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Alcohol Determination in Protein Fractionation Intermediates by Steam Distillation and Digital Refractometry

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Abstract

Almost 75 years after implementing the industrial ethanol fractionation process, based on the pioneering work of Edwin J. Cohn's research group, this niche biotechnology process has not lost its importance in helping to supply patients with life-saving biotherapies. Clearly, the focus has shifted from albumin, which was first used, to the indispensable immunoglobulin preparations produced for the effective, long-term treatment of patients suffering from immunodeficiencies. In addition, the widespread and safe therapeutic use of immunoglobulins has paved the way for the development of monoclonal antibodies, now used not only for the treatment of various autoimmune diseases, but also for cancer treatment. The Cohn fractionation process, based on the different solubilities of plasma proteins, depends on the five parameters of ethanol concentration, pH, temperature, protein, and salt concentration, which are the basis of this development. Ethanol concentration can clearly be considered an essential critical parameter for this process. Therefore, it is surprising that even after the advent of process analytical technology, there is still no fast, precise, and accurate procedure at hand to determine the alcohol content of Cohn fractionation intermediates. In this paper, we will describe the implementation of an old methodology for a new purpose, which is designed to close this gap.

Introduction

Alcohol precipitation still constitutes the backbone of blood plasma processing. Only the precipitates may be purified further with advanced techniques like biochromatography. However, their composition will depend on the content of alcohol added, which then could be considered to be the essentially critical parameter. Even 75 years after Edwin J. Cohn *et al*. published their work on the development of industrial plasma fractionation[1], there is still no fast, precise, and accurate procedure at hand to determine the alcohol content of the respective Cohn fractionation intermediates. If we look outside the biotech box and into the analytical tool kit of alcoholic beverage processing, we are transported to the heydays of refractometry a century ago. Steam distillation with gravimetric sample dosage and refractometric alcohol measurement seizes on Cohn's initial refractometric concept and speeds it up to an in-process technique. This paper focuses on the fundamental analytical parameters of accuracy (recovery), resolution, and precision, as well as the applicability on pure ethanol and denatured alcohols. It is primarily demonstrated on model fractions with known compositions but can be applied to plasma fractionation method scouting.

Alcohol:
Not just the historical reagent of choice in protein fractionation

While alcohol precipitation at reduced temperatures was already established for the processing of immune sera before any industrial-scale plasma fractionation[2], the advantages of ethanol or denatured alcohols were particularly welcomed after the technology of freeze-drying was introduced.[1,3] Ethanol, readily available from industrial fermentation and distillation, was well-characterized in its physicochemical properties. By vacuum sublimation in the cold, this volatile solvent could then easily be removed from any precipitate, even without an ultra- or diafiltration step.

Recently, alcohol precipitation has been proposed for the downstream processing of monoclonal antibodies (MAbs) with ethanol, methanol, and 2-propanol as potential precipitants.[4] For plasma, the target alcohol concentration ranges from 8–40% by volume, while for MAbs, 25% was set as established for the intramuscular IgG precipitate from plasma.[3]

Alcohol Quantitation in (Plasma) Protein Fractionation and Processing:
A neglected point?

Published data based on actual verification of the alcohol content of plasma fractions are surprisingly scarce. Only in his fundamental publication did Edwin J. Cohn mention the quantitation of the added ethanol content by a laboratory-scale vacuum sublimation-condensation technique using a cold trap.[2] Once the condensate was obtained, the ethanol content (by volume) was then measured with an immersion

refractometer (**Table 1**).[5,6] The reference temperature was defined as 25 °C, even though the actual processing temperatures needed to be kept at sub-zero to avoid denaturation.

Alcoholometry and Alcohol Determination:

Not a straightforward solution

For alcoholic beverages, distillation and density measurement constitute the analytical reference methods.[7] Usually the sample is defined by its volume, and an equivalent volume of distillate must be obtained. The alcohol content in the latter can then be measured through the density[8] by a graduated areometer immersed into the liquid, a calibrated pycnometer filled with the liquid and weighed on a balance, or in an oscillating tube/digital density meter. For all such volume and weight-based procedures, an accurate thermostatization or temperature correction is essential. Furthermore, the respective reference temperature may depend on national legislation, as (historically) it is 15°C in France, 15.56°C (60°F) in the USA, and 20°C in the European Union.

The sample volume and the processing time required are considerable: 100–200 mL must be distilled to enable the use of an areometer in a volumetric flask, which takes at least 30 minutes. The specified accuracy may depend on the respective class of beverages. Those produced by fermentation (*e.g.*, beer and wine) may be biased by volatile “congeners” in the distillate or spirit such as methanol, aldehydes, fusel oils, and esters. Grain spirits and spirit liqueurs will contain virtually pure ethanol from the main distillation fraction without “heads” and “tails.”

In cream and emulsion liqueurs (*e.g.*, egg liqueur or eggnog), the matrix, which is rich in sugar, protein, fat, and emulsifiers, poses a special challenge as any local overheating, flocculation, and “burn-in” of the solids must be avoided.[9,10] Such materials can be distilled only very slowly. Plasma fractions resemble the egg liqueurs (in their protein content) and wine (in the presence of volatile acetic acid). The latter must be neutralized through the addition of mild alkali because NaOH or suspended quicklime added to wine would favor the degradation of protein and the generation of ammonia. In practice, the high sample consumption and considerable sample processing time will rule out any direct distillation technique for alcohol determination in plasma fractionation intermediates.

Steam Distillation with Gravimetric Dosage:

Circumventing the direct shortcomings

Originally introduced in the late 1920s for the determination of alcohol in forensic samples[11], analytical steam distillation was proposed for alcohol, volatile acids, or ammonia in wine in 1950[12], but apparently gained further attention only about 20 years ago for emulsion liqueurs.[9,10]

At (theoretically) constant sample volume, the distribution coefficient of a (highly diluted) substance between steam and liquid, termed the “water vapor number” C[13], determines the efficiency in the distilled analyte yield y for quantitative recovery and the necessary distillate volume z (1 = to the sample volume) according to the equation (1) as:

Equation (1): $$\ln(1-y) = -Cz$$

The yield y may thus be calculated as:

Equation (2): $$y = 1 - e^{-Cz}$$

For ethanol ($C \sim 12$), a yield of >99.999 % will be theoretically obtained at an equivalent distillate volume. In contrast, acetic acid ($C \sim 0.65$) would require a 10-fold distillate volume. (In practice, steam will condense in the sample volume during the initial heat-up, and then C gradually increases only to the theoretical value.)

A gravimetric dosage of both the sample and the distillate (in g rather than mL)[14] avoids thermostatization, improves the overall measurement accuracy, and reduces the sample consumption to ~ ≤25 g with a distillation time of only a few minutes. Measuring the sample density may still be necessary to calculate the sample volume and the alcohol content by volume. The distillate’s alcohol content (by weight) and the total alcohol weight in the sample will be measurable either through the density or the refractive index, which both constitute intrinsic properties.

A Bitter Spirit of Invention?

Alcohol, specially denatured for the general public‘s health

Considerable taxes are usually charged on undenatured ethanol, and even the reclaiming of spent ethanol through distillation may be practiced only under the scrutiny of taxation

TABLE 1. Selected physicochemical characteristics of key intermediates in the Cohn fractionation process.

% Ethanol (v/v) (25 °C)	Mole Fraction	Refractive Index n_D^{25}	% Ethanol (w/w)	Cohn Fraction	Protein Content (g/L)	pH	Temperature (°C)
8.0	0.0266	1.3366	6.53	I	51.1	7.2	–3
10.0	0.0328	1.3377	7.98	N/A	N/A	N/A	N/A
15.0	0.0509	1.3405	12.06	N/A	N/A	N/A	N/A
18.0	0.0624	1.3422	14.54	IV–1	15.8	5.2	–5
25.0	0.0907	1.3463	20.32	II	N/A	N/A	–5
				II + III	30.1	6.8	
40.0	0.1630	1.3537	33.24	IV – 4	10.1	5.8	–5
				V	7.5	4.8	
53.3	0.2462	1.3583	45.51	N/A	N/A	N/A	N/A

authorities. Denatured alcohol, rendered unfit for human consumption (and abuse), can be a substitute for ethanol in plasma fractionation without any deleterious influence on the product, as long as the denaturant can be removed without any residue.[15] The toxic methanol may now be considered a "legacy" additive in the "specially denatured alcohol" (SDA)-3A (or 30) in the USA, as far less toxic denaturants such as 2-propanol and methyl ethyl ketone (MEK) will ensure the safe handling, usage, and reclaiming of such fractionation alcohols in the same way (**Table 2**). Density and refractive index calibrations need to be established specifically for every such denatured alcohol.

In the Wake of Steam:
It may not be just alcohol alone coming along

Denaturants added to ethanol should not be separable by distillation. For the steam distillation of fractionation alcohol, this peculiar property is essential for a quantitative "entrainment" and recovery from the samples. The other volatile additive, acetic acid for pH adjustment, must not pass into the distillate at any amount (**Table 3**). Of note, upon prolonged storage, esters from alcohols and acetic acid will build up in the form of an opalescent distillate. This results in a possible bias in the alcohol content because such esters can no longer be neutralized. Other than for wine, strongly alkaline additives must be avoided for protein solutions. As denaturing proteins tend to exhibit excessive frothing and foaming from off-boiling alcohol and bubbling steam, a highly effective antifoaming agent must be employed to break down foaming and suppress any spillover from the bubbler vessel.

The Ideal Steam Still for Bioprocess Samples?
Size matters — but smaller is better

In conjunction with the gravimetric technique, refractometry requires only a small sample volume, even smaller than digital density measurement, so that a few mL of distillate should

TABLE 2. Properties of commonly used denaturants for fractionation alcohol.

Denaturant	Boiling Point[a] (°C)	Toxicity	Absorbance at 280 nm	Formula	Additive Ratio (v+v)	Azeotrope Boiling Point (°C)
Methanol	64.5	High	No	SDA-3A[b]	5+100	No
				SDA-30[b]	10+100	
2-propanol	82.2	Moderate	No	SDA-3C[b]	5+100	80.4[e]
Acetone	56.1	Moderate	Yes (262 nm)	SDA-23A[b]	8+100	No
MEK	79.6	Moderate	Yes (274 nm)	AT, DE[c]	1+100	Refer to[f]
				CH[d]	1+50	
Diethyl ether	34.4	Moderate	No	SDA-13A[b]	10+100	34.2[e]
				SDA-32[b]	5+100	

REMARKS: (a) Taken from reference[16]. (b) Formula valid for United States. (c) Formula valid for Austria (AT) and Germany (DE). (d) Formula valid for Switzerland (CH). (e) Applies to mixture with water. (f) Azeotrope boiling points: MEK/ethanol/water (73.2°C), MEK/water (73.4°C), and MEK/ethanol (74.8°C).

TABLE 3. Probable volatile compounds present in fractionation alcohols and steam distillates.

Compound	Occurrence/Origin	Refractive Index n_D^{20}[a]	Density ρ^{20} (g/mL)[a]	Boiling Point (°C)[a]	Water Vapor Number C[b]
Ethanol	Base compound	1.36143	0.78924[c]	78.3	12
Methanol	SDA-3A and SDA-30[d]	1.32840	0.79104	64.5	8
2-propanol	SDA-3C[d]	1.37720	0.78545	82.2	N/A
Acetone	SDA-23A[d]	1.35868	0.78998	56.1	N/A
MEK	Denaturant[e]	1.37880	0.80490	79.6	N/A
Diethyl ether	SDA-13A and SDA-32[d]	1.35243	0.71361	34.4	N/A
Methyl acetate	Esterification upon storage	1.36140	0.93420	56.9	N/A
Ethyl acetate	Esterification upon storage	1.37239	0.90063	77.1	N/A
2-propyl acetate	Esterification upon storage	1.37730	0.87730	88.6	N/A
Acetic acid	pH adjustment additive	1.37190	1.04955	117.9	0.65
Ammonia	Alkaline protein digestion	N/A	N/A	N/A	N/A

REMARKS: (a) Taken from reference[16]. (b) Taken from reference[13]. (c) The OIML (International Organization of Legal Metrology) value. (d) Formula valid for United States. (e) Formula valid for Austria, Germany, and Switzerland.

suffice. The commonly applied steam distillation apparatus derived from Kjeldahl instrumentation have all been optimized for a high vapor generation rate and a short distillation time, but cannot handle sample volumes below 25 mL, as these would result in an insufficient alcohol recovery. Furthermore, both for commercial in-process or quality controls, and even more for downscaled pilot or laboratory fractionation runs, the sample volume should remain as small as possible.

The semi-miniaturized Polydest steam still (Gravitech) was originally developed to handle small sample volumes in the analysis of spirits and spirit liqueurs. Dewar bubbler vessels are available in sizes ranging from 100 to 250 mL. The standard 250 mL size can only hold about 25 mL of protein-containing matter. The remaining headroom should accommodate the intermittent buildup of foam and minimize any potential spill-over into the bubble trap. Adding an effective anti-foam suspension will help to suppress frothing and foaming.

The current version of the Polydest steam still is time-controlled. Of the two heating rods in the steam reservoir bottle, one keeps the water hot while the other is switched on for the set boiling time to generate the steam. With the standard 250 mL bubbler vessel, about 15–25 g of sample can be processed. The higher the alcohol content, the lower the sample weight necessary. The combined Allihn-Graham type of cooler avoids any backing up of the distillate, which may be collected on an ice bath to dissipate any mixing heat upon the dilution of the alcohol. (To prevent users from coming into physical contact with steaming hot surfaces, it is advised that a protective shield be constructed for the respective steam still components, particularly the reservoir bottle and the vapor conduits.)

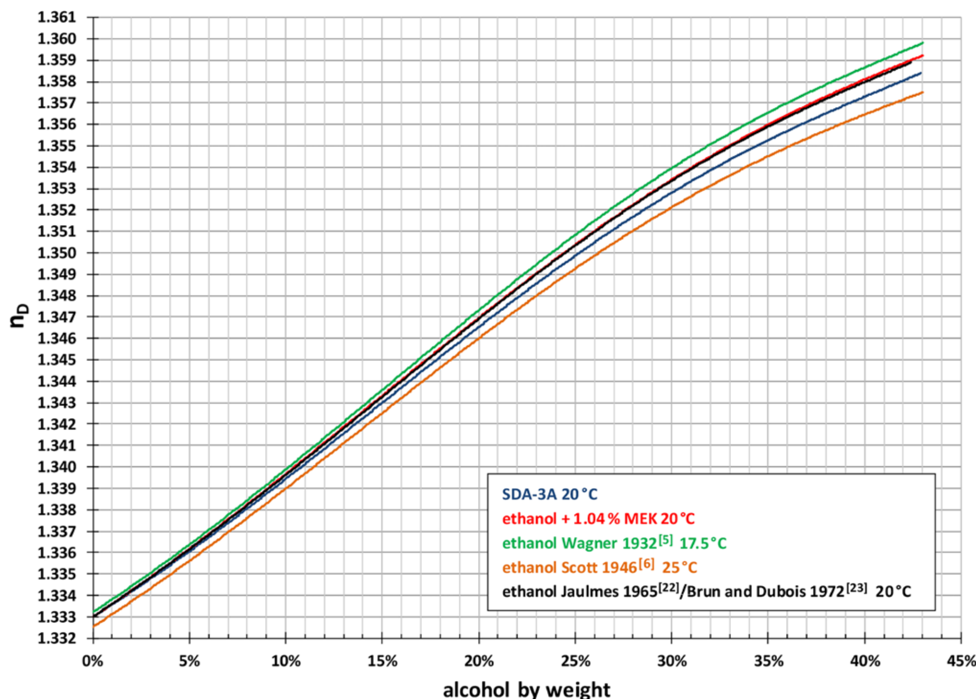

FIGURE 1. Refractive indices of ethanol/water and denatured alcohol/water mixtures. The concentration-dependent increase of the refractive index is shown for ethanol at three reference temperatures (17.5, 20, and 25 °C) from literature data.

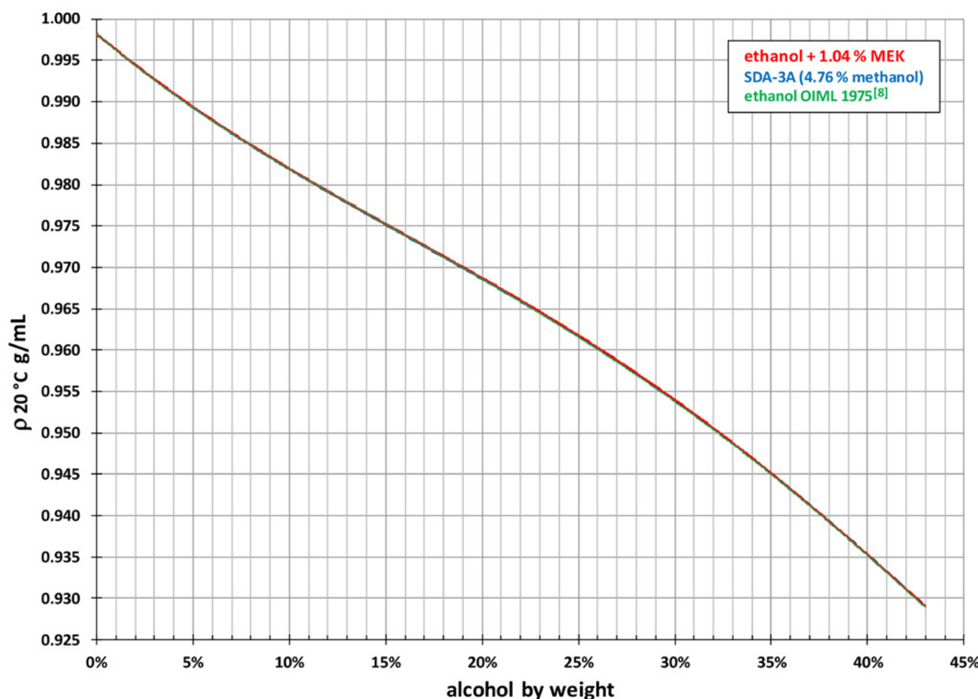

FIGURE 2. Densities of ethanol/water and denatured alcohol/water mixtures. The concentration-dependent density decrease is shown at the reference temperature of 20 °C.

Materials and Methods

Fractionation alcohols were mixed from anhydrous reagents (ethanol, methanol, MEK). For calibration, alcohol/water mixtures were prepared by weighing. Refractive indices (17.5 °C for ethanol, 20 °C for other alcohols) and densities (20 °C) were measured with a Rudolph Research J157 or J357 refractometer and a DDM2911 density meter (Rudolph Research Analytical) respectively (**Figures 1 and 2**). The refractive index calibration plots were segmented and approximated by second-order polynomial regression to enable an automated calculation of the alcohol content in the distillate.

Surrogate samples mimicking the respective Cohn fractions (**Table 4**) were prepared by weighing the respective alcohol with either pure components (*i.e.*, water, NaCl, [unstabilized] albumin concentrate [~ 26% protein], and IgG concentrate [10%]), or with the plasma intermediate cryo/DEAE supernatant[17] on a precision balance to an accuracy of 0.001 g (**Table 5**). The protein content of the Cohn fractions was determined by permeate-corrected refractometry[18] calibrated on Dumas or Kjeldahl measurements.[19,20] Precipitate pastes were simulated by mixing low-fat (<0.5%) cottage cheese (14% protein, 5% carbohydrate) with 96% (v/v) ethanol (94.26% w/v as determined by density measurement[8]) on an ice bath (**Table 6**).

TABLE 4. Characteristics of Cohn fractionation surrogate samples I (protein matrix purified albumin and IgG).

Fraction	Protein (≈ g/L)	Kjeldahl Factor	Protein dn/dc (mg/mL)[a]	% Ethanol (target w/w)	Addition of Acetic Acid
Plasma (thawed/pooled)	55	6.25	0.000194	None	None
Cryo/DEAE supernatant	53	6.54	0.000195	None	None
Cohn I supernatant	47	6.54	0.000193	6.5	pH adjusted to >7.0
Cohn II + III supernatant	29	6.25	0.000195	17	pH adjusted to 6.0–7.0
Cohn IV–1 supernatant	24	6.25	0.000186	17	pH adjusted to 4.8–6.0
Cohn IV–4 supernatant	13	6.25	0.000175	33	pH adjusted to 4.8–6.0
Fraction V suspension	73	6.25	0.000185	≈7–12	pH adjusted to 4.8–6.0
Fraction V depth filtrate	63	6.25	0.000184	≈7–12	pH adjusted to 4.8–6.0

TABLE 5. Composition of Cohn fractionation surrogate samples II (protein matrix plasma intermediate).

Surrogate Sample	Net Weight (g)							% Alcohol (w/w)
	NaCl	Albumin	IgG	Cryo/DEAE[a]	Water	Alcohol	Total	
Crude Cohn I low, ethanol	N/A	N/A	N/A	50.524	N/A	2.132	52.656	4.05
Pseudo Cohn I, ethanol	1.630	33.834	8.282	N/A	143.894	14.000	200.010	7.00
Crude Cohn I high, ethanol	N/A	N/A	N/A	98.770	N/A	8.003	106.773	7.50
Pseudo Cohn II + III, ethanol	1.185	26.576	N/A	N/A	138.229	34.010	200.000	17.01
Pseudo Cohn IV–4, ethanol	N/A	9.360	N/A	N/A	122.660	67.970	199.990	33.99
Pseudo Cohn V suspension, ethanol	N/A	57.758	N/A	N/A	124.252	18.020	200.030	9.01
Crude Cohn I low, ethanol + MEK	N/A	N/A	N/A	98.760	N/A	6.865	105.625	6.50
Crude Cohn I high, ethanol + MEK	N/A	N/A	N/A	98.620	N/A	7.458	106.078	7.03
Pseudo Cohn II + III, ethanol + MEK	1.182	26.753	N/A	N/A	138.045	34.010	199.990	17.01
Pseudo Cohn IV–4, ethanol + MEK	N/A	10.561	N/A	N/A	121.409	68.050	200.020	34.02
Pseudo Cohn IV–4 high, ethanol + MEK	N/A	2.528	N/A	N/A	28.468	19.004	50.000	38.01
Crude Cohn I low, SDA-3A	N/A	N/A	N/A	99.870	N/A	6.713	106.583	6.30
Crude Cohn I high, SDA-3A	N/A	N/A	N/A	99.510	N/A	7.269	106.779	6.81
Pseudo Cohn II + III, SDA-3A	1.173	26.756	N/A	N/A	138.061	34.019	200.009	17.01
Pseudo Cohn IV–4, SDA-3A	N/A	10.567	N/A	N/A	121.463	67.980	200.010	34.00

REMARKS: (a) Taken from reference[17].

TABLE 6. Preparation of paste surrogate samples.

Paste Type	Net Weight (g)			Appearance	% Alcohol (w/w)
	Cottage Cheese	96% Ethanol	Total		
Low alcohol	191.45	8.49	199.94	Paste	4.00
Medium alcohol	170.09	29.70	199.79	Sludge	14.01
High alcohol	140.63	59.41	200.04	Slurry	27.99

FIGURE 3. The semi-miniaturized Polydest steam still (with protective shield) is shown in the laboratory setting.

Depending on the alcohol content, 15–25 g of the respective samples were distilled in a Polydest steam still (**Figure 3**) at a steam generation rate equivalent to about 10 mL water/min to obtain an about 2.5 to 4-fold amount of distillate. Before distillation, about 0.5 g of solid $NaHCO_3$ was added to neutralize acetic acid, and depending on the protein load, 1–3 drops of Silicon Antischaum US (C. Schliessmann Kellerei-Chemie GmbH & Co.KG) anti-foam emulsion. The weighed paste surrogate samples were suspended in about double the volume of 0.8 M trisodium citrate/0.8 M $NaHCO_3$ to keep the calcium in suspension.

Results

In the surrogate fractionation intermediates, a quantitative recovery of 99–101% was achieved for all alcohols at all concentrations with high precision (relative standard deviation [RSD] <1%) (**Table 7**). The distillation time had to be adapted to the

TABLE 7. Alcohol concentrations determined for the surrogate fractionation intermediates.

Surrogate Sample	Nominal Alcohol (%)	Alcohol Concentration (%)							RSD (%)	Recovery (%)
		Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Mean		
Crude Cohn I low, ethanol	4.05	4.05	4.08	N/A	N/A	N/A	N/A	4.07	N/A	100.4
Pseudo Cohn I, ethanol	7.00	7.02	7.03	7.05	7.03	7.03	7.01	7.03	0.20	100.4
Crude Cohn I high, ethanol	7.50	7.55	7.56	7.55	N/A	N/A	N/A	7.55	0.06	100.8
Pseudo Cohn II + III, ethanol	17.01	17.07	17.09	17.07	17.10	17.10	17.07	17.08	0.08	100.5
Pseudo Cohn IV–4, ethanol	33.99	34.16	34.12	34.14	34.09	34.07	34.04	34.10	0.12	100.4
Pseudo Cohn V suspension, ethanol	9.01	9.00	9.01	8.99	9.06	9.04	9.03	9.02	0.25	100.2
Crude Cohn I low, ethanol + MEK	6.50	6.56	6.46	6.53	6.48	N/A	N/A	6.51	0.61	100.1
Crude Cohn I high, ethanol + MEK	7.03	6.99	6.98	6.97	6.98	N/A	N/A	6.98	0.08	99.3
Pseudo Cohn II + III, ethanol + MEK	17.01	17.02	17.03	16.95	16.97	16.96	16.95	16.98	0.19	99.9
Pseudo Cohn IV–4, ethanol + MEK	34.02	33.83	33.90	33.91	33.88	34.04	33.96	33.92	0.20	99.7
Pseudo Cohn IV–4 high, ethanol + MEK	38.01	38.00	37.94	38.01	N/A	N/A	N/A	37.98	0.07	99.9
Crude Cohn I low, SDA-3A	6.30	6.35	6.31	6.31	N/A	N/A	N/A	6.33	0.28	100.4
Crude Cohn I high, SDA-3A	6.81	6.82	6.86	6.88	6.81	6.82	N/A	6.86	0.40	100.7
Pseudo Cohn II + III, SDA-3A	17.01	16.99	16.99	16.95	16.99	16.99	16.99	16.98	0.11	99.9
Pseudo Cohn IV–4, SDA-3A	34.00	34.36	33.95	33.96	33.93	33.94	33.91	34.01	0.46	100.0

REMARKS: The percent recovery was calculated according to mean found alcohol concentration/nominal alcohol concentration ×100.

alcohol amount to avoid either an insufficient recovery or an excessive dilution of the distillate to <2% alcohol by weight. Linearity was assessed by increasing the distillate amount with the sample size (**Table 8**) and robustness by keeping the sample amount constant with the variation of distillation time (**Table 9**). The technique was proven to tolerate such variations within a reasonably narrow range. In addition, the whole measurement cycle took less than ten minutes, which qualifies the method as "process analytical technology" because it provided process control data close to real-time. This is especially valuable and essential when changes in the process have to be evaluated. Thus, the method can be seen as an essential asset to be used for additional characterization testing, supporting process performance qualification runs whenever concerned, or as required.

For the paste surrogate samples, the recovery was borderline low (<98%) at the lowest alcohol content and highest protein load, and acceptable to good (≥98%) at the higher alcohol concentrations (**Table 10**). Such protein-rich samples required a generous addition of anti-foaming agent and an ample bubbler headspace to suppress foaming. Of note, the fraction V depth filtrate in **Table 9** reflects a suspended, diluted, and filtered Cohn V paste, suggesting an easy applicability on this late-stage, high-protein fraction.

Two supplemental tables on refractive index versus mass concentration are located on page 9 of this article.

TABLE 8. Linearity investigation where increasing amounts of a given sample were measured and the recovery of alcohol was determined.

Sample Weight (g)	Distillation Time (min:sec)	Distillate Weight (g)	Distillate Refractive Index n_D^{20}	(%) Alcohol in Distillate	(g) Alcohol in Distillate	(%) Alcohol in Sample	Recovery (%)
10.057	4:00	33.49	1.33980	10.18	3.41	33.91	99.68
14.009	5:20	45.78	1.33996	10.41	4.76	34.01	99.97
17.808	6:40	68.39	1.33885	8.85	6.05	33.99	99.89
21.786	8:00	78.91	1.33924	9.40	7.42	34.04	100.06

TABLE 9. Robustness investigation.

Sample Weight (g)	Distillation Time (min:sec)	Distillate Weight (g)	Distillate Refractive Index n_D^{20}	(%) Alcohol in Distillate	(g) Alcohol in Distillate	(%) Alcohol Concentration in Sample
21.280	4:30	38.94	1.33585	4.53	1.77	8.30
21.298	5:00	46.18	1.33538	3.82	1.76	8.28
21.697	5:30	50.51	1.33521	3.56	1.80	8.29
21.296	6:00	57.58	1.33489	3.06	1.76	8.29
21.310	6:30	66.00	1.33464	2.67	1.76	8.28
21.305	7:00	68.68	1.33457	2.56	1.76	8.26
					Mean	8.28
					RSD (%)	0.11

TABLE 10. Recovery results for the paste surrogate samples.

Paste Type	Nominal Alcohol (%)	Alcohol Concentration (%)				RSD (%)	Recovery (%)
		Sample 1	Sample 2	Sample 3	Mean		
Low alcohol	4.00	3.82	3.93	3.94	3.90	1.40	97.42
Medium alcohol	14.01	13.88	13.79	13.75	13.81	0.39	98.55
High alcohol	27.99	27.76	27.78	27.79	27.78	0.04	99.24

Discussion and Conclusion

At all concentrations, a resolution of 0.1% alcohol by weight can be achieved at an absolute accuracy of ±0.1%. The method is equally applicable for both pure ethanol and the common denatured fractionation alcohols. While variations in the denaturant content, as specified, may shift the refractive index, the resulting bias may still be negligible for the highly dilute distillates. Furthermore, the actual volume additive range usually meets the target value fairly well, as verified in-house for MEK in 32 alcohol batches by UV absorbance measurement[21] (data not shown).

For a volumetric approach, optical differential refractometry was shown 40-plus years ago to achieve a resolution of 0.05% alcohol by volume.[22,23] In the present gravimetric technique, the refractometric resolution of 0.00001 n_D would correspond to 0.03–0.05% by weight, depending on the dilution of the distillate. If a (digital) refractometer is not at hand, a digital density meter may deliver the same accuracy and precision.[24,25] However, the measurement cycle will be much slower. Both properties are intrinsic and absolute, as determined by the material composition only, so the gravimetric steam distillation method can be transferred between laboratories without any further recalibration. In conclusion, steam distillation enables a hitherto unmet accuracy that is essential for a reference method in quality control, in-process analysis, and process development and optimization.

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Supplemental Tables

TABLE A. Refractive index versus mass concentration for pure ethanol at 20°C (taken from literature).

% by Weight Ethanol	Refractive Index n_D^{20}
0.00	1.33299
1.11	1.33365
2.19	1.33430
3.21	1.33497
4.16	1.33562
5.18	1.33627
6.13	1.33690
7.05	1.33755
7.97	1.33819
8.88	1.33884
9.77	1.33947
10.67	1.34010
11.52	1.34073
12.41	1.34136
13.28	1.34199
14.13	1.34262
15.00	1.34324
15.85	1.34388
16.71	1.34453
17.56	1.34516
18.41	1.34579
19.24	1.34642
20.20	1.34706
21.18	1.34774
22.14	1.34842
23.13	1.34910
24.14	1.34978
25.21	1.35046
26.26	1.35113
27.33	1.35181
28.19	1.35235
29.37	1.35303
30.57	1.35370
31.84	1.35437
33.22	1.35505
34.62	1.35571
36.10	1.35638
37.63	1.35704
39.26	1.35771
40.60	1.35823
42.42	1.35889

REMARKS: % by weight ethanol values were converted from the given volume % ethanol data based on the tables for 15°C[22] and 20°C[23]. (Tables VIII A and VIII B by K. Windisch and J. Grossfeld, respectively, in: *Handbuch der Lebensmittelchemie [handbook of food chemistry], Vol. 2, part II*, Berlin: Springer 1935.)

TABLE B. Established refractive index versus mass concentration table for methanol-denatured alcohol (SDA-3A) at 20°C.

% by Weight SDA-3A	Refractive Index n_D^{20}
0.00	1.33299
1.05	1.33359
2.05	1.33419
3.05	1.33479
3.96	1.33537
5.09	1.33610
6.00	1.33671
7.05	1.33741
8.08	1.33811
8.97	1.33872
9.88	1.33937
11.01	1.34015
12.04	1.34089
13.13	1.34167
14.16	1.34237
15.22	1.34316
16.00	1.34369
16.98	1.34432
17.91	1.34508
18.97	1.34578
20.10	1.34662
21.13	1.34737
21.94	1.34787
23.05	1.34859
24.07	1.34930
24.98	1.34988
26.82	1.35098
27.11	1.35117
28.23	1.35181
29.06	1.35228
30.10	1.35285
31.05	1.35337
32.11	1.35390
33.05	1.35436
33.90	1.35477
34.90	1.35523
36.03	1.35569
37.03	1.35616
38.06	1.35654
39.00	1.35691
39.92	1.35726
41.00	1.35770
41.90	1.35801
42.90	1.35838