

Nontargeted nuclear magnetic resonance (NMR) analysis to detect hazardous substances including methanol in unrecorded alcohol from Novosibirsk, Russia

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Nuclear magnetic resonance (NMR) spectroscopy was applied to the analysis of alcoholic products in the context of health and safety control. A total of 86 samples of unrecorded alcohol were collected in Novosibirsk and nearby cities in Russia. Sampling was based on interviews with alcohol dependent patients, and unrecorded alcohol thus defined included illegally or informally produced alcoholic products (e.g., counterfeit or home-made alcoholic beverages) or surrogate alcohol in the form of cosmetic or medicinal products and industrial non-beverage alcohol such as antifreeze (antifreeze windshield washer fluid). For sample preparation, addition of buffer and of a water/ethanol mixture was required as single step. To detect potentially harmful samples, a nontargeted approach based on principal component analysis (PCA) was applied. The PCA scores plot shows six conspicuous samples with highly divergent scores from the rest. These samples are antifreeze windshield washer fluids containing high amounts of methanol, with concentrations in a range between 7% and 48% vol. The antifreeze products were bought in regular retail sale and were claimed as “not containing methanol” on five out of six labels. Additionally, formic acid (1.1%) was observed in four of the alcohol-containing medicinal products. The major advantage of NMR over conventional methods is the fact that it not only provides the same quantitative data for specific compounds, but also allows rapid nontargeted screening for unknown contaminants.

Introduction

The increasing use of nuclear magnetic resonance (NMR) spectroscopy for the analysis of beverages is attributed to the ability of the technique to selectively determine individual components in complex mixtures and providing much richer chemical information than conventional analyses^{1,2}. The technique has gained popularity in the screening of beverages for components for authentication purposes³, as well as routine analysis and rapid detection of potentially unsafe substances⁴⁻⁷. As a result of the minimal sample preparation and the short analysis time, the sample output is considerably increased compared to other approaches^{1,8-10}.

The consumption of surrogate alcohol, a subgroup of unrecorded alcohol, continues to endanger public health in Russia¹¹, mainly because of high ethanol content and lower prices compared to recorded alcohol. Surrogate alcohol is officially not intended for human consumption, but is consumed as a surrogate for alcoholic beverages, often by people with a low socioeconomic status and alcohol dependence¹¹⁻¹⁴. The World Health Organisation (WHO) estimated that out of the 15.1 L total adult per capita consumption of pure alcohol in the Russian Federation, 3.6 L were unrecorded (24%)¹⁵. Other estimates were considerably higher¹¹.

Previous studies conducted on surrogate alcohol from Russia detected high levels of disinfectants and denaturing agents in some samples, which pose an additional risk beyond ethanol^{16,17}. The aim of this research was to use principal component analysis (PCA) of NMR spectral data to rapidly detect potentially dangerous and harmful substances in surrogate alcohol samples obtained from Novosibirsk and surroundings.

Materials and methods

A total of 86 samples consisting of different types of unrecorded alcohols were collected from typical sale locations of unrecorded alcohol, such as kiosks, taxi cabs, pharmacies or private homes in Russia in the context of an international project designed to explore drinking patterns and quality of unrecorded alcohol and their consequences for the consumer's health. The samples included home-made beverages, food flavouring agents based on ethanol, smuggled alcohol, alleged counterfeits, obvious counterfeits, medicinal and cosmetic products containing alcohol as well as diluted non-beverage alcohol such as antifreeze washer fluids, which may also be drunk by people of low socioeconomic status¹⁸.

Samples for NMR spectroscopy were prepared by diluting 300 µl of the beverage with 240 µl of a water/ethanol mixture (190/50)

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and 60 μl of NMR buffer (pH 7.45, 1.5M KH_2PO_4 in D_2O , 0.1% 3-(trimethylsilyl)-propionate acid- d_4 (TSP), 3mM NaN_3) before measurement on a Bruker Avance 400 Ultrashield spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5-mm SEI probe with Z-gradient coils using a Bruker Automatic Sample Changer (Sample Xpress). The spectra were automatically acquired at 300.0K under the control of ICON-NMR (Bruker BioSpin, Rheinstetten, Germany), requiring about 25 min per sample. A suppression of eight frequencies (around 4.70ppm for OH-group signal for both water and ethanol, 3.66ppm for ethanol CH_2 quartet, and 1.18ppm for the corresponding ethanol CH_3 triplet) was conducted. The measurement protocol was previously described in detail^{8,9}. All ^1H -NMR spectra were automatically phase and baseline corrected using the Topspin 3.2 software package (Bruker BioSpin, Rheinstetten, Germany).

Principal component analysis (PCA) was performed using the Unscrambler X software (CAMO Software AS, Oslo, Norway). Bucketing of spectra data was done with Amix 3.9.14 (Bruker BioSpin, Rheinstetten, Germany) with a 0.01 ppm width as previously described for alcoholic beverages³. The chemical shift regions of -0.5 to 0.3, 1.0 to 1.39, 3.43 to 3.87 and 4.78 to 5.07 ppm containing TSP, ethanol and the water signals, respectively, were excluded. Validation of the PCA model was done by full cross validation with 20 segments, each segment containing three samples.

Results and discussion

Nontargeted and targeted analysis of unrecorded alcohol samples

The scores plot of the PCA with all samples shows a clear grouping of six outliers (Figure 1). The highest PC1 values on the scores plot are attributable to six antifreeze products. Examination of the loadings plots (Figure 2) and corresponding NMR spectra shows that only the methanol signal at 3.36ppm has significant influence on the PC1 score. After the methanol-containing antifreeze samples are eliminated as outliers from the PCA analysis, the scatter plot of

the remaining unrecorded alcohols gives another definite cluster of divergent samples (Figure 3). As depicted by the loadings plot of PC2 (Figure 4), the score of PC2 is primarily negatively determined by two NMR signals at 8.15ppm (ethyl formate) and 8.48ppm (formic acid). All four medicinal alcohols, containing formic acid as labeled, are grouped with a high PC2 value. The loadings plot of PC2 indicates that the value of PC2 is determined by the amount of formic acid in the samples.

Following the non-targeted PCA analysis, quantification of the samples for the identified compounds methanol, formic acid and ethyl formate was conducted by use of the internal standard TSP using the signals at 3.36, 8.15 and 8.48ppm for methanol, formic acid and ethyl formate, respectively. The quantitative results are separately discussed in the following sections.

Methanol in antifreeze samples

The antifreeze compounds were found to contain 7% vol, 26% vol, 26% vol, 27% vol, 28% vol, and 48% vol methanol. The content of methanol in these antifreeze samples was alarmingly high and exceeded the maximum tolerable concentration (MTC) of methanol of 2% vol in all cases¹⁹. There was no label information about methanol as an ingredient in any of the six products, and five of them claimed to "not containing methanol" on the labels. This poses a particular risk to the consumer, who may be exposed to such products accidentally because of lack of information on the label or because of incorrect and misleading labelling of the products. The incorrect and deceptive labelling is perhaps to avoid scrutiny by the Russian authorities since the use of methanol in antifreeze has been banned since 2000 in Russia¹⁸. Such alcohol products continue to pose considerable threat to the health of consumers of surrogate alcohol, a majority of whom belong to low socio-economic strata, including but not limited to marginalized groups such as people with alcohol dependence, who may be attracted to these products because of their low cost^{11,18}. This is corroborated by a recent study highlighting the widespread consumption of cheap unrecorded

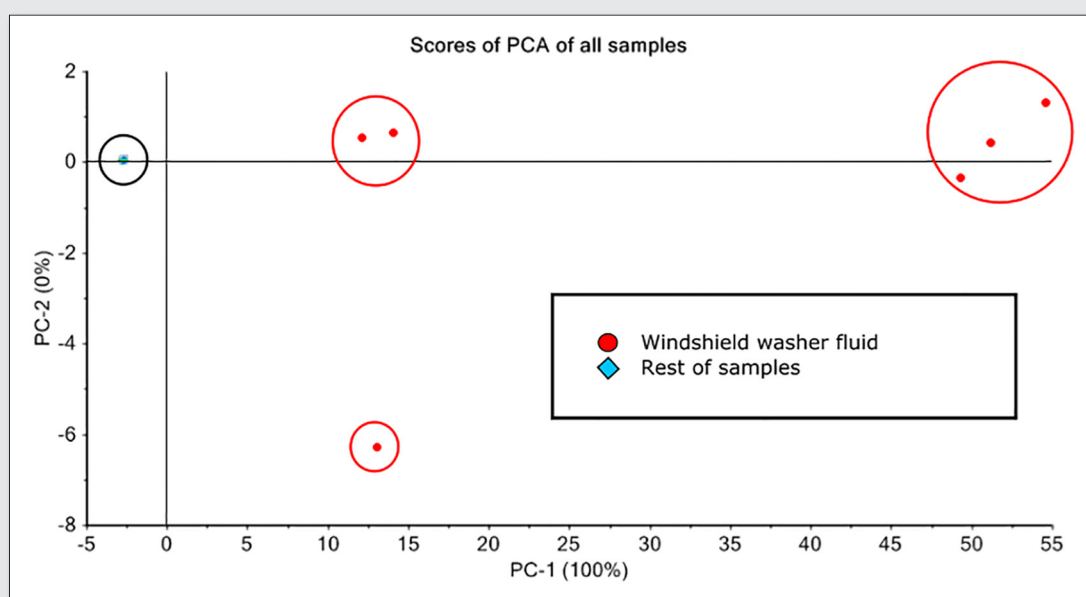


Figure 1. PCA scores plot of all measured samples. All unrecorded alcohols containing methanol (red circles) show a different PC-1 value compared to the rest of the samples (black circle, including vodka, homemade alcohol, cosmetic alcohol, medicinal alcohol, beer, food flavouring agent, counterfeited alcohol). (Note: the scores of the rest of the samples in the green circle are so similar that individual samples cannot be distinguished in this visualization).

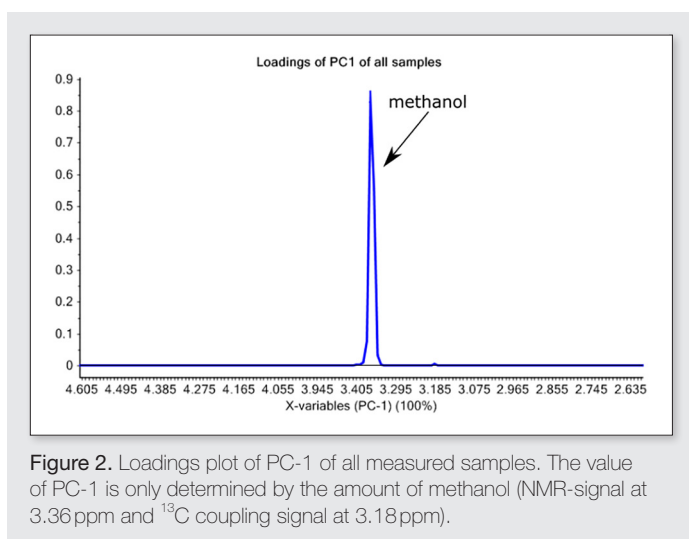


Figure 2. Loadings plot of PC-1 of all measured samples. The value of PC-1 is only determined by the amount of methanol (NMR-signal at 3.36 ppm and ^{13}C coupling signal at 3.18 ppm).

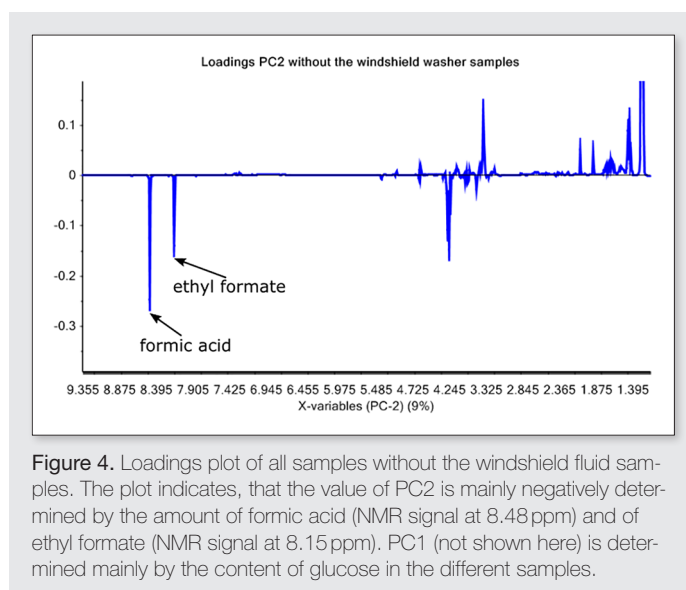


Figure 4. Loadings plot of all samples without the windshield fluid samples. The plot indicates, that the value of PC2 is mainly negatively determined by the amount of formic acid (NMR signal at 8.48 ppm) and of ethyl formate (NMR signal at 8.15 ppm). PC1 (not shown here) is determined mainly by the content of glucose in the different samples.

beverages such as medicinal or cosmetic alcoholic tinctures by marginalized groups¹⁸.

Formic acid in medicinal surrogates

The amounts of formic acid and ethyl formate were detected at 1.1 g/100g and 0.5 g/100g, respectively. The four samples, containing formic acid, are medicinal tinctures called "Formic alcohol" and are meant for medicinal purposes (e.g. as local antiseptic and claimed for treatment of myositis, neuralgia and specific types of arthritis). However, due to its cheap price and relatively large vessel size, formic alcohol is also frequently consumed as surrogate alcohol by alcohol dependent individuals. While methanol has been a common element occasionally occurring in unrecorded alcohol and leading to a number of documented poisoning cases (see review in Lachenmeier et al.²⁰), formic acid so far has never before been appreciated as potential constituent of unrecorded alcohol. Formic acid is an oxidation product of methanol, while ethyl formate is a

flavor compound of rum and raspberry²¹ that arises from the esterification of formic acid in excess ethanol, a reaction favored by the high content of ethanol in the medicinal products. The use of formic acid (E 236) as food additive has been banned in the European Union (EU) since 1998. Formic acid is also not currently approved in any medicinal product in the EU, while it has been formerly applied in hair tonics to stimulate hair growth (5-10% formic acid solution), especially in *alopecia areata*²². The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has suggested a group acceptable daily intake (ADI) for formic acid and ethyl formate of 3 mg/kg bodyweight per day²³. For a 60 kg person, this would be 180 mg per day. For the surrogate alcohol containing 1.4 g/100g (sum of formic acid and ethyl formate, expressed as formic acid), approximately 13 g (about 1/4 of a bottle) need to be consumed to exceed the ADI. It should be noted that the JECFA ADI is conservatively estimated using safety factors of 100, so that acute toxic effects

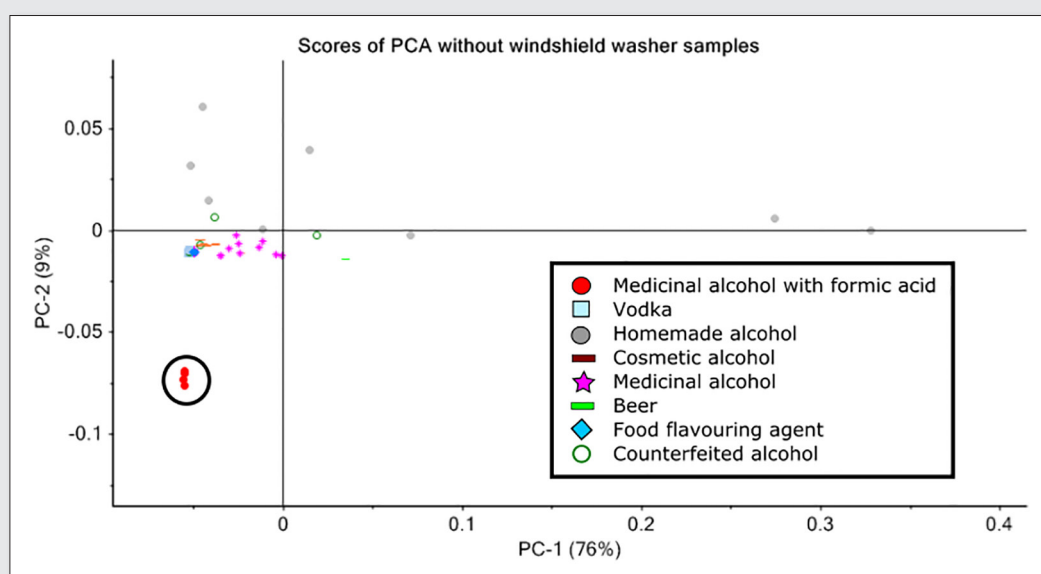


Figure 3. Scores plot of all samples without the six windshield fluid samples. All four samples, containing formic acid and ethyl formate as labelled, shows the same behaviour in their PC-2 value.

are not expected even at this level of intake. Serious formic acid poisoning (reversible intravascular coagulation and acute renal failure) was only observed following intake of 30g of formic acid or more, and high mortality (14 out of 15 patients) was recorded for ingestion of 45 to 200g of formic acid²⁴. These levels of intake cannot be reached with the alcohol surrogates. Chronic effects may be the exacerbation of the effects of ethanol contributing to metabolic acidosis, which appears to be a common denominator for consumers of surrogate alcohol. For example, we have previously determined that consumption of mouthwash may contribute to metabolic acidosis due to methyl salicylate contents²⁵. For this reason, we believe that the benefits of medicinal products containing formic acid (and ethyl formate) do not outweigh their risks. Similar to the prohibition of unsafe compounds such as diethyl phthalate (DEP) and polyhexamethyleneguanidine (PHMG) in alcohol-containing disinfectants due to the risk of human ingestion²⁶, we suggest that regulators in Russia should reassess the safety and efficacy of medicinal products containing formic acid and potentially phase-out their marketing authorisation or at least reduce the approved bottle sizes or increase their price.

Conclusion

NMR-spectroscopy was found to be a suitable technique for rapid nontargeted analysis of a large number of spirit samples. A similar study conducted in 2010 on surrogate alcohol samples from Russia utilizing multivariate data analysis detected two samples out of 22 to contain the toxic denaturant diethyl phthalate (DEP) and the disinfectant polyhexamethyleneguanidine (PHMG)¹⁶. In contrast, in this study methanol in windshield fluid samples and formic acid in medicinal surrogates are detected as hazardous substances. However, none of the current 86 samples contained DEP and PHMG. As all measured antifreeze samples contained considerable amounts of methanol, which is a banned substance for antifreeze in Russia, quality control of corresponding products is strongly advised. The current findings reveal that surrogate alcohol continues to pose significant hazards to the lives of problem drinkers and alcohol dependent people from low socio-economic strata in Russia.

With the possibility of simultaneous analysis of a large number of samples, the PCA approach is highly effective in the field of industrial analysis and quality control. The combination of NMR and PCA for data analysis is therefore a fast and easy-to-implement method for the investigation of a large number of unknown components within a certain concentration in alcohol samples without *a priori* knowledge of their chemical structure. Although infrared spectroscopy as an alternative may be less time consuming, NMR spectra show less overlap of signals and achieve improved identification based on specific chemical shifts and coupling patterns. Due to the minimal sample preparation effort and the possibility of multiple solvent suppression of ethanol and water signals, NMR spectroscopy is the method of choice in the field of nontargeted analysis of potentially hazardous substances in alcoholic beverages. The technique will be hopefully applied by governmental and industrial quality control institutions in the future to increase consumer protection.

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