Solvent-free tissue processing using supercritical carbon dioxide

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Aims: Xylene is most often employed in tissue processing protocols for paraffin embedding, but poses a health hazard. The aim of this study was to evaluate a solvent-free processing protocol that uses supercritical carbon dioxide \((\text{scCO}_2)\) as an intermediate.

Methods and Results: A series of tests (with bovine tissues) was run, evaluating dehydration and tissue shrinkage in our new \(\text{scCO}_2\)-based protocol as compared with routine processing using a graded ethanol and xylene series. A series of tests was then run to evaluate the significance of processing parameters for the outcome. Finally, a validation series was performed with optimal conditions, testing various human tissues with several staining methods. The tissue water content after paraffination was the same with our new \(\text{scCO}_2\)-based protocol and the routine xylene-based protocol. Tissue shrinkage was similar with the two methods, at \(\sim 15\%\), which is also similar to values in the literature. In the validation series, the human tissues showed good morphology with strong staining, probably because of stronger antigenicity.

Conclusions: This \(\text{scCO}_2\)-based protocol has been shown to be a good solvent-free, alternative form of tissue processing. Although not the focus of this article, the time needed for tissue processing with this new protocol is within 4 h, and there is no need to change macroscopy/sectioning protocols.

Keywords: dehydration, paraffination, solvent-free, supercritical carbon dioxide, tissue processing, tissue shrinkage, xylene-free

Introduction

Most laboratories still employ xylene in tissue processing protocols for paraffin embedding. Since the late 1970s, there has been accumulating evidence of xylene toxicity.1–3 Therefore, research is focused on developing xylene-free tissue processing.4 In most xylene-free protocols, substitutes such as vegetable oils, terpenes and alkanes are used, with variable success and, sometimes, also with personnel safety concerns.2 In the review by Buesa,2 tissue processing without a clearing agent was referred to as a ‘drastic option’. Here, we want to demonstrate another way of processing tissue without xylene or other solvents/clearing agents. Therefore, we developed and patented (WO 2005001437) a supercritical carbon dioxide \((\text{scCO}_2)\)-based protocol for tissue processing, which is applicable in general pathology.5

Besides the well-known phases solid, liquid, and gas, the so-called supercritical phase exists (Figure 1). A supercritical fluid is defined as any substance that is above its critical pressure \((P_c)\) and critical temperature \((T_c)\), i.e. the critical point. \(T_c\) is the highest temperature at which a gas can be converted into a liquid by any increase in pressure. \(P_c\) is the highest pressure at which a liquid can be converted into a traditional gas by any increase in the liquid temperature. \(P_c\) and \(T_c\) are unique physical properties; for carbon dioxide, \(T_c = 31^\circ\text{C}\) and \(P_c = 73.7\) bar (72 atm).

In the supercritical phase, a substance has the physical properties of both a gas and a liquid.6 Liquid-
like solvency and gas-like volatility make scCO₂ a versatile ‘solvent’ with which to replace organic solvents in all kinds of processes, such as ethylacetate or dichloromethane in the decaffeination of coffee, or hexane in the extraction of essential oils. Although scCO₂-based processes are widely used in the pharmaceutical and food industries, this technique is new for tissue processing. In a series of pilot studies, solvent-free tissue processing with scCO₂ resulted in good-quality impregnated tissues with good sectioning and staining characteristics. Routine (immuno)histochemistry and molecular diagnostics could be performed without the need to change protocols, e.g. for gross sectioning.

The aims of the current study were: (i) to compare dehydration and shrinkage of tissue samples with an scCO₂-based protocol with standard tissue processing using a graded ethanol series and xylene as a solvent; (ii) to evaluate which parameters of the scCO₂-based protocol have the greatest influence on dehydration and shrinkage, and thus on the quality of sectioning, haematoxylin and eosin (H&E) staining, and morphology; and (iii) to validate the new protocol in a test series of human tissues with standard (immuno)histochemical staining.

**Materials and methods**

**Tissue processing**

In this study, two processing methods were compared. One uses a conventional graded ethanol series followed by xylene as a solvent. The process that is routinely used in our laboratory for diagnostics is performed in a Shandon Excelsior ES (A78410106; Thermo Electron Corporation, Basingstoke, UK), and is performed at night, with nearly 14 h of processing time. In comparison, the scCO₂ processing takes place in a reaction vessel, whereby the tissue samples are immediately exposed to 100% ethanol under supercritical conditions at 60°C and 150 bar. Supercritical conditions increase the diffusion rates, and thus the exchange of water and ethanol, in the tissues. Furthermore, scCO₂ functions as a solvent, and increases the paraffination rate. This process takes place in a TISPA processor for nearly 4 h. A comparison of the processing steps in these two protocols is shown in Figure 2.

**Measurements for percentage remaining water**

The amount of residual water in tissue samples was determined at three different stages during the course of processing (after the ethanol step, after the xylene step, and after paraffination).

To make the method robust, both tissue processors were maximally filled with tissue cassettes. Slices of bovine liver and mammary tissue of thickness 2–2.5 cm were fixed for 24 h in 4% Neutral Buffered Formalin. With a Siemens Universal Slicer (MS65500) (Siemens Netherlands NV, The Hague, Netherlands), 4-mm-thick slices were prepared, from which standardized samples of 2.18 × 2.18 cm (4.75 cm²) were taken with a metal punch with sharp edges, resulting in a tissue sample of 2.5–3.0 g. Samples were then dehydrated in either the routine graded ethanol series or in the scCO₂ tissue processor in 100% ethanol under supercritical conditions for 2 h. Samples were obtained after the xylene step by the use of routine protocols and after paraffination in both processing methods.

For the evaluation of residual water content in the tissue samples of the different groups, different methods were necessary.

Dehydrated tissue samples (from both routine and scCO₂-based protocols) and xylene-cleared tissue samples (only routine processing protocol) were ground for 30 s with a Krups 75 rotating blade grinder. The grinder was emptied on a folded piece of paper, and the tissue was transferred to a 20-ml scintillation vial, which was filled completely with methanol and capped immediately thereafter. The weight of the mixture was ~25 g. The vial was then shaken vigorously every 15 min for 1 h.

The paraffinized samples of both routine and scCO₂-based protocols were allowed to harden thoroughly for 1 h, and this was followed by scraping of excess

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**Figure 1.** Graph showing the solid, liquid, gaseous and supercritical phases as a result of temperature and pressure.
paraffin from the surface, before grinding as mentioned above. The tissue was then transferred to a 20-ml scintillation vial, and subsequently filled entirely with 2-propanol.

From the different groups of samples, a 1-ml syringe was filled with extraction fluid. The needle was dried before being pushed through the septum of the Metrohm 756KF Coulometer (Metrohm Applikon B.V., Schiedam, Netherlands). Depending on the extraction method, two to six droplets of extraction fluid were injected. The measurements were performed in triplicate.

SHRINKAGE TESTS

After the various stages of tissue processing, the tissue samples (bovine mammary and liver tissues) were measured with a camera (HP Photosmart 707, 5.1MPixel, ×24 zoom, Macro mode; Hewlett-Packard Nederland BV, Amstelveen, Netherlands) with a fixed position and light source. The surface area of the tissue fragments was measured with Image J (http://rsb.info.nih.gov/ij/) analysis software. By comparison of the size of the tissue fragments at various stages of processing with the original size of the standardized samples of 2.18 × 2.18 cm, the percentage shrinkage can be calculated.

EFFECT OF DEHYDRATION VARIABLES DURING scCO₂ PROCESSING ON PERCENTAGE WATER CONTENT, PERCENTAGE SHRINKAGE, AND HISTOLOGICAL OUTCOME

In a series of 32 separate scCO₂ runs, the effects of several processing parameters on remaining water content, shrinkage, sectioning quality and histological outcome were evaluated. The set-up of this experiment (bovine tissues) is shown in Table S1. Per run, three tissue samples were placed high in the reaction vessel, and three were placed low in the vessel. The other spaces were filled with dummies (i.e. cassettes filled with tissues that were not evaluated) to robustly test this tissue processor for its maximum capacity. This set-up was chosen because filling of the reaction chamber with ethanol or liquid paraffin starts at one end, giving a theoretically longer reaction time with the tissues than at the opposite end. The largest part of the reaction process, however, was performed under supercritical conditions, thereby creating diffusion characteristics of a gaseous substance instead of the slower diffusion characteristics of a liquid. The theoretical extremes in positioning were compared.

The variables used in this experiment were as follows: loading density, 30 or 45 cassettes per reaction vessel; amount of ethanol, 840 or 1200 ml; ethanol temperature, 6°C or 18°C; temperature of the reaction vessel, 54°C or 64°C; vessel pressure, 120 or 150 bar; alcohol/CO₂ holding time, 35 or 45 min; CO₂ filling rate, 7 or 10 l/h; and CO₂ flush temperature, 20°C or 60°C. The outcome parameters tested were: percentage remaining water after paraffination, percentage shrinkage, sectioning, and histological quality. Minitab 15 (Minitab, Coventry, UK) was used for evaluation. Sectioning was performed by a single experienced histotechnician using the same microtome (Thermo HM 355S; Thermo Electron Corporation, Basingstoke, UK) during this experiment. A single pathologist (W.D.) evaluated all of the automatically stained H&E sections (Tissue-tek Prisma). The evaluation process was blinded for the specific variables tested.
VALIDATION SERIES WITH HUMAN SAMPLES

Human samples were evaluated in a series of 32 consecutive scCO\textsubscript{2} runs with the ideal processing parameters. The tissues tested were normal skin, breast, stomach, colon, liver, lymph node, tonsil, and spleen. The samples were fixed for at least 24 h and for a maximum of 72 h. Samples were mostly taken from surgical resections (e.g., breast reductions or sleeve resections of the stomach) or from autopsies (skin, liver, and/or spleen). Sectioning was performed by a single experienced histotechnician with the same microtome (Thermo HM 355S) during this experiment. One pathologist (W.D.) evaluated all of the automatically stained sections. All tissue samples were H&E-stained. In addition, liver, stomach and colon samples were stained with periodic acid–Schiff (PAS) and Masson trichrome, skin and breast samples with cytokeratin (CK)/8/18, CK AE1/3 and oestrogen receptor (ER) antibodies, and lymph node, tonsil and spleen samples with Giemsa, CD3, CD20, and Ki67. Histochemical staining was performed in a Tissue-tek Prisma and immunohistochemistry on a Ventana BenchMark Ultra. Besides these test tissues, which were placed at different positions in the reaction vessel, the racks were again completely filled with dummies (i.e. tissue samples that were not further evaluated) in order to robustly test this processing method.

Results

SOLVENT AND Scco\textsubscript{2}-BASED PROTOCOLS RESULT IN SIMILAR DEHYDRATION

The results for bovine liver and mammary tissues were not significantly different, and are therefore grouped together. After the dehydration step, the tissue samples processed with scCO\textsubscript{2} contained 8% water [95% confidence interval (CI) 7.5–8.5]. After routine dehydration in a graded ethanol series with fresh fluids, the samples contained 6% water (95% CI 5–7.5). The use of old fluids in the routine tissue processor, however, resulted in much more remaining water and a much wider range of results (median 23%, 95% CI 17–26). These results were significantly different (Kruskal–Wallis test, \( P < 0.001 \)) (Figure 3A,B).

After the xylene step in the Excelsior tissue processor, the water content further decreased to 5.5% (95% CI 4.8–6.1) and 5.0% (95% CI 4.6–5.1) with the use of old and fresh liquids, respectively (Figure 3A,C). These results were no longer statistically significant (Mann–Whitney \( U \)-test, \( P = 0.132 \)). It is interesting that xylene seemed to be able to dehydrate significantly when an old ethanol series was used.

Finally, after paraffinization, the percentage water contents for the different processing methods were not significantly different (Kruskal–Wallis test, \( P = 0.476 \)), whereby the median outcome for the scCO\textsubscript{2}-processed tissues was in the same range as that for routinely processed tissues with the use of old or fresh liquids (Figure 3A,D).

SHRINKAGE OF TISSUES AFTER DIFFERENT PROTOCOLS

After the dehydration step, the tissue samples had shrunk significantly. Tissue samples shrunk by 9.1% after dehydration in an old ethanol series. In fresh fluids in the routine tissue processor or in the scCO\textsubscript{2}-based protocol, shrinkage was similar (16.3% and 15.7%, respectively), but significantly more than with old fluids (Kruskal–Wallis test, \( P < 0.001 \)) (Figure 4). Within the series of tests using scCO\textsubscript{2}-based tissue processing, liver samples shrunk significantly more after the ethanol step than did mammary tissue (19.41% versus 12.19%, \( P < 0.001 \)), but the differences in percentage shrinkage disappeared after paraffination (15.54% versus 15.49%, \( P = 0.49 \)).

INFLUENCE OF PROCESSING PARAMETERS ON RESIDUAL WATER PERCENTAGE AND SHRINKAGE

The residual water percentage after dehydration could be well predicted (\( R^2 = 87\% \)). All of the tested tissue processing parameters except rack design had a statistically significant effect on the percentage of remaining water after paraffinization. In descending order, the main influencing parameters were: loading density, ethanol temperature, amount of ethanol, tissue type, \( CO_2 \) filling rate, reaction vessel pressure, ethanol/\( CO_2 \) holding time, reaction vessel temperature, and \( CO_2 \) flushing time (Figure 5A).

Shrinkage also had a high predictability (\( R^2 = 96\% \)) within the boundaries of this experiment. The type of tissue was by far the main influencing parameter. In descending order, the other main influencing parameters were: reaction vessel pressure, amount of ethanol, loading density, ethanol/\( CO_2 \) holding time and rack design (Figure 5B).

The visual histological appearance did not seem to be affected by changes in the processing parameters. In general, most histological tissue sections had a good visual appearance, within the boundaries of routinely processed tissues used for routine diagnostics.

With these results, a response optimizer was performed for residual percentage water and shrinkage, but not for histological appearance, as no significant
The maximum differences in remaining water and tissue shrinkage were 1.5% (Figure 5). The following were found to be favourable: a 45-rack design, a loading density of 30 cassettes, a reaction vessel temperature of 64°C, an ethanol temperature of 18°C and fill rate of 1200 ml, a CO₂ fill rate of 7 kg/h, a CO₂ temperature of 60°C, and an ethanol/CO₂ holding time of 45 min.

The human samples showed more variation among tissue types than the bovine samples described above. For instance, in the human breast tissue there was much more fat, and when samples from the gastrointestinal tract were evaluated, there was variation in mucosal lining, submucosa with fat, and muscle tissue. In general, H&E sections showed clear pictures with uniform staining intensity throughout the

**Validation test with human samples**

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section (Figures 6 and 7). Nuclear morphology and chromatin had the same appearance as in samples processed with the standard method. Furthermore, cytoplasmic details could be normally appreciated. Within the fat cells, there still seemed to be some content, as there was still some vague eosinophilic staining present. In the scCO₂-processed gastrointestinal samples, there appeared to be slightly more retraction of the muscle fibres.

PAS staining of liver samples showed sufficient granular cytoplasmic staining, and in the gastrointestinal samples, normal staining of mucus-producing cells was observed. Masson trichrome and Giemsa also gave normal staining patterns.

The immunohistochemical staining of membranous (CD3 and CD20) and nuclear antigens (ER and Ki67) appeared crisp. False-positive staining was not encountered. The cytoplasmic antigens tested here (CK8/18 and CK AE1/3) resulted in much stronger staining than is normally seen (Figure 6).

Discussion

From the results described in this article, it can be concluded that tissue processing with scCO₂ results in well-dehydrated and paraffinized tissue samples with histological quality that is similar to that of tissues routinely processed with xylene as a solvent. The main focus here is on the development of solvent-free tissue processing, without the need to change protocols for grossing methods. This will form the main focus of the discussion. The scCO₂-based method, however, is less time-consuming than routine tissue processing, which may also be beneficial.

On comparison of remaining water percentage and shrinkage of tissue samples in the different protocols, it can be concluded that the results obtained with the solvent-free, scCO₂-based method lie within the same range as those obtained with routinely used, xylene-based protocols. A literature search on PubMed did not reveal publications discussing remaining water percentage at various stages of tissue processing. Furthermore, as far as we know, the methods applied here to measure remaining water percentage in processed tissues are also new. We therefore have to rely on the comparison between the differently processed tissues, coming from the same batch.

The results from the scCO₂-based method are less variable, because, with every run, fresh ethanol and paraffin were used. These results are most similar to those obtained with routine tissue processing with fresh liquids. On comparison of solvent-based tissue processing with old and fresh liquids, huge differences can be seen with regard to residual water percentage after the ethanol step. It is interesting that, in the xylene and paraffination steps, extra water is removed from the tissue samples, ultimately resulting in similar outcomes with the different methods studied here. Because, in most laboratories, more processing runs are performed with the same liquids before they are refreshed, one can assume that a slightly wider range in remaining water content may be expected, and that pathologists/histologists are used to a certain ‘range’ in outcome (e.g. staining intensity/crispness of the histological slides).

Shrinkage of tissues was variable among the different protocols tested here. The use of routine tissue processing with a fresh graded ethanol series and xylene resulted in similar shrinkage as in the scCO₂-based method (i.e. 16–17%). The use of ‘old’, more diluted liquids resulted in less shrinkage, of ∼9%. Probably, the more gradual dehydration in a more diluted ethanol series leads to less shrinkage.

The literature on tissue shrinkage during tissue processing is scanty, and when the percentage is mentioned, it is not always clear whether this refers to the amount in the patient or the amount in the excised sample with or without fixation. In this study, we decided to cut standardized samples from fixed tissues. One study that described shrinkage in the different steps of tissue processing very well used human cervices after hysterectomy. As compared with the fixed state, shrinkage after the paraffin wax embedding ranged from 11.0% to 15.1%. In the discussion of that article, a reference was made to a study from 1908, where the volume of liver samples

![Figure 4. Use of fresh fluids in either the Excelsior or the supercritical carbon dioxide (scCO₂) tissue processor resulted in more shrinkage (16–17%) than tissue processing with an old series of ethanol in the Excelsior.](image-url)
Figure 5. Evaluation of remaining water content (A) and tissue shrinkage (B) after changing various supercritical carbon dioxide processing variables with Minitab.
was found to shrink to 68% of the fresh volume after formalin fixation, ethanol dehydration, and paraffin embedding. From this, one can calculate a linear shrinkage of 14%. In a more recent study on prostatesctomy samples, linear shrinkage was 4.1% after fixation and 14.5% after tissue processing. These are similar to those from our current study.

The influence of the parameters of the scCO₂-based protocol on the remaining water content and tissue shrinkage could be well predicted. Within the boundaries in which this test was performed, the variation in the parameters did not cause large enough changes to be observed in H&E microscopy. Looking at all the results for the remaining water percentage (maximal difference of 1.5%) and tissue shrinkage (also maximal difference of 1.5%), this is not surprising. With routinely processed tissues, pathologists are used to a certain range in the quality of sections and staining, and the results from the scCO₂-processed tissues appear to lie in the same range.

After our studies with bovine tissues, it was decided to perform a test with human samples with subsequent immunohistochemical staining for membranous, cytoplasmic and nuclear epitopes. In general, the results were good, without false negatives or false positives. Sometimes, much stronger staining was observed than with routinely processed tissues. Other studies that have used alternatives for xylene with or without microwave fixation have mentioned similar and sometimes even better immunohistochemical results. Besides the fact that microwaves are used for antigen retrieval, and thus the use of microwaves during fixation may improve antigenicity, the use of an alternative for xylene may be beneficial for the integrity of membranous/cellular structures. Xylene has been found to have an extracting effect on the cytoplasm, as observed ultrastructurally by electron microscopy. After application as a clearing agent in frozen sections, xylene appeared to decrease immunoreactivity. Moreover, in comparison with chloroform and inhibisol, clearing
Figure 7. A, Haematoxylin and eosin overview with regularly distributed follicles, with clearly visible light and dark zones of the follicular centre. B–D, Immunohistochemistry for CD3, CD20, and Ki67, respectively. E, Giemsa stain of the same area. F, Higher magnification of a follicular centre. G, H, Further details of (F).
with xylene during tissue processing resulted in a 50% reduction in the immunohistochemical signal of plasma cells.\textsuperscript{13} The fact that our processing method is solvent-free might explain the much stronger staining of both the CKs tested. Therefore, some immunohistochemical techniques will have to be retitred when this new processing method is used.

For all the tests in this study (both bovine and human tissue samples), the same cassettes and the same amounts of tissue in the cassettes were used as in the routine diagnostic setting. The advantage of this is that no specific changes in protocols for grossing are necessary, which is the case in the continuous-throughput processing method, where a dissection board is used to obtain tissue slices no thicker than 1.5 mm.\textsuperscript{14} Furthermore, the use of regular, ‘thicker’ tissue samples is beneficial to obtain archival material and for the production of tissue microarray blocks.

The racks in the scCO\textsubscript{2} processor were filled to maximum capacity (2 \times 50 cassettes) to test the robustness of this system. Even then, the whole processing scheme took \textless 4 h. This is comparable with the Milestone Pathos automated microwave-assisted tissue processor, which can process up to 210 cassettes with 5-mm-thick specimens in 3–4 h. The Sakura Xpress can process 40 prefixed specimens with a thickness of 1.5 mm in 1 h. Thicker slices can be processed by increasing the time. A review of microwave-assisted tissue processing has been presented by Buesa.\textsuperscript{15} In this study, we chose to test uniform, ‘thick’ tissue samples. It is conceivable that smaller tissue samples such as needle biopsies can be processed much more quickly. This was, however, beyond the scope of this study, and is a subject for future research.

Finally, some additional remarks concerning the use of high-pressure vessels need to be made. High-pressure equipment needs to comply with the Pressure Equipment Directive (97/23/EC).\textsuperscript{16} Also, IEC 61010\textsuperscript{17} concerning safety requirements for laboratory equipment has a chapter that regulates high-pressure laboratory equipment. Both directives must be complied with to act in accordance with the EC marking. The Tispa Processor complies with both the Pressure Equipment Directive (97/23/EC) and IEC 610101, obtained the CE mark, and is registered as an In Vitro Diagnostic Class I item of laboratory equipment.\textsuperscript{18} Therefore, the system is safe and simple to use in any environment, according to IEC 62366:2008.\textsuperscript{19} After basic training (2 h), personnel of any level can operate the processor and do not require any protective gear or otherwise. The processor cannot start the run if not all process requirements are met.

In conclusion, solvent-free, scCO\textsubscript{2}-based tissue processing results in well-dehydrated and paraffinized tissue blocks with histological and immunohistochemical properties that are similar to those of routine xylene-based tissue processing.

References

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Table showing the set up of the dehydration and shrinkage experiments with bovine tissues. In this table the different settings for all the parameters are given.

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