sample using gas chromatography was developed.

- 1) The results indicated that SPME was more effective sample injection method comparing to direct injection. SPME extracted low concentration compounds, such as esters, on its coating and enable gas chromatography to detect these low concentration compounds in ethanol.
- 2) Adsorptive type SPME coatings, Carboxen/PDMS and PDMS-DVB, extracted impurities from ethanol more efficient. Between these two adsorptive coatings, Carboxen/PDMS was the best coating to extract volatile by-products from ethanol

sample due to its comprehensive extraction and high extraction capacities for acetaldehyde and ethyl vinyl ether.

- 3) Highly proof ethanol samples needed to be diluted with water to avoid the interferences of ethanol on adsorption of target impurities to SPME. The effect of ethanol was more significant on higher molecular weight compounds. It is recommended that ethanol concentration should be adjusted to 10% (v/v) to maximize the SPME adsorption efficiency.
- 4) Carboxen/PDMS coating showed high linearity between extraction time and mass extracted for all the volatile by-products between extraction times of 10 to 20 s. It is recommended to utilize 20 s extraction time to maximize extraction efficiency and to avoid displacement of compounds.
- 5) Salt addition resulted in increases in extracted amount of all target compounds except ethyl octanoate and ethyl decanoate. The decreases in the extracted amount of ethyl octanoate and ethyl decanoate lessened the advantage of SPME, pre-concentration of esters. Therefore, no salt addition is recommended to avoid decreasing the mass extracted of ethyl octanoate and ethyl decanoate, while increasing experimental error by addition of one extra sample preparation step.
- 6) The calibration curves for ten target compounds were prepared. The linearity of each calibration curve, correlation coefficient (R^2), ranged between 0.93 to 1.00, the RSD range for each compounds ranged between 0.10 to 11.96%, and MDLs ranged between 9.7×10^{-8} to 9.5×10^{-4} mol/L. This indicated that SPME-GC-MS were a proper technique to quantify volatile by-products in ethanol sample. Lastly, ten volatile by-products in the real industrial ethanol sample were quantified with the developed method. Acetaldehyde and isoamyl alcohol were the most dominant impurities, and styrene and 2-pentylfuran were the least dominant impurities in the industrial ethanol sample.
- 7) To our knowledge (supported by extensive literature review presented in Supporting Information) this is the most comprehensive quantification method for volatile impurities in raw ethanol to date.

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Author Contributions

All authors contributed to this manuscript. SO was a M.S. student and performed research. JAK, HvL, LC, WSJ served on thesis committee, helped SO with experimental design, data analyses, and manuscript writing. SR helped with manuscript writing.

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Notes

The authors declare no competing financial interest.

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Figure Captions

Figure 1. Origin of volatile by-products of corn-based ethanol fermentation.

Figure 2. Typical chromatograms for the separation of impurities in industrial ethanol sample with SPME preconcentration and with direct injection. (Direct injection conditions: injection volume, 1 μ L; splitless mode. SPME conditions: 85 μ m Carboxen/PDMS fiber; headspace extraction; extraction time, 20 s extraction temperature, 40 $^{\circ}$ C; agitation time, 10 min; agitation speed, 850 rpm; ethanol dilution ratio, 1/10. 1=acetaldehyde; 2=ethanol; 3=1, 1-diethoxyethane; 4=Isobutyl alcohol; 5=Isoamyl alcohol; 6=Isoamyl acetate; 7=styrene; 8=2-pentylfuran; 9=ethyl hexanoate; 10=ethyl octanoate; 11=ethyl noanoate, 12=ethyl decanoate; 13=ethyl dodecanoate; 14=ethyl tridecanoate)

Figure 3. Effect of SPME fiber coating on extraction efficiency of impurities in industrial ethanol. (SPME conditions: 85 μ m Carboxen/PDMS fiber; 85 μ m PA fiber; 100 μ m PDMS fiber; 65 μ m PDMS-DVB fiber; headspace extraction; extraction time, 20 s; extraction temperature, 40 $^{\circ}$ C; agitation time, 10 min; agitation speed, 750 rpm; ethanol concentration, 10 %, v/v).

Figure 4. Effect of ethanol concentration on extraction efficiency of impurities in the industrial ethanol sample. (SPME conditions: 85 μ m Carboxen/PDMS fiber; headspace extraction; extraction time, 30 s extraction temperature, 35 $^{\circ}$ C; agitation time, 10 min; agitation speed, 850 rpm)

Figure 5. Effect of extraction time on extraction efficiency of impurities in the industrial ethanol sample. (SPME conditions: 85 μ m Carboxen/PDMS fiber; headspace extraction; extraction temperature, 40 $^{\circ}$ C; agitation time, 10 min; agitation speed, 750 rpm; ethanol dilution ratio, 10%)

Figure 6. Effect of NaCl addition on extraction efficiency of impurities in the industrial ethanol sample (SPME conditions: 85 μ m Carboxen/PDMS fiber; headspace extraction; extraction time, 30 s extraction temperature, 35 $^{\circ}$ C; agitation time, 10 min; agitation speed, 850 rpm; ethanol concentration 10%.)

Table 1. Parameters of calibration curves and MDLs for volatile by-products in the ethanol sample

Compound	Equation of calibration curve	Correlation coefficient	RSD range (%)	MDL (mol/L)
Acetaldehyde	$y = 6.79 \times 10^7 x + 2.62 \times 10^5$	0.99	0.52 to 6.35	9.5×10^{-4}
Ethyl vinyl ether	$y = 9.68 \times 10^8 x + 5.04 \times 10^4$	0.99	1.18 to 5.26	2.2×10^{-5}
1,1-Diethoxyethane	$y = 9.94 \times 10^8 x + 4.69 \times 10^5$	0.93	0.25 to 7.71	2.6×10^{-4}
Isoamyl alcohol	$y = 1.30 \times 10^8 x + 4.15 \times 10^4$	1.00	1.03 to 3.20	8.2×10^{-5}
Isoamyl acetate	$y = 1.90 \times 10^{10} x + 1.31 \times 10^6$	0.98	0.63 to 5.12	1.4×10^{-6}
Styrene	$y = 1.82 \times 10^{11} x - 2.71 \times 10^5$	1.00	2.53 to 9.87	9.7×10^{-8}
2-Pentylfuran	$y = 2.65 \times 10^{11} x - 3.44 \times 10^5$	1.00	0.10 to 2.33	3.8×10^{-7}
Ethyl hexanoate	$y = 4.84 \times 10^{10} x - 1.45 \times 10^4$	1.00	0.18 to 3.33	2.4×10^{-7}
Ethyl octanoate	$y = 1.38 \times 10^{11} x - 1.10 \times 10^6$	1.00	0.35 to 8.34	9.1×10^{-7}
Ethyl decanoate	$y = 1.19 \times 10^{11} x - 2.69 \times 10^6$	0.97	0.10 to 11.96	1.6×10^{-6}

(Concentration range from 1.70×10^{-6} to 8.52×10^{-2} mol/L; headspace SPME extraction; x = concentration (mol/L); y = MS detector response)

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Table 2. Concentrations of volatile by-products in the industrial ethanol determined by SPME-GC-MS

Compound	Concentration (mol/L)	RSD (%)
Acetaldehyde	4.83×10^{-2}	2.29
Ethyl vinyl ether	4.55×10^{-5}	3.41
1,1-Diethoxyethane	1.99×10^{-3}	0.04
Isoamyl alcohol	7.31×10^{-2}	0.66
Isoamyl acetate	1.14×10^{-5}	2.06
Styrene	2.44×10^{-6}	1.15
2-Pentylfuran	1.86×10^{-6}	0.62
Ethyl hexanoate	8.14×10^{-6}	2.01
Ethyl octanoate	2.89×10^{-5}	0.06
Ethyl decanoate	1.01×10^{-4}	1.70

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